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Special Topic for 973 Program

Effect of moxibustion at Shenshu (BL 23) on the ethology, corticosterone and glucocorticoid receptor in aging rats

艾灸肾俞穴对衰老大鼠行为学、皮质酮及糖皮质激素受体的影响

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Abstract

Objective: To observe the effect of moxibustion on learning and memory abilities, corticosterone and glucocorticoid receptor (GR) in subacute aging rats.

Methods: Twenty four Sprague-Dawley (SD) rats were randomly divided into a normal group, a model group and a moxibustion group, 8 rats in each group. Rats in the model group and the moxibustion group were subcutaneously injected with 25% D-galactose [125 mg/(kg·bw)] for 40 d continuous; rats in the normal group were injected with saline at the same position for 40 d continuous. Rats in the moxibustion group were given mild moxibustion at bilateral Shenshu (BL 23) at the same time of modeling; rats in the normal group and the model group were only identically grabbed without moxibustion for 40 d. The learning and memory abilities of rats were observed using the Morris water maze at the end of the experiment. Abdominal aorta blood and thymus were collected after water maze experiment. Enzyme-linked immunosorbent assay (ELISA) was used to detect serum corticosterone level, and immunohistochemical method was used to detect the expression of thymus GR.

Results: Compared with the normal group, rats in the model group showed that a significantly longer escape latency time ($P<0.01$) on the third and the fourth days; number of times crossing the platform in 70 s significantly reduced ($P<0.01$); activity times in the fourth quadrant significantly decreased ($P<0.05$); serum corticosterone levels increased ($P<0.01$); thymus GR expression decreased ($P<0.05$). Compared with the model group, rats in the moxibustion group showed that the escape latency times were significantly shorter on the third, the fourth and the fifth days ($P<0.01$, $P<0.05$); number of times crossing the platform in 70 s significantly increased ($P<0.05$); activity times in the fourth quadrant significantly increased ($P<0.05$); serum corticosterone levels decreased ($P<0.05$); thymus GR expression increased ($P<0.05$).

Conclusion: Moxibustion could improve the learning and memory abilities of subacute aging rats, down-regulate serum corticosterone levels, and increase thymus GR content.

Keywords: Moxibustion Therapy; Mild Moxibustion; Point, Shenshu (BL 23); Aging; Corticosterone; Receptors, Glucocorticoid

【摘要】目的：观察艾灸对亚急性衰老大鼠学习记忆能力和皮质酮及糖皮质激素受体(glucocorticoid receptor, GR)的影响。**方法：**将24只(Sprague-Dawley, SD)大鼠按随机数字表分为正常组、模型组和艾灸组，每组8只。模型组和艾灸组按每日125 mg/(kg·bw)于颈背部皮下注射25%的D-半乳糖，连续40 d；正常组大鼠同部位注射等量生理盐水，连续40 d。艾灸组大鼠造模同时给予温和灸双侧肾俞，正常组和模型组只做相同抓取，不艾灸，共40 d。实验结束，采用Morris水迷宫实验观察大鼠学习记忆能力，水迷宫实验结束后取腹主动脉血和胸腺，酶联免疫吸附测定(enzyme-linked immunosorbent assay, ELISA)法检测血清皮质酮含量，免疫组化法检测胸腺GR表达情况。**结果：**与正常组比较，模型组大鼠逃避潜伏期时间在第3天和第4天显著延长($P<0.01$)，70 s内穿越平台次数显著减少($P<0.01$)，在第四象限的活动时间明显减少($P<0.05$)；血清皮质酮含量升高($P<0.01$)；胸腺GR表达下降($P<0.05$)。与模型组比

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较，艾灸组大鼠逃避潜伏期时间在第 3 天、第 4 天和第 5 天显著缩短($P<0.01$ 或$P<0.05$)，70 s内穿越平台次数显著增多($P<0.05$)，在第四象限的活动时间明显增多($P<0.05$)；血清皮质酮含量降低($P<0.05$)；胸腺GR表达升高($P<0.05$)。**结论：**艾灸可改善亚急性衰老大鼠学习记忆能力，下调血清皮质酮含量，上调胸腺GR含量。

【关键词】灸法；温和灸；肾俞；衰老；皮质酮；受体，糖皮质激素

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Aging is a natural process and the overall phenomenon of gradual decline in the whole body's morphology, structure and function with age[1]. The average human life has been increasing with an expanded proportion of elderly population due to improvement of living standards and medical conditions. China has entered an aging society at present[2]. Aging and age-related diseases become problems which cause more and more attentions worldwide, thus to study the mechanisms of aging and explore effective anti-aging methods become the hotspot research at home and abroad. Memory ability declining of the elderly with age causes great inconvenience for the life of older people. Glucocorticoid is an important medium of nerve-endocrine-immune interaction. It can significantly inhibit the immune system and is one of the important reasons for a weakened immune system. Thymus is an important immune organ of the immune system; its functional status is directly related to the immune function. Traditional Chinese medicine (TCM) believes that 'the kidney stores essence, dominates bone and generates marrow'. Deficiency of kidney essence plays an important role in aging. In this study, mild moxibustion over bilateral Shenshu (BL 23) was used to observe the effect of moxibustion on the learning and memory abilities, serum corticosterone and thymus glucocorticoid receptor (GR) in the subacute aging rats. These are summarized as follows.

1 Materials

1.1 Animal feeding and modeling

Twenty-four clean grade male Sprague-Dawley (SD) rats weighing (200 ± 20) g were purchased from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine [animal license number: SYXK (Shanghai) 2009-0069].

Breeding environment was 12 h alternate circadian rhythm with room temperature at 18-22 ℃ and relative humidity of 55%-65%. The cages and rooms were disinfected once a week. Rats were free access to foods and water. After adaptive feeding for a week, rats were completely randomly divided into a normal group, a model group and a moxibustion group ($n=8$), by a random number table generated with SPSS 18.0 version software for Windows. Rats in the model group and the moxibustion group were subcutaneously injected with 25% D-galactose (125 mg/kg·bw) at the nape for 40 d incessantly; rats in the normal group were injected with equal volume saline at the same position for 40 d continuously[3-4].

1.2 Reagents and equipments

D-galactose [Amresco Corporation, USA]; GR antibody (Abcam, UK); corticosterone EIA kit (Cayman, USA); SABC kit (SA1021, Wuhan Boster Biological Technology Co., China); computer automatic tissue hydroextractor [ATP700 (ST), Changzhou Haosilin Medical Instrument Co., Ltd., China]; paraffin embedding machine and slicer (Leica, Germany); Morris water maze and related software (provided by Shanghai Geriatrics Institute of TCM, China); optical microscope (BX53, Olympus, Japan); cellSens Dimension image analysis system (Olympus, Japan).

2 Methods

2.1 Intervention methods

Rats in the moxibustion group were intervened at the same time of modeling. Hair of bilateral Shenshu (BL 23) points areas of the rats was shaved with a natural prone position. Gently grasped the side of rat's head by the left hand and held a special moxa stick (0.3 cm in diameter, 20 cm in length) by the right hand. Lit the moxa stick and performed suspended moxibustion about 3-5 cm away from Shenshu (BL 23) (adjusted the height according to whether the rats struggled)[5], once daily. Moxibustion was performed over bilateral Shenshu (BL 23) at the same time, 5 min each time, continuously for 40 d.

Rats in the normal group and the model group were only grabbed same as those in the moxibustion group without moxibustion for 40 d, 5 min every time.

2.2 Specimen collection

2.2.1 Serum

On the second day after the water maze test, rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital at 2 mL/(kg.bw). After fixation, cut the abdominal wall to fully expose the abdominal aorta. 5-8 mL blood from the abdominal aorta was collected with syringe. Sit at room temperature for 2 h and centrifugated at 5 000 r/min for 15 min to separate serum for corticosterone level assay.

2.2.2 Thymus

Cut the chest using scissors to detach the thymus. Weighed the thymus and recorded after removal of the connective tissues. Part of the thymus was placed into 10% formalin solution and fixed (less than 24 h). Embedded in paraffin after tissue dehydration, and then

performed the immunohistochemisty assay for GR expression after slicing.

2.3 Observation items and methods

2.3.1 The overall situation and body weights of rats in each group

Mental states, activity informations, fur appearances, diet statuses and body weight changes of rats in each group were observed and recorded during the experiment.

2.3.2 Spatial learning and memory abilities

In this study, we used Morris water maze test to observe the spatial learning and memory abilities of rats[6]. Water maze tests were immediately conducted for rats in each group after continuous intervention for 40 d. Schematic diagram of Morris water maze test apparatus is shown in Figure 1.

The water maze was a circular pool of 50 cm in height and 130 cm in diameter. Clean water was added into the pool (at least 30 cm in depth) before the beginning of experiment, and then appropriate amount of Indian ink was added into the water to make the water opaque. Water temperature was maintained at (20±1) ℃. Opened the water maze information collection software in the computer and adjusted the position of the camera to make the whole water maze keep in the camera's field of vision. Markers were made at the edge of the water maze according to the quadrant set by the software in advance. Different shapes of patterns were used as references by sticking them onto the inner wall of each water maze quadrant. Then a circular platform (29 cm in height, 9 cm in diameter) was installed in the fourth quadrant at 33 cm from the wall of the pool on the connection line between center and inner wall of the pool, which was 1 cm below the surface to ensure that the platform cannot be seen. Rats (facing the wall of pool) were put into the water from the two quadrants far away from the platform. Information collection time was set to 70 s. Recorded the times that rats used to find and climb to the platform. Results of two times were averaged. The interval of each rat to get into water (two times) was at least 5 min. Positions of other references (including experimenter) remained unchanged during the whole period of test.

2.3.3 Place navigation test

Place navigation test (PNT) was used to measure the learning and memory abilities of rats to water maze. Rats (facing the wall of pool) were put into the water from two quadrants. Times used for rats to find and climb to the platform were escape latencies. The routes were recorded. Rats would be guided to the platform and stayed there for 15 s if they could not find the platform within 70 s, and the escape latency was denoted as 70 s. Same experiments were conducted for 5 d.

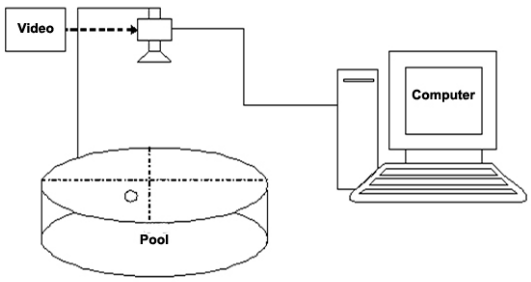


Figure 1. Morris water maze test apparatus

2.3.4 Spatial probe test

Spatial probe test (SPT) was used to check the memory abilities of rats to the platform spatial position. After the platform was demolished on the sixth day and the rats were put into the water from the same site, swimming trajectory and residence time of rats in each quadrant within 70 s were observed and recorded; the numbers crossing the original platform location in 70 s were recorded. After removal of the underwater platform, the more times of rats passing through the underwater platform position in 70 s indicated the stronger memory abilities after learning; otherwise the memory abilities were weaker. Another evaluation index of this experiment was residence times of rats in the fourth quadrant, the longer they stayed indicated the stronger memory abilities.

2.3.5 Serum corticosterone levels

Serum corticosterone levels were determined by ELISA according to the instruction manual.

2.3.6 Thymus GR expression

Thymus GR expression was detected by immunohistochemical (IHC) SABC assay according to the instruction manual.

2.4 Statistical methods

Statistical analyses for all the experimental data were performed using SPSS 18.0 version statistical software. Data fit the normal distribution were statistically described by mean ± standard deviation ($\bar{x} \pm s$). Means of multiple samples were compared using One-way ANOVA. Least significant difference (LSD) was used for pairwise comparison of data with homogeneity of variance; Dunnett's T3 method was used for data with heterogeneity of variance. Non-normal distribution data were statistically described by median (inter-quartile range) [M (Q_{25}, Q_{75})]. Means of multiple samples were statistically compared using non-parametric tests. Repeated measurement data were statistically analyzed using repeated measure design variance analysis of general linear model (GLM). Correlation of data was analyzed according to Mauchly's test results: $P>0.05$ indicated that the data had no correlation and could use One-way ANOVA; $P<0.05$

indicated that the data had correlation and multivariate tests were performed. Size of test was set as $\alpha=0.05$, and $P<0.05$ was considered statistically significant.

3 Results

3.1 The overall situation of rats in each group

Rats in the normal group had shiny and smooth fur, normal diet, agile movement, and dry and clean padding in the cages.

Rats in the model group had parch blight and shineless fur, bradykinesia, lethargy, slow reaction, serious hair loss, and damp and dirty padding in the cages.

Compared with rats in the model group, the fur, activity, spirit and reaction of rats in the moxibustion group were all improved at different degrees; hair loss phenomenon, status of damp and dirty padding in the cages were all slightly better.

3.2 Weight changes of rats in each group

After statistical analysis, body weights of rats in each group showed no significant difference ($F=1.968$, $P=0.165$) before the experiment. At the end of the experiment, body weights of rats in the model group were lower than those in the normal group, and the difference between the two groups was statistically significant ($P<0.01$); growth rates of body weights in the model group were also lower than those in the normal group ($P<0.01$). Compared with the model group, at the end of the experiment, rats in the moxibustion group were heavier ($P<0.01$), and the growth rates of body weights were also faster ($P<0.01$), (Figure 2).

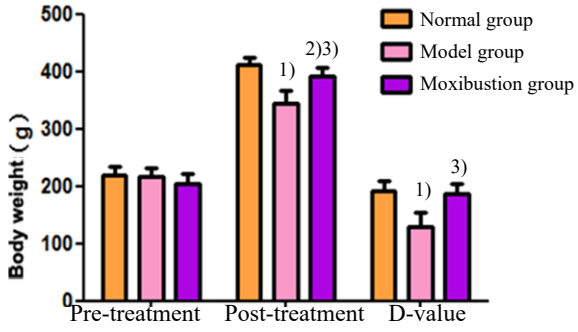


Figure 2. Weight changes of rats in each group [Note: Compared with the normal group, 1) *P*<0.01, 2) *P*<0.05; compared with the model group, 3) *P*<0.01]

3.3 Results of water maze test

3.3.1 Results of place navigation tests

Place navigation tests were conducted for 5 d in total. Rat escape latencies showed a decreasing trend with experimental days ($P=0.0004$) during the experimental days, among the groups, at the interaction between experimental days and groups. Interaction between experimental days and groups (day × group) was not statistically significant ($P=0.735$), which indicated that the role of time factor did not vary among groups. Comparison of the results among groups showed the differences among data, of rat escape latency on the 5th day, were statistically significant ($F=7.828$, $P=0.003$). Differences among the three groups showed no statistical significance on the first and the second days, however, differences between the model group and the normal group were significant on the third day ($P=0.002$) and the fourth day ($P=0.009$); differences between the moxibustion group and the model group were significant on the third day ($P=0.004$), the fourth day ($P=0.033$) and the fifth day ($P=0.042$), (Figure 3).

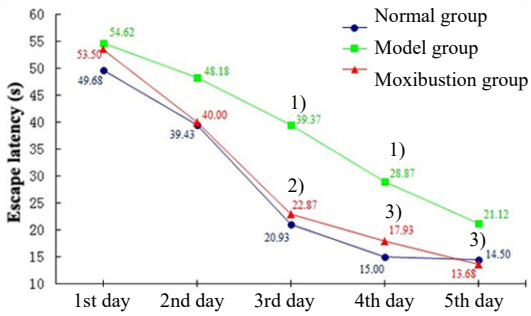


Figure 3. Variance trends of escape latency of each group in 5 d [Note: Compared with the normal group, 1) *P*<0.01; compared with the model group, 2) *P*<0.01, 3) *P*<0.05]

3.3.2 Results of SPT

Compared with the normal group, times of rats in the model group passing through the platform in 70 s were significantly reduced, and the difference between the two groups was statistically significant ($P=0.005$). Compared with the model group, times of rats in the moxibustion group passing through the platform in 70 s significantly increased, and the difference between the two groups was statistically significant ($P=0.034$), (Figure 4).

The fourth quadrant (the platform quadrant) activity time: compared with the normal group, activity time of the model group rats in the fourth quadrant was significantly reduced, and the difference between the two groups was statistically significant ($P=0.014$). Compared with the model group, activity time of the moxibustion group rats in the fourth quadrant was significant increased, and the difference between the two groups was statistically significant ($P=0.036$), (Figure 5).

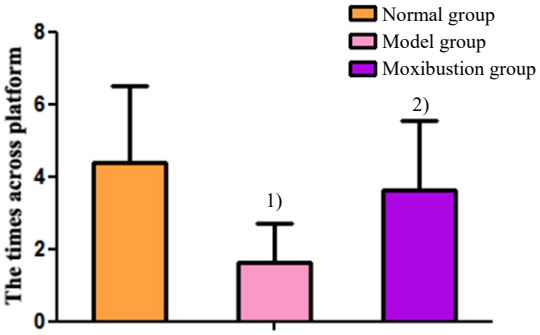


Figure 4. Comparing times of each group rats passing through the platform in 70 s [Note: Compared with the normal group, 1) *P*<0.01; compared with model group, 2) *P*<0.05]

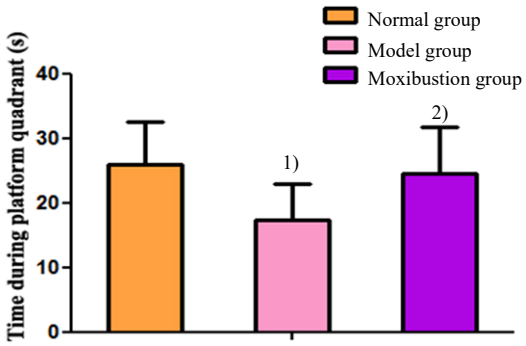


Figure 5. Comparing activity times of each group rats in the fourth quadrant [Note: Compared with the normal group, 1) *P*<0.05; compared with the model group, 2) *P*<0.05]

3.4 Comparing serum corticosterone levels of rats in each group

Compared with the normal group, serum corticosterone levels of rats in the model group increased significantly, and the difference between the two groups was statistically significant ($P = 0.004$). Compared with the model group, serum corticosterone levels of rats in the moxibustion group decreased. There was a statistically significant difference between the two groups ($P = 0.03$). This indicated that serum corticosterone levels of aging rat models were significantly higher, while moxibustion could reduce the serum corticosterone levels of aging rat models (Figure 6).

3.5 Thymus GR expressions of rats in each group

GR expression in rat thymus was detected by IHC SABC assay and observed under OLYMPUS BX53 microscope. Integrated optical density (IOD) value was analyzed using OLYMPUS cellSens Dimension image analysis system. Yellow, brown-yellow, and brown indicated the positive expression (Figure 7).

Compared with the normal group, thymus GR expressions of rats in model group were significantly reduced, and the difference between the two groups was statistically significant ($P=0.018$). Compared with the model group, thymus GR expressions of rats in the moxibustion group increased, and there was a statistically significant difference between the two groups ($P=0.042$), (Figure 8).

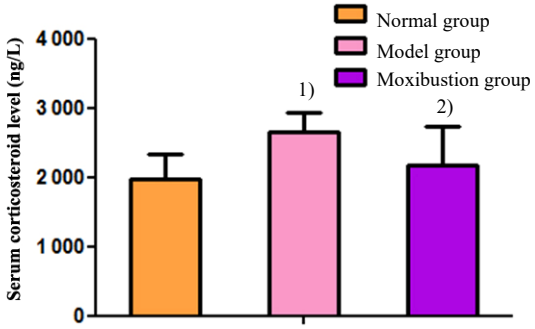
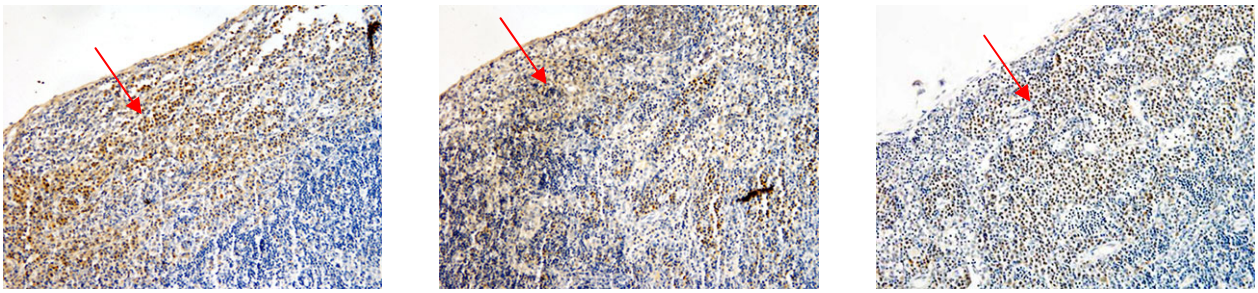


Figure 6. Comparing serum corticosterone levels of rats in each group (Note: Compared with the normal group, 1) *P*<0.01; compared with the model group, 2) *P*<0.05



Normal group **Model group** **Moxibustion group**

Figure 7. IHC staining of rat thymus GR in each group (IHC, ×200)

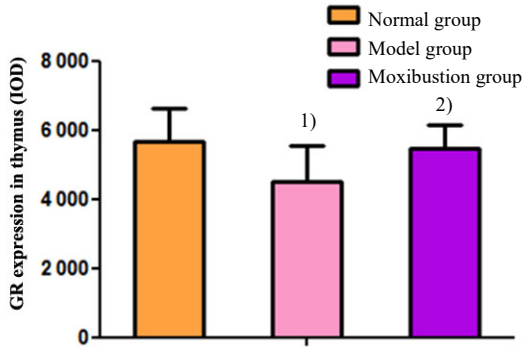


Figure 8. Comparing thymus GR expressions of rats in each group [Note: Compared with the normal group, 1) *P*<0.05; compared with the model group, 2) *P*<0.05]

4 Discussion

In Chinese medicine, the kidney is the congenital foundation of the human body. It stores essence and dominates human growth and development. Kidney essence and qi is directly associated with human birth, growth, maturity, aging and death. Deficiency and exhaustion of kidney essence and qi are related to human aging and death. Deficiency of kidney essence and qi is a major factor contributing to aging. In addition, it also decides the life span. Consequently, it's of great significance to tonify kidney essence and qi to delay aging.

The aging model induced by D-galactose was first proposed by Chinese scholars in 1985. A large dose of D-galactose is continuously injected into an animal in a short time to accelerate the aging of animal, and ultimately to form the animal model similar to natural aging. Currently, this method is the most widely used one to prepare the aging animal model[7]. D-Galactose is a normal metabolite in vivo. Under normal conditions, it can be converted to glucose to participate the processes of glucose metabolism in vivo. However, it would cause the body's metabolism disorders if over-loaded[8], including manifests of biochemical changes similar to natural aging, immunocompromise, abnormal gene expression and regulation, decreased cell growth and reproduction ability, cell degeneration and cognitive impairment[9]. Glucocorticoids are final secretion products of hypothalamus pituitary adrenal (HPA) axis, and have versatile inhibitory effects on the immune system[10]. Studies have found that down regulation of GR in hippocampus or increased cortisol is closely related to the decline of hippocampus-dependent cognitive function[11], suppression of gonads and immune functions[12]. Prescriptions with the effect of warm reinforcing kidney yang can improve the suppression status of HPA axis in corticosterone rats[13]. *Zuogui* pill, the prescription with the effect of tonifying the kidney yin, down-regulate the hyperactivity of the HPA axis in laevoclination monosodium glutamate rats[14]. Prescriptions with the effect of tonifying kidney and replenishing qi can improve age-related phenomenon of high corticosterone[15]. These research results suggest that HPA axis function status is closely related to the kidney. The theory of 'kidney deficiency causing senescence' holds that kidney essence continues loss due to various factors during the life time, which will result in the age-related degeneration of organ function[16].

In this study, rats were subjected to continuous subcutaneous injection with D-galactose into the nape to make subacute aging models. The general conditions were worse, the growth of body weights was slower, and the spatial learning and memory abilities were significantly poorer in rats of the model group than those of the normal group. The evidences above indicated that these models were successful. In this study, mild moxibustion at bilateral Shenshu (BL 23) was used to observe the effects of mild moxibustion on spatial learning and memory abilities of subacute aging rats. The experimental results showed that the spatial learning and memory abilities of rats in the model group were significantly lower than those in the normal group. The spatial learning and memory abilities of rats were significantly increased in the moxibustion group than those in the model group, which indicated that the brain of rats appeared aging and functional decline after injection of D-galactose, and moxibustion at Shenshu (BL 23) could significantly improve the spatial learning and memory abilities of subacute aging rats.

Thymus is an important immune organ of the immune system and closely related to the maturation and secretion of lymphocytes. Its function is also affected by glucocorticoid. Glucocorticoid induces cell death by disrupting the integrity of thymocyte DNA[17], which leads to significantly decreased thymocyte number and reduced thymus weight[18]. High concentration glucocorticoids are combined to GR of target organs to form glucocorticoid-receptor complexes. Studies have found that serum corticosterone levels in old rats were significantly higher than that in the young control group[19]. The results of this study also showed that the serum corticosterone levels in subacute aging rats significantly increased compared with that in the normal rats, while the expression of thymus GR decreased. The possible reason is that the high concentrations of serum corticosterones, presented in subacute aging rats, formed glucocorticoid-receptor complexes by binding to thymocyte GR, so that the content of thymocyte GR decreased[20]. Decreased GR content led to accumulation of corticosterone by feedback, which further increased serum corticosterone level.

The results of this study showed that moxibustion at Shenshu (BL 23) could reduce serum corticosterone

levels of subacute aging rats and up-regulate the expression of thymus GR. This indicated that moxibustion could delay senility by improving age-related high corticosterone phenomenon, mitigating the adverse effects of corticosterone on the immune system, and improving immune functions of subacute aging rats, which provided an experimental basis for the further research on anti-aging by moxibustion.

Conflict of Interest

There was no potential conflict of interest in this article.

Acknowledgments

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

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