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Basic Study

Influence of electroacupuncture on histomorphology of lacrimal glands and ocular surface in experimental dry eye syndrome

电针对实验性干眼症泪腺和眼表组织形态学的影响

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Abstract

Objective: To observe the influence of electroacupuncture (EA) on histomorphologies of lacrimal glands, cornea and conjunctiva in experimental dry eye syndrome, and to explore the repair effects of EA on lacrimal glands and ocular surface damage.

Methods: Twenty-four healthy male New Zealand rabbits were randomly divided into a normal group, a model group, an EA group and a medication group, 6 rabbits in each group. Experimental dry eye syndrome models were prepared in rabbits by using 0.1% benzalkonium chloride for eye drops. Tear secretion volume, break-up time of tear film (BUT) and corneal fluorescein staining score were observed before and after the treatment. Periodic acid Schiff (PAS) staining method was used to observe the changes of conjunctival goblet cells in rabbits. After hematoxylin eosin (HE) staining, morphological changes of rabbit cornea, conjunctiva and lacrimal gland tissues were observed under light microscope.

Results: Compared with the normal group, tear secretion volume and BUT were significantly reduced (both $P<0.01$), while the corneal fluorescein staining score was significantly increased ($P<0.01$) in the model group. Compared with the model group, tear secretion volume and BUT were significantly increased, while the corneal fluorescein staining score was significantly decreased in the EA group and the medication group (all $P<0.01$). Compared with the normal group, the number of conjunctival goblet cells in the model group was significantly reduced; compared with the model group, the numbers of conjunctival goblet cells were all relatively higher in the EA group and the medication group. Pathological lesions of cornea, conjunctiva and lacrimal glands all showed improvement by HE staining in the EA group and the medication group after the intervention.

Conclusion: EA can improve tear secretion and tear film stability of rabbit dry eye syndrome, and repair the pathologic lesions of conjunctival goblet cells, corneal epithelia, cornea, conjunctiva and lacrimal glands.

Keywords: Acupuncture Therapy; Electroacupuncture; Dry Eye Syndrome; Anatomy & Histology; Rabbit

【摘要】目的：观察电针对实验性干眼症兔泪腺、角膜和结膜组织形态学的影响，探讨电针对干眼症泪腺和眼表损伤的修复作用。**方法：**健康雄性新西兰兔 24 只，随机分为正常组、模型组、电针组和药物组，每组 6 只。采用 0.1%苯扎氯铵滴眼制备兔实验性干眼症模型，观察治疗前后实验性干眼症兔的泪量、泪膜破裂时间(break-up time, BUT)和角膜荧光素钠染色评分；应用 PAS 染色法观察兔结膜杯状细胞的变化；采用苏木精伊红 (hematoxylin eosin, HE)染色，光镜下观察兔角膜、结膜、泪腺 组织形态学的变化。**结果：**与正常组比较，模型组泪量、BUT 均显著减少(均 $P<0.01$)，角膜荧光素钠染色评分显著增加($P<0.01$)。与模型组比较，电针组和药物组的泪量、BUT 均显著增加(均 $P<0.01$)，角膜荧光素钠染色评分均显著降低(均 $P<0.01$)。与正常组比较，模型组结膜杯状细胞数量显著减少；与模型组比较，电针组和药物组结膜杯状细胞数量均相对较多。HE 染色结果显示，电针组、药物组干预后角膜、结膜、泪腺组织的病理损伤均有一定改善。**结论：**电针对干眼症兔泪液分泌和泪膜稳定性均具有一定的改善作用，对结膜杯状细胞、角膜上皮损伤和角、结膜及泪腺的病理损伤均具有一定的修复作用。

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【关键词】针刺疗法; 电针; 干眼症; 解剖学和组织学; 兔
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Dry eye syndrome (DES) is a generic name of a group of conditions characterized by lesions of ocular surface, due to abnormal tear secretion and poor tear film stability[1]. DES is defined as eye discomfort, visual disturbance, poor tear film stability, ocular surface inflammation and other potential lesions caused by abnormal tear secretion and other factors at the international seminar on DES in 2007[2]. The main symptoms of DES are dry eyes, foreign body sensation, burning sensation, itching sensation and blurred vision. Long-term lesions can lead to a decline in corneal transparency and visual extinction, thus affect work, study and life, and even bring about blindness. With the popularity of computer, changes of lifestyle and living habit, the incidence of DES has gradually increased and presented a trend of development in younger population. Epidemiological studies have shown that the incidence of DES in China is about 21%-30%, higher than that in the US and European countries[3]. How to effectively treat the DES has become a hot topic[4]. Currently, tear substitute and trying to keep the tears in the eyes are commonly used for the treatment of DES, which are passive methods to increase tear. Cyclosporine A (CsA), lacrimal point embolism and autologous submandibular gland transplantation show certain effects, however, these therapeutic methods still have limitations[5]. Acupuncture can increase tear secretion by stimulating the acupoints[6], which is expected to fundamentally cure DES. In recent years, literatures about the treatment of DES by acupuncture are gradually increased. Acupuncture can improve the signs and symptoms of dry eye patients at different degrees and is more effective than artificial tears therapy[7-11]. However, studies on the mechanisms of acupuncture treatment for DES are seldom reported in China. This study was to establish models of rabbit experimental DES and to study the influence of electroacupuncture (EA) on lacrimal glands and tissue damages of ocular surface in rabbit experimental DES models.

1 Materials and Methods

1.1 Experimental animals and grouping

Twenty-four healthy male New Zealand white rabbits, weighing (2.2±0.2) kg, were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine. Feeding environment: indoor temperature (20±2) ℃, relative humidity of 50%-70%, 12 h of light-dark cycle, free access to food and water. Animals were adaptively fed for 1 week. Only those rabbits with normal ocular surface function, determined by slit lamp microscope, were used for experiments. The 24 rabbits were then randomly divided into a normal group (NG), a model group (MG), an EA group (EAG) and a medication group (MDG), with 6 rabbits in each group.

1.2 Reagents and instruments

Benzalkonium chloride (Sigma Company, USA); dehydrated alcohol, formaldehyde, paraformaldehyde, xylene, neutral gum binder, dodecahydrate disodium hydrogen phosphate, potassium chloride, potassium dihydrogen phosphate, 0.9% sodium chloride injection (Sinopharm Chemical Reagent Co., Ltd., China); hematoxylin and eosin (Nanjing Jiancheng Biotechnology Co., Ltd., China); polyethylene glycol eye drops (Alcon Laboratories, Inc., USA); fluorescein ophthalmic test strip and tear detection filter strips (Tianjin Jingming New Technological Development Co., Ltd., China); filliform needles (Wujiang City Cloud & Dragon Medical Device Co., Ltd., China); slit lamp microscope (Suzhou 66 Vision Tech Co., Ltd., China); optical microscope and analysis software (OLYMPUS Corporatio, Japan); Han's EA device (Nanjing Jisheng Medical Devices Co., Ltd., China).

1.3 Model preparation and identification

Both eyes of New Zealand rabbits were used for the experiment. Tear secretion volume, break-up time of tear film (BUT) and corneal fluorescein staining score were observed before modeling. After the examination, excepting the NG, rabbits in the other three groups were prepared for DES models using 0.1% benzalkonium chloride for eye drops[12-13], with one drop per eye, twice a day, for 4 weeks. After modeling, rabbit tear secretion volume, BUT and corneal fluorescein staining score were observed to identify if the models were successful.

1.4 Intervention method of each group

Therapeutic intervention was performed in rabbits with successful modeling.

1.4.1 EAG

Rabbits in the EAG were treated with EA after the successful modeling.

Acupoints: Bilateral Cuanzhu (BL 2), Taiyang (EX-HN 5), Sizhukong (TE 23) and Fengchi (GB 20).

Methods: The *Laboratory Animal Acupoint Atlas*, developed by China Association of Acupuncture and Moxibustion, were referenced for acupoint positioning. The filliform needles of 0.25 mm in diameter and 25 mm in length were used for acupuncture. Lifting, thrusting and twisting were manipulated after insertion of the needles. Bilateral acupoints of Cuanzhu (BL 2) and Taiyang (EX-HN 5) were respectively connected to Han'S EA device after the arrival of qi. The continuous wave was selected, with a frequency of 2 Hz and

intensity of 1 mA. The needles were retained for 20 min. The treatment was continued for 7 d, once a day.

1.4.2 MDG

After the successful modeling, rabbits in the MDG were treated with artificial tears. Polyethylene glycol eye drops were used, with 1 drop/time, 4 times a day, for 7 d.

1.4.3 NG

Rabbits in the NG didn't perform modeling and treatment.

1.4.4 MG

Rabbits in the MG accepted the same grasping and fixation as in the EAG, but without any therapeutic intervention.

1.5 Specimen collection

Rabbits were sacrificed by air embolism. Corneal specimen was isolated and cut into two fractions along the central line. One was frozen, and the other one was fixed in 4% paraformaldehyde. Bulbar conjunctiva was isolated (the upper half of the eyeball) and cut by the middle. The nasal side was frozen and the temporal side was fixed in 4% paraformaldehyde. Lacrimal glands were separated (the main lacrimal glands) and divided into two pieces. One was frozen and the other one was fixed in 4% paraformaldehyde.

1.6 Indicator detection

1.6.1 Schirmer test I (ST I)

The head of the Schirmer test strip was folded at 5 mm from the end, and then placed in the bilateral conjunctival sac around the 1/3 between the intermediate and the outer of the rabbit lower eyelids. Five minutes later, the strips were taken out and observed. The wetted length of the test strip was recorded and accurate to 0.5 mm, measured three times repeatedly and averaged.

1.6.2 BUT measurement

The fluorescein test paper was wetted with saline. Gently touched the inside of the lower eyelid by the top of the test paper. Closed the rabbit eyes and gently massaged, so that the fluorescein was evenly distributed on the surface of the eyes. Both eyes were placed under the slit lamp in turn. The time of the first black spot appearing, which was calculated from the time of eyes opening, was recorded as BUT. Measured three times and averaged.

1.6.3 Corneal fluorescein staining score

The rabbits were fixed in the fixing boxes. Fluorescein test strip was wetted with 1 drop of saline. Gently touched the inside of the lower eyelid by the top of the test strip and stayed a while. Closed the rabbit eyes and gently massaged, so that the fluorescein was evenly distributed on the surface of the eyes. Both eyes were placed under the slit lamp in turn. Corneal staining was observed and recorded as the corneal fluorescein staining score. The score was calculated by dividing the cornea into four quadrants. No dyeing was designated 0 point; dyeing was divided into 3 grades of mild, moderate and severe, and then scored as 1 point, 2 points and 3 points, respectively. 1 point indicated that dyeing was less than 5 spots; 3 points indicated appearing massive staining or filaments; 2 points were in between. The lowest score was 0 point, and the highest score was 12 points.

1.6.4 Observation of conjunctiva goblet cells

The rabbits were fixed in the fixing boxes, 1% lidocaine local anesthetic was dropped in eyes, and the excess liquid on the ocular surface was wiped with cotton balls. Autoclaved cellulose acetate membrane filter paper was placed gently on the upper half of the bulbar conjunctiva. Pressed evenly and kept it stay on the ocular surface for 15 s. Gently peeled off the filter paper, and placed it in 4% paraformaldehyde and fixed it for 24 h. Performed periodic acid Schiff (PAS) staining and recorded the results.

1.6.5 Morphological examination of rabbit cornea, conjunctiva and lacrimal gland tissues

Rabbit cornea, conjunctiva and lacrimal gland tissues were paraffin-embedded and sliced. Morphological changes of these tissues in each group were observed under light microscope with hematoxylin eosin (HE) staining.

1.7 Statistical analysis

The SPSS 19.0 version statistical software was used for the statistical analysis of the experiment data. Normal distribution analyses were conducted for the measurement data. The data, meeting the normal distribution and homogeneity of variance, were presented as mean ± standard deviation ($\bar{x} \pm s$) and analyzed using one-way ANOVA analysis. The comparison between the two groups used least significant difference (LSD); non-normal distribution or heterogeneity of variance data were presented as median (min, max), and analyzed using non-parametric tests. Statistical significance level was $\alpha = 0.05$, and $P < 0.05$ indicated a statistically significant difference.

2 Results

2.1 Effects of EA on rabbit tear secretion and the stability of tear film

2.1.1 Baselines of tear secretion and tear film stability

Before modeling, tear secretion volume and BUT of rabbits in each group were statistically analyzed. The difference between groups was not statistically significant ($P > 0.05$), which indicated that the inter-group baseline data in rabbit tear secretion volume and BUT before modeling were comparable (Figure 1).

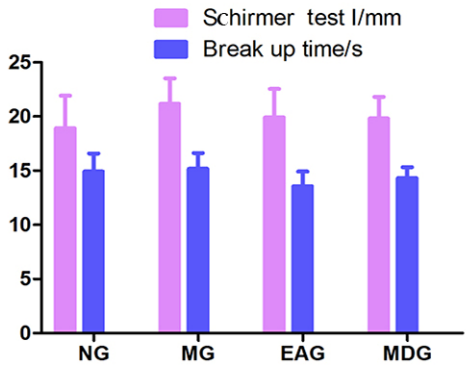


Figure 1. Comparison of tear secretion volume and BUT before modeling

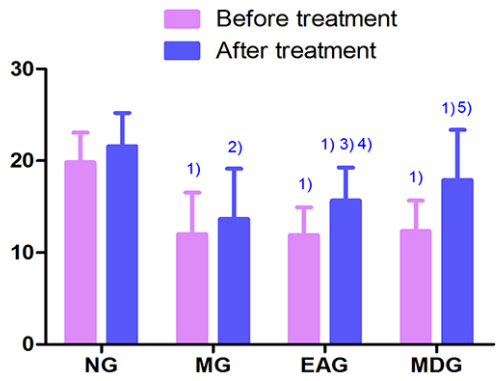


Figure 2. Comparison of the tear secretion volume (mm) [Note: Compared with the NG, 1) $P<0.05$, 2) $P<0.01$; compared with the MG, 3) $P<0.01$; intra-group comparison, 4) $P<0.05$, 5) $P<0.01$]

2.1.2 Effect of EA on tear secretion

As shown in Figure 2, compared with the NG, tear secretion volumes in the MG, EAG and MDG were significantly decreased before the treatment ($P<0.05$); the differences among the MG, EAG and MDG were not statistically significant (all $P>0.05$).

Intra-group comparison after treatment: The differences were not statistically significant in the NG and the MG (both $P>0.05$); the differences were statistically significant in the EAG ($P<0.05$) and the MDG ($P<0.01$).

Inter-group comparison after the treatment: Compared with the NG, tear secretion volume in the MG was significantly decreased ($P<0.01$). Compared with the MG, tear secretion volume in the EAG was significant increased, and the difference was statistically significant ($P<0.01$), which indicated that EA could improve tear secretion and increase tear secretion volume in experimental rabbit DES (Figure 2).

2.1.3 Effect of EA on the stability of tear film

Compared with the NG, BUTs in the MG, EAG and MDG were significantly shortened before the treatment (all $P<0.05$); the differences were not statistically significant among the MG, EAG and MDG ($P>0.05$), (Figure 3).

Intra-group comparison after the treatment: The differences were not statistically significant in the NG and MG ($P>0.05$). The differences were statistically significant in the EAG and MDG ($P<0.01$).

Inter-group comparison after the treatment: Compared with the NG, BUTs decreased significantly in the MG, EAG and MDG (all $P<0.01$); compared with the MG, BUTs were significantly longer in the EAG and MDG ($P<0.01$). The results indicated that both EA and polyethylene glycol drops could increase BUT and improve the tear film stability of experimental rabbit DES (Figure 3).

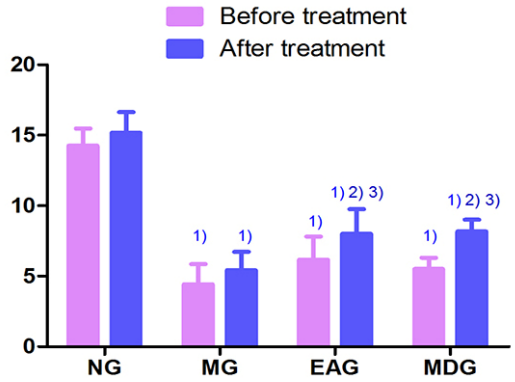


Figure 3. Comparison of BUT in each group (s) [Note: Compared with the NG, 1) $P<0.01$; compared with the MG, 2) $P<0.01$; intra-group comparison, 3) $P<0.01$]

2.2 Effects of EA on corneal fluorescein staining score

As shown in Figure 4, before treatment, compared with the NG, corneal fluorescein staining scores in the MG, EAG and MDG were significantly increased (all $P<0.01$); while the MG, EAG and MDG showed no significant differences (all $P>0.05$).

Intra-group comparison after the treatment: The NG showed no significant difference ($P>0.05$); the difference was statistically significant in the MG ($P<0.05$); there were significant differences in the EAG and MDG (both $P<0.01$).

Inter-group comparison after the treatment: After the treatment, compared with the NG, corneal fluorescein staining scores in the MG, EAG and MDG were significantly increased (all $P<0.01$); compared with the MG, corneal fluorescein staining scores in the EAG and MDG were significantly decreased (both $P<0.01$), which indicated that EA and polyethylene glycol drops both could improve rabbit corneal injury in experimental DES (Figure 4).

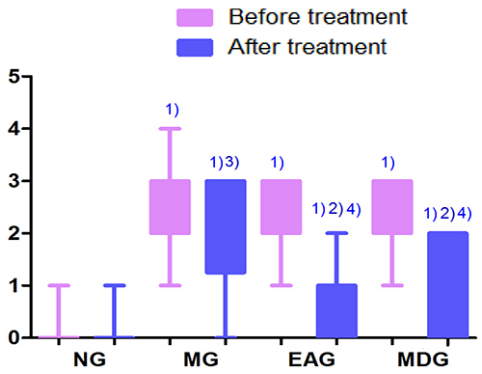


Figure 4. Comparison of corneal fluorescein staining score (point) [Note: Compared with the NG at the same period, 1) $P<0.01$; compared with the MG, 2) $P<0.01$; intra-group comparison, 3) $P<0.05$, 4) $P<0.01$]

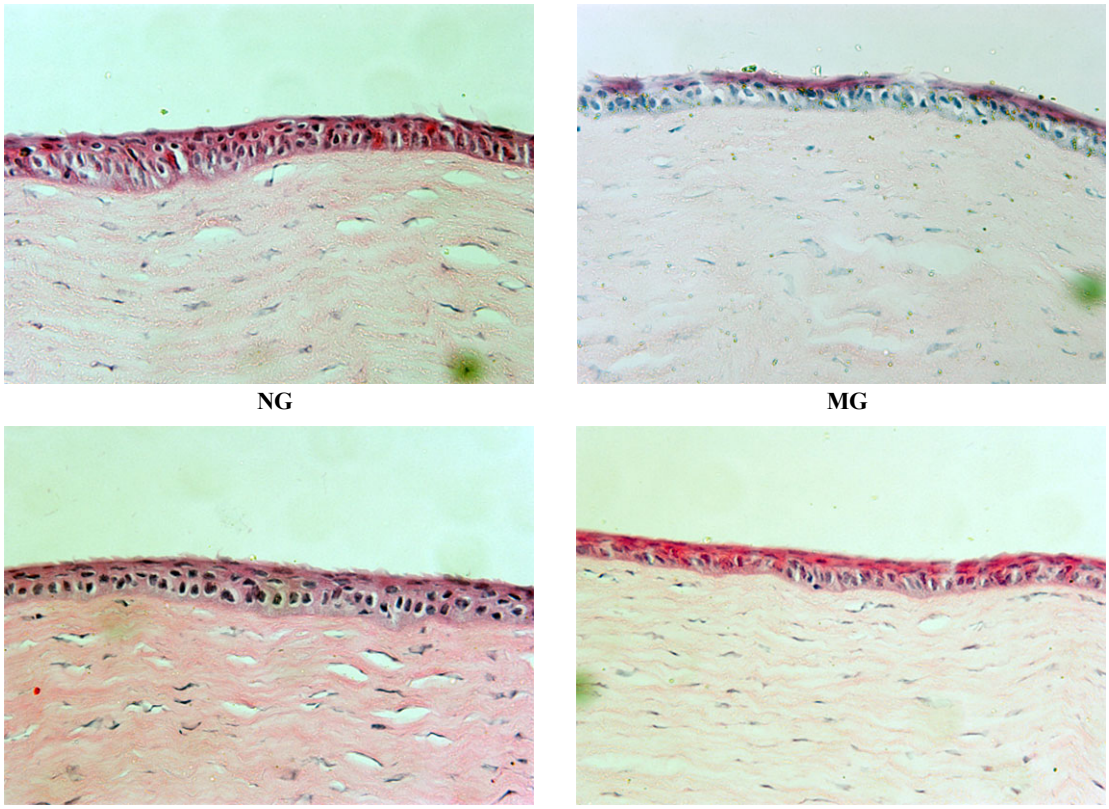
2.3 Effect of EA on the bulbar conjunctival goblet cells

Bulbar conjunctival goblet cells were observed using imprint cytology and PAS special staining. The results showed that the goblet cells were rich with well-distributed and satiated morphology, at rabbit bulbar conjunctiva, in the NG. Cells showed orange after PAS staining, indicating the rich mucus; and the number of conjunctival epithelial cells was significantly more than that in the MG. Compared with the NG, conjunctival goblet cell numbers were relatively less in MG, EAG and MDG after the treatment, however, the numbers were relatively higher in the EAG and MDG than that in the MG.

2.4 Effects of EA on rabbit cornea, conjunctiva and lacrimal gland morphology

2.4.1 Observation of rabbit's corneal histomorphology in each group

Cornea was observed under microscope after HE staining. In the NG, rabbit cornea was complete in the structure of each layer and clearly distributed. The epithelial lamina was constituted with 5-6 layers of cells, mainly including flat cells, wing-like cells and basal cells, with a normal number and distribution. Compared with the NG, corneal epithelium in the MG was significantly thinner with obvious edema. Compared with the MG, corneal tissue lesions were improved in EAG and MDG, evidenced by the increased cells of the epithelial lamina, however, the cell numbers were still less than that in the NG. Distribution of each layer was clear, and basal cell edema was alleviated (Figure 5).



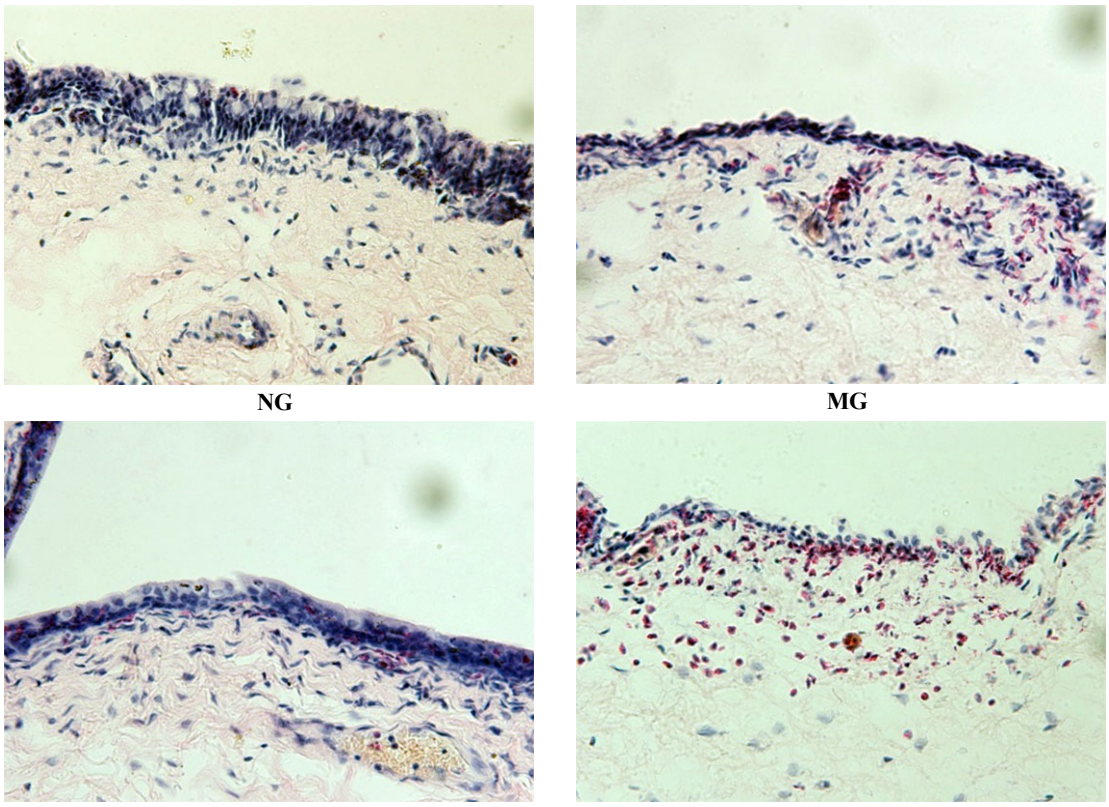
NG MG

EAG MDG

Figure 5. HE staining of rabbit cornea tissue in each intervention group (HE, ×400)

2.4.2 Morphological observation of rabbit conjunctival tissue in each group

In the NG, rabbit bulbar conjunctival surface was stratified columnar epithelium with cell surface microvillus, columnar nucleus and goblet cells distribution, observed under light microscopy, after HE staining of the rabbit conjunctival tissues, and the goblet cells were rich in mucus. Compared with the NG, columnar epithelium lamina of rabbit bulbar conjunctival surface was thinner, with eosinophil infiltration, in the MG. After the treatment with EA or polyethylene glycol eye drops, the pathological changes of rabbit conjunctival tissues were improved, evidenced by mild thinning of the surface epithelium with infiltration of subcutaneous inflammatory cells. The therapeutic effect of EA was better than medication based on improving inflammatory cell infiltration in conjunctival tissues (Figure 6).



NG MG

EAG MDG

Figure 6. HE staining of rabbit conjunctiva in each intervention group (HE, ×400)

2.4.3 Morphological observation of rabbit lacrimal gland tissue in each group

After HE staining of lacrimal glands, microscopic observation in the NG showed that rabbit lacrimal gland tissue surface was coated with a thin layer of connective tissue; the substance of the lacrimal glands contained different sizes of lacrimal gland lobules; tissues among the lobules were mainly dense connective tissues containing numerous fibroblasts, scattered lymphocytes, and blood vessels; the acinar sizes were uniform; a certain amount of mucus secretion could be seen in acinar cavities; the acinar epithelial cells were satiated; mucus was rich inside the cells; the nuclei were regular and distributed on the base. Compared with the NG, we could see increased gaps of rabbit lacrimal gland lobules; connective tissue with scattered lymphocytes, macrophages, plasma cells and neutrophils; vasodilation and edema; atrophy of the lacrimal gland epithelial cells; obvious decrease of mucus in cavities, in the MG. Compared with the MG, pathological changes of rabbit lacrimal glands were improved, evidenced by mild edema of the lacrimal gland tissues with the infiltration of a small amount of lymphocytes and plasma cells; intact morphologies of lacrimal gland epithelial cells; tight connections among cells; regular acinar structure and more intracavitary mucus, in the EAG and MDG (Figure 7).

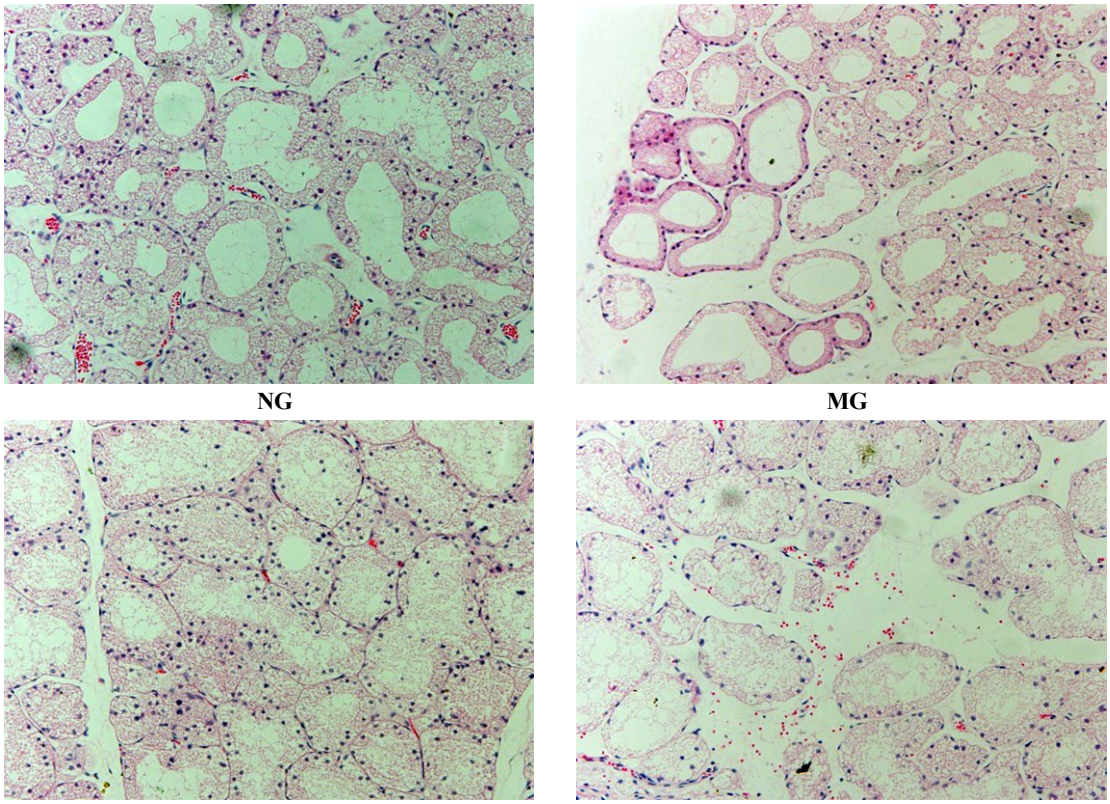


Figure 7. HE staining of rabbit lacrimal gland tissues in each group (HE, ×200)

3 Discussion

DES is the damage of tear film stability and ocular surface function, caused by various factors, belonging to the category of *Bai Se Zheng* in traditional Chinese medicine. Inflammation is one of the most critical factors in the pathogenesis of DES in current opinions[14]. A variety of immune cells and inflammatory factors are involved in the occurrence and development of DES. Apoptosis, abnormal neural regulation and gonadal hormone imbalance also participate in the pathogenesis of DES[15]. Therapeutic mechanisms of acupuncture in treating DES have not been fully carried out and reported in the literatures. By reviewing the existing research results, studies were mainly focused on improving lacrimal gland forms and functions, regulating apoptosis and sex hormones[16], and have acquired preliminary data. However, further researches and explorations are required.

Our pre-clinical studies confirmed that acupuncture is effective in the treatment of DES[17-18]. Clinical acupuncture prescription contains the major points of Cuanzhu (BL 2), Taiyang (EX-HN 5), Sizhukong (TE 23) and Fengchi (GB 20), which was the effective acupoint prescription, based on our long-term clinical practice in the treatment of DES, functioning to unblock qi and blood flow in eyes to relieve symptoms of dry eye and pain in DES patients. Therefore, Cuanzhu (BL 2), Taiyang (EX-HN 5), Sizhukong (TE 23) and Fengchi (GB 20) were selected as the acupuncture prescription in this study, followed by connecting the points of Cuanzhu (BL 2) and Taiyang (EX-HN 5) to EA device by the stimulation parameters with the frequency of 2 Hz, current strength of 2 mA and continuous wave for the intervention of rabbit experimental DES. This study showed that EA could significantly improve the tear secretion volume and BUT, which indicated that EA had certain improvement effect on tear secretion and tear film stability in experimental rabbit DES.

Corneal fluorescein staining is a method to stain the living cells of ocular surface. Positive staining indicated the integrity of corneal epithelial cells was destroyed[3]. Rabbit corneal fluorescein staining scores were determined in this study. The results suggested that EA could repair corneal epithelial damage in rabbit experimental DES. However, it had not yet reached the normal status.

Imprint cytological examination of conjunctiva is commonly used for clinical detection of DES, as it can discover the signs of ocular surface damage in patients with DES. It can be used to examine the changes and squamous metaplasia of conjunctival epithelium goblet cells in ocular surface lesions, and analyze the pathological changes of the entire ocular surface epithelial cells[19]. Imprint cytological examination of conjunctiva could provide a reliable basis for the

diagnosis and treatment of DES[20]. In this study, imprint cytological examination together with PAS special staining were used to observe the changes of goblet cells in rabbit bulbar conjunctiva, before and after the treatment. The results suggested that EA could partially repair rabbit conjunctival goblet cells in experimental DES. Morphological observations of rabbit cornea, conjunctiva and lacrimal glands suggested that EA and polyethylene glycol eye drops could partially repair and improve the pathological lesions of rabbit cornea, conjunctiva and lacrimal glands in DES. This further confirmed the intervention effects of EA on DES based on the morphological findings. However, the specific mechanism needs further investigation by more basic researches. Immune-associated inflammation is considered to be the most important pathogenesis of DES[14]. Researches on anti- inflammation will help to further reveal the mechanisms of acupuncture in improving ocular surface and lacrimal gland damages of DES.

Conflict of Interest

The authors declared that 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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