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Original Article

Intervention of electro-acupuncture and acupuncture on expression of related genes on lacrimal gland cholinergic signaling pathway in treating dry eye rabbits

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Abstract: Purpose: To determine expression of related genes on lacrimal gland cholinergic signaling pathway at day 17 of electro-acupuncture and acupuncture interventions in dry eye rabbit models. Methods: 18 healthy 3-4 month old New Zealand white rabbits were randomly divided into control group (CON group), model group (MOD group) and electro-acupuncture and acupuncture treated group (EA group). Observing times of tear flows, tear break-up time and corneal staining scores in 3 groups. Assaying the contents of acetylcholine (Ach) and detecting the effects to type 3 muscarinc acety-lcholine receptor (M3AChR), p44/p42 mitogen-activated protein kinase (MAPK) and 1,4,5-inositol trisphosphate receptor (IP3R). Results: Compared with control group, MOD and EA groups were significantly lower in tear flows and tear film break-time; and were significantly higher on Day 4. From Day 7 to Day 17, tears flows and tear film break-time of EA group were significantly higher than MOD group. About ach contents, MOD group was significantly lower than control group; EA group was significantly higher than MOD group in day 17. The acinar epithelial cells of the lacrimal gland were columnar, with small round cell nuclei in the basal part and abundant vesicular mucus in the glandular lumens in CON group. In day 17, M3AchR gene expression of EA was significantly lower than MOD group; Comparing with MOD group, MAPK gene expression of EA was significantly decreased. Conclusions: Repeated EA and acupuncture interventions have a time-dependent cumulative positive effect in dry eye rabbits, which is closely associated with its regulatory effects on M3AchR and MAPK levels.

Keywords: Electro-acupuncture, acupuncture, dry eye, M3AchR, MAPK, IP3

Introduction

Dry eye is a common ocular surface disease, which can cause instability of the tear film and ocular surface damage. Its pathogenesis is complex and is mainly related to inflammation, cell apoptosis, abnormal neural regulation, the imbalance of sex hormones and other factors of related disorders at present [1]. In recent years, many studies had reported neural regulation played an integral role regulating lacrimal gland protein, electrolyte, and water secretion and hence tear volume and composition. The following two signal transduction pathways, MAPK signal transduction pathway and IP3 signal transduction pathway, had gradually attracted people's attention [2]. Those reports were rare about the specific intervention effect of electro-acupuncture and acupuncture. Acupuncture had a significant neural regulation and protection effects [3-5], and could relieve dry eye symptoms [6]. Then, the mechanism of electro-acupuncture and acupuncture in the treatment of dry eye was not very clear. This study was through the observations of electro-acupuncture and acupuncture on dry eye model of rabbit lacrimal gland tissue M3AChR and the key gene expression effect during its mediated signal transduction to further explore the possible mechanism of electro-acupuncture for the treatment of dry eye.

Materials and methods

Animals and grouping

A total of 18 3-4-month-old healthy New Zealand white rabbits (from the animal reproduction base of Qinglong hill, Nanjing, SCXK-

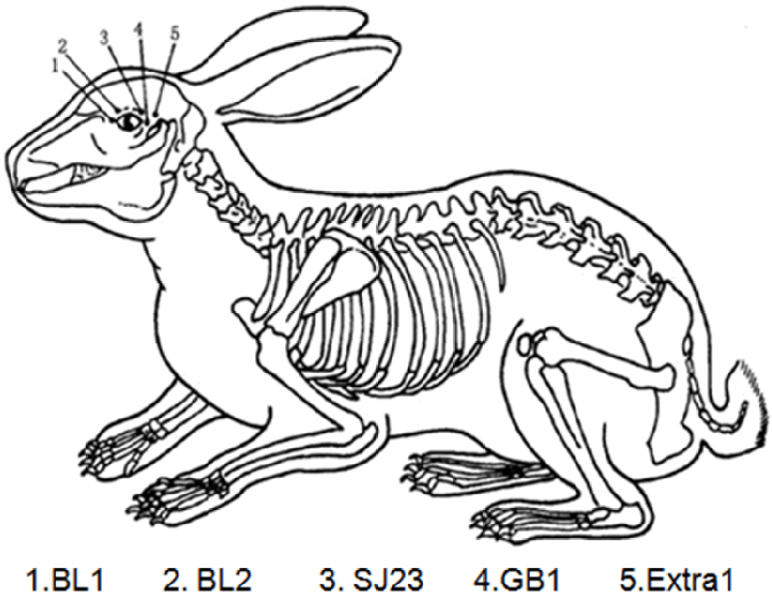

Figure 1. Experimental acupuncture points image.

su2012-0008), received regular feeding and body weight were 1.5~2.0 Kg. Anterior segment slit lamp microscope examination showed no abnormality. Schirmer I test ≥ 10 mm/5 min. Animals were randomly assigned (n = 6 in each group) to control (CON), dry eye model (MOD) and model plus electro-acupuncture and acupuncture treatment (EA) groups. All experimental procedures were approved by the Institute of Animal Center of Jiangsu provincial hospital of TCM, and performed according to the "Guidelines for Laboratory Animal Care and Use" of the Chinese Ministry of Science and Technology.

CON group: no treatment

MOD group: 1% atropine sulfate eye drops four times a day (8:00, 12:00, 16:00, 20:00) for a total of 17 days until the end of the experiment. Dropping three days later, Schirmer I test < 5 mm/5 min, the experimental dry eye rabbit model was made.

Electro-acupuncture and acupuncture group: The fourth days of model replication, rabbits were given electro-acupuncture and acupuncture treatment. And 1% atropine sulfate eye drops continued until the end of the trial. Acupoints: ching ming (BL1), cuan zu (BL2), sizukong temple (TE23), temporal (Extra-1), tongziliao (GB1), reference to "experimental acupuncture of laboratory animals" [7]. Ching Ming hole needlepoint inward inclined to pierce the skin below 3 mm, cuan zu flat spines down 3 mm, tongziliao stab 3 mm, temporal stab 3 mm. The acupoints were no needle retaining needle for 15 min. The rerearch chose WQ1002 asaps cupping therapy apparatus, using the density wave, the frequency of 2 Hz/15 Hz, pulse width 0.5 MS, intensity of 1 mA, retaining needle for 15 min. EA group was treated with 17-day continuous 1% atropine sulfate eye drops four times a day and 14 consecutive days of electro-acupuncture and acupuncture treatments one time a day. Specific points are shown in **Figure 1**.

Detection methods

Schirmer I test: Rabbits were kept immobile by intraperitoneal injection of 3 mg pentonbarbital. The lower eyelid was pulled down slightly, then tear detection filter strips (Tianjin Jing Ming Pharmaceutical Co., Ltd.) were placed on the palpebral conjunctiva at a specified point approximately 1/3 of the distance from lateral canthus of the lower eyelid. Each eye in three groups was individually tested with the eyes open for 5 min. The length of moist folds is measured in centimetres. Each eye was tested 3 times, and the average length of moist folds was considered as the final length. After the test, eyes were turned closed to avoid excessive exposure and irritation of the ocular surface.

Tear film break-up time

One microliter of 0.1% liquid sodium fluorescein was dropped into the conjunctival sac. After 3 blinks, BUTs were recorded in seconds.

Corneal staining

One microliter of 0.1% liquid sodium fluorescein was dropped into the conjunctival sac. Ninety

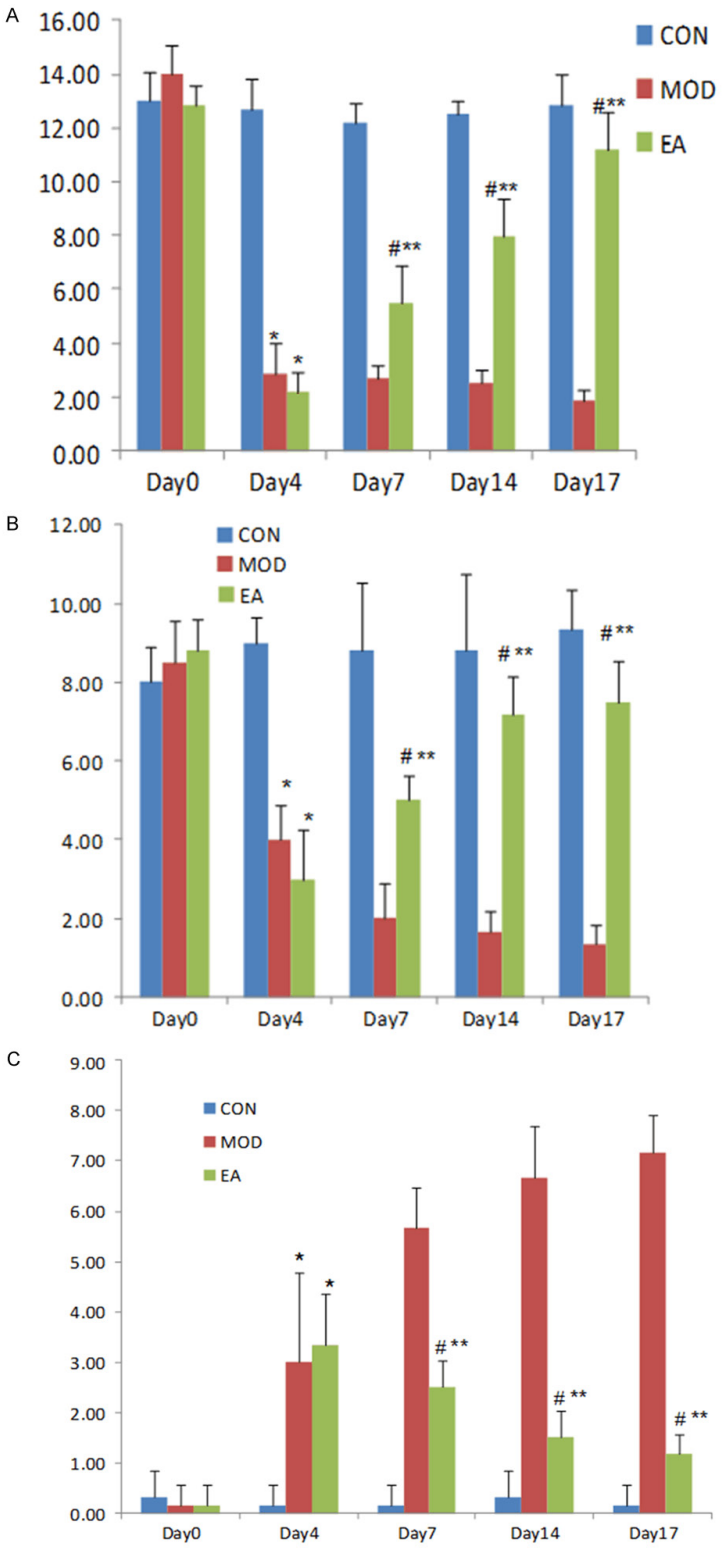

Figure 2. A. Tear flows of the rabbits in each group at different time-points. (*P < 0.001 vs. day 0, #P < 0.001 vs. Day 4 **P < 0.001 vs. MOD group). B. Tear film break-time of the rabbits in each group at different time-points. (*P < 0.001 vs. day 0, #P < 0.001 vs. day 4, **P < 0.001 vs. MOD group). C. Corneal staining scores of the rabbits in each group at different time-points. (*P < 0.001 vs. day 0, #P < 0.001 vs. day 4, **P < 0.001 vs. MOD group).

seconds later, corneal epithelial damage was graded with a cobalt blue filter under a slit-lamp microscope (Kanghua Science & Technology Co., Ltd, Chongqing, China). The cornea over the cornea center to do two vertical and horizontal lines was divided into 4 equal portions, which were scored respectively. The 4 scores were added to arrive at a final grade (total: 16 points). The fluorescein score was analyzed as previously described [8] with essential modification, briefly, as follows; each decile 0-3 degree, 0, no staining, 1; slightly punctate staining less than 5 spots; two dyed into five or more, divided into three massive stained, and finally scored each decile of the sum out of 12 points.

Sampling

The normal group, model group, and EA group were given Schirmer I test; tear film break-up time, corneal staining scores on days 0, 4, 7, 10, 17, at a similar time of the day (7AM) in the standard environment. Rabbits were killed humanely at 17 days after dry eye rabbit models were made, along with controls. Following sacrifice, whole blood and sera were collected and stored at -80°C. Each lacrimal gland (right and left) was divided into three pieces: one fixed in 10% formalin solution for histological analysis, one for ELISA analysis, and one stored at -80°C. All inspections were conducted by the same person in the same environment in a double-blind way.

Enzyme-linked immunosorbent assay (ELISA) in lacrimal gland

On day 17, lacrimal gland tissues were measured by enzyme-linked immunosorbent assay ACh content immediately after removing the right side of the sacrificed rabbit. Homogenizati-

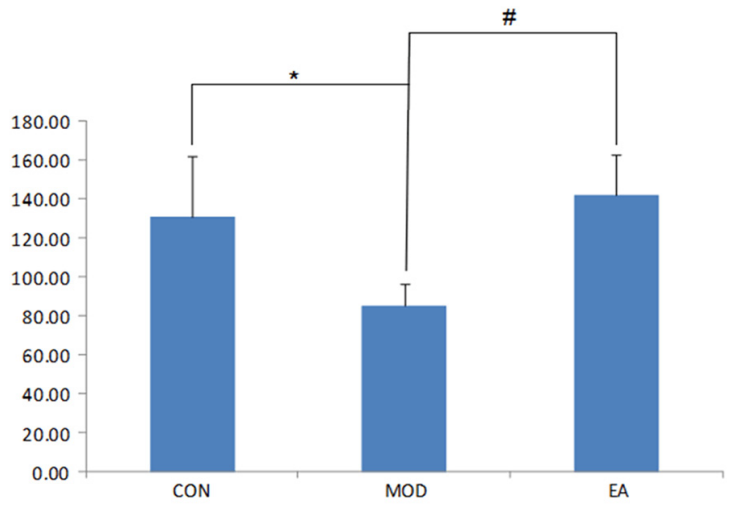

Figure 3. The contents of Ach in each group on day 17. (*P < 0.05 MOD vs. CON, #P < 0.05 EA vs. MOD).

on was carried out on ice using a tissue homogenizer and incubated for 1 min at 4°C with shaking. Homogenates were centrifuged and supernatants were collected. Protein concentrations were estimated by the procedure of Taylor et al. [9] with BSA as the standard. The Ach concentration was measured by a competitive enzyme-linked immunoassay (ELISA) using a rabbit polyclonal Ach antibody (Nanjing sai yan Technology Development Co. Ltd., Nanjing, China) according to the manufacturer's protocol. Samples (or standard) and conjugate were added to each well, and the plate was incubated for 1 h at room temperature without blocking. After wells were washed several times with buffers and proper color developed, the optical density was measured at 450 nm using an ELISA reader (MutiRead 400; Authos Co., Vienna, Austria).

Histologic analysis

Lacrimal gland tissues for light microscopy were fixed in 10% neutral buffered formalin and embedded in paraffin using routine procedures. Four-micrometer thick sections were cut from the tissue blocks and stained with hematoxylin-eosin (H&E). For H&E staining, the lacrimal gland histologic evaluation revealed morphological change in lacrimal gland after acupuncture and electro-acupuncture treatment.

Immunohistochemical analysis

The expression of anti-M3AchR, anti-MAPK, and anti-IP3 antigens (MaxVisionTM, Fuzhou Maixin Biotech. Co., Ltd) was examined in histological paraffin sections. For this, sections measuring 4 μm in thickness were collected on silanized slides and subsequently dewaxed in xylene and hydrated in decreasing concentrations of ethanol. Tissues were then placed in citrate buffer (pH 6.0) and processed for heat-induced epitope retrieval for 10 minutes at 95°C. Slides were washed in deionized water, followed by buffer. A primary antibody (1:50-100dilution, Fuzhou Maixin Biotech. Co., Ltd) was applied for 60 minutes followed by buffer washes (2 washes × 5 minutes each). A commercial secondary kit (MaxVisionTM, Fuzhou Maixin Biotech. Co., Ltd) was applied according to the manufacturer's recommendations (30 minutes) and followed by buffer washes (2 washes × 5 minutes each). Chromogen (DAB) was applied to tissues for 5 minutes followed by buffer rinses. Tissues were then counterstained with hematoxylin. As a negative control, sections incubated with non-specific immunoglobulin were used in place of the primary antibody. Tissues were examined by a pathologist. For immunohistochemical evaluation of M3AchR, MAPK, and IP3, we used semi-quantitative scoring analysis (H) as described by A. Scharl D [10]. 0 for negative reactivity and 1 to 3 according to the degree of intensity of reactivity to M3AchR, MAPK, and IP3. All indicators are membrane and (or) light yellow to brown appearance of fine particles in the cytoplasm, was higher than the background color colored positive. Classification criteria: according to the depth of the lacrimal gland tissue staining and the percentage of positive cells one by one score for each slice, the mean was chosen for the staining intensity. Staining intensity scoring criteria: percentage of positive cells < 10% is negative (-), 10%-25% as a pale brown weakly positive (+), 25%-75%, dark brown positive (++), > 75% tan strong positive (+++). For statistical convenience, semi-quantitative scoring was given as 0, 1, 2, 3, respectively -, +, ++, +++. P < 0.05 was considered statistically significant.

Real-time quantitative RT-PCR of mRNA

Total RNA was extracted from the lacrimal gland samples using an RNAprep Pure Tissue

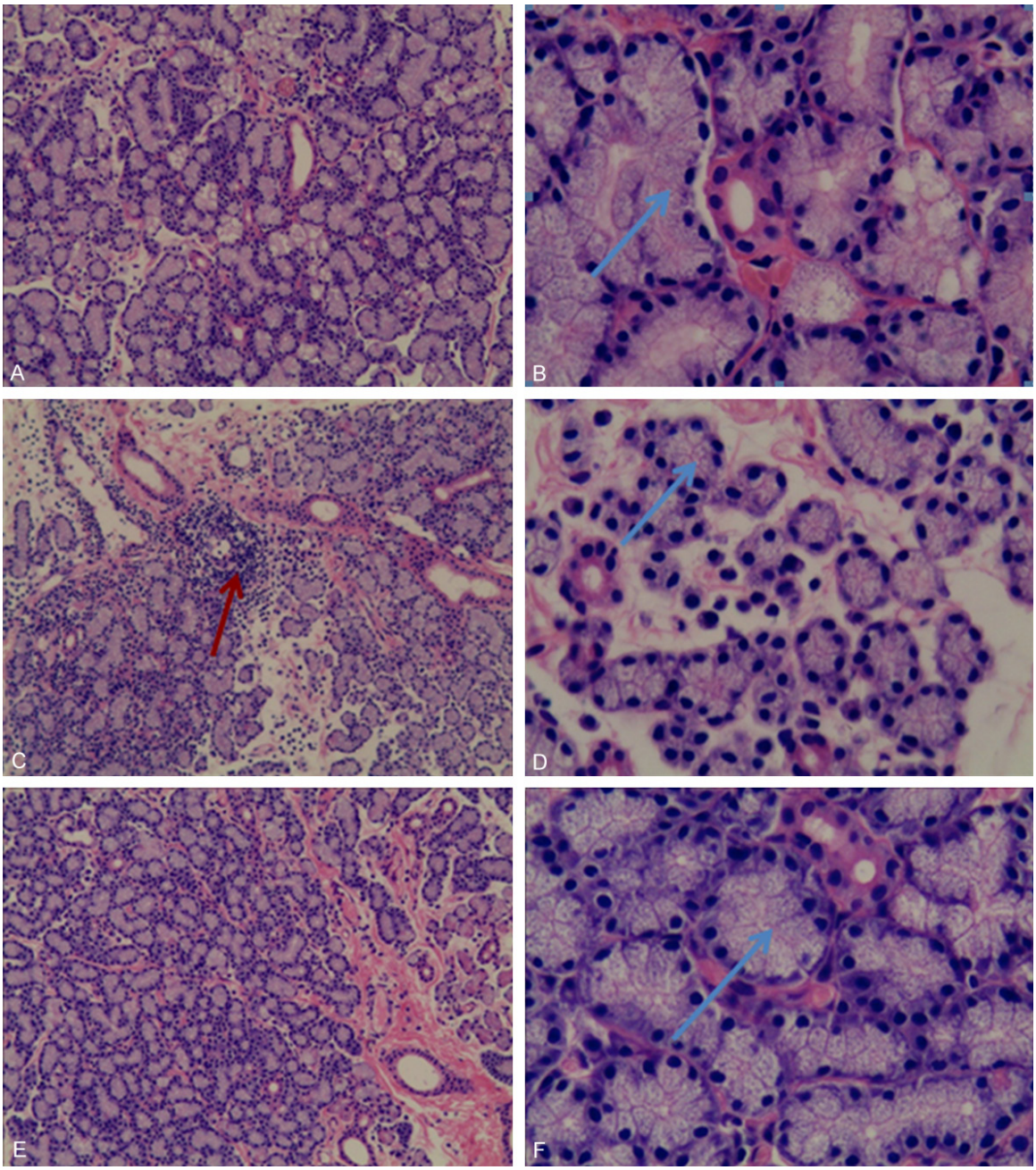

Figure 4. HE staining results of the lacrimal gland acinar epithelial cells. In the pictures, the red arrow shows lymphocyte cell, while the blue arrow shows acinar cell. A. CON group × 100; B. CON group × 400; C. MOD group × 100; D. MOD group × 400; E. EA group × 100; F. EA group × 400.

kit (Tiangen Biotech Co. Ltd., Beijing, China). RNA quality and concentration were assessed and confirmed using the Eppendorf BioSpectrometer (Eppendorf, Germany). Extracted RNA was converted to cDNA through reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen). RT-PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen). For the amplification of the desired cDNA, the following gene-specific primers were used: GAPDH (housekeeping gene), M3-AchR, MAPK, and IP3. Primers were synthesized by Shanghai Sangon Biological Engineering (Shanghai, China). GAPDH as an internal refer-

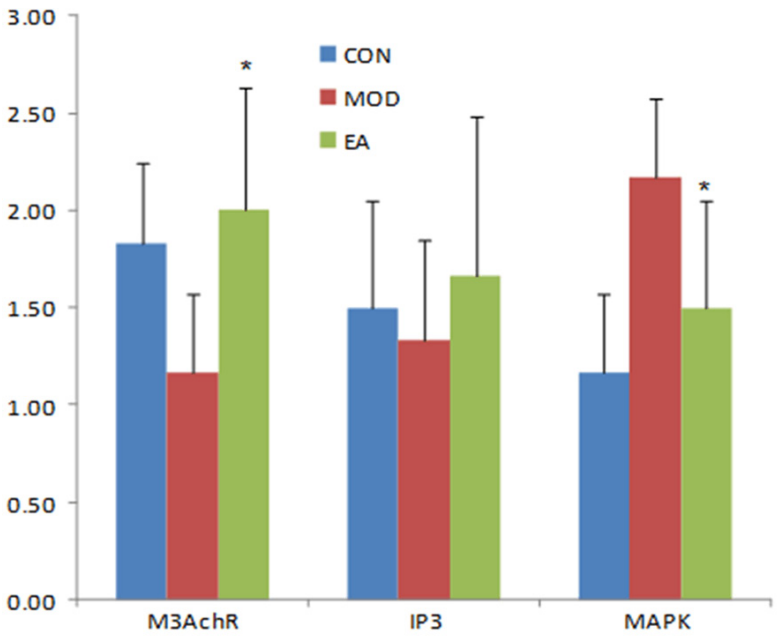

Figure 5. The number of positive cells of M3AchR, IP3 and MAPK in each group on day 17. (*P < 0.05 EA vs. MOD).

ence, the upstream primer 5 'CAC GGT CAA GGC TGA GAA CG 3', downstream primer 5 'GTA CTC GGC ACC AGC ATC AC 3'. CHRM3 upstream primer 5 'TTG ACA GGT ACT TTT CCA TC 3', downstream primer: 5 'CAA GCT AGA CCA ATC ATC AC 3'; IP3R upstream primer: 5 'TAG CTG ACC GAA AGC AGA AT 3', downstream primer: 5 'GCA AGC TCT TTG GGC TTC TC 3'; MAPK upstream primer: 5 'CCT CCA ACA TCC TGG TCA AC 3', downstream primer: 5' CCT GGT GCC CAC GAA GGA GT 3'. RT-PCR was conducted by bringing the temperature up to 95°C for 15 minutes, then 40 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. The reactions for mRNA were automated by a 7900HT Real-Time PCR. For all RT-PCR experiments, sterile water was used as a negative control, and the murine housekeeping gene, GAPDH, was used as a positive control. All quantitative PCR reactions, including no-template controls, were performed in triplicate. All calculations and analyses were performed using SDS RQ Manager 1.1 software using the 2-ΔΔCt method [11].

Statistical analysis

Data from all groups were expressed as mean ± standard error of the mean. Statistical analyses were performed using SPSS 17.0. Repeated measures analysis of variance was performed for atropine sulfate eye drops, the tear volume and BUTs, while one-way ANOVA analysis with a post hoc test was performed for the contents of Ach and the number of positive cells of M3AchR, MAPK and IP3. A Kruskal-Wallis rank test was performed for ranked data. *P* values < 0.05 were considered significant.

Results

Ocular surface changes

In day 0, there were no significantly differences among three groups (CON, MOD and AE groups) in schirmer test, tear film break-time and corneal staining score. However, in day 4, MOD and AE groups were significantly lower in schirmer test and tear film break-time and higher in corneal staining score compared with day0. Comparing with day 4, AE group was significantly increased in schirmer test and tear film break-time and decreased in corneal staining score. Moreover, in day 7, 10 and 17, AE group was also significantly improved schirmer test and tear film break-time, and significantly attenuated corneal staining score compared with MOD group (data was shown in **Figure 2A-C**).

The contents of ach

About ach contents, MOD group was significantly lower than control group; EA group was significantly higher than MOD group in day 17 (*P* < 0.05, respectively) (**Figure 3**).

HE staining results

The acinar epithelial cells of the lacrimal gland were columnar, with small round cell nuclei in the basal part and abundant vesicular mucus in the glandular lumens in CON group (**Figure 4A**, **4B**). In MOD group, we found the acinar was atrophied, lacking enough nuclei. We also found some inflammatory cells located in focal

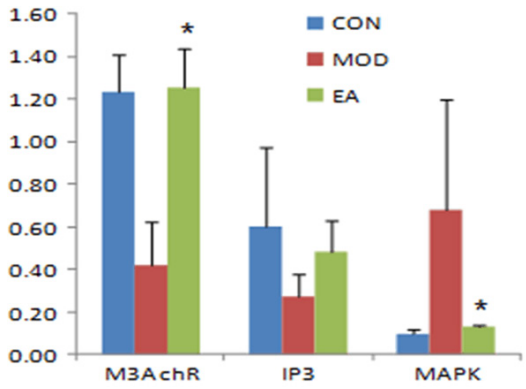

Figure 6. M3AchR, IP3 and MAPK in each group on day 17 were assessed by Real-time PCR. 2-ΔΔCt was as the relative content. (*P < 0.05 EA vs. MOD).

areas (**Figure 4C**, **4D**). In EA group, columnar epithelial cells expanded with abundant nuclei without inflammatory cells (**Figure 4E**, **4F**).

The number of positive cells of M3AchR, MAPK and IP3

Immunohistochemiscal assay results showed that the number of positive cells in M3AchR was significantly increased in the electro-acupuncture and acupuncture rabbits on day 17 (P < 0.05 EA vs. MOD). The number of positive cells in MAPK was significantly decreased in the electro-acupuncture and acupuncture rabbits on day 17 (P < 0.05 EA vs.MOD). But the number of positive cells in IP3 had no obvious change in the electro-acupuncture and acupuncture rabbits on day 17 (**Figure 5**). Classification criteria: 1) positive cells < 25% negative (-); 2) positive cells in 25%-50% for weakly positive (+); 3) positive cells in 51%-75% positive (++); 4) positive cells > 75% as strongly positive (+++). Its statistics, negative, weakly positive, positive, strong positive respectively denoted as 0, 1, 2, 3.

Gene expression of M3AchR, MAPK and IP3

In day 17, M3AchR gene expression of EA was significantly lower than MOD group; Comparing with MOD group, MAPK gene expression of EA was significantly decreased (**Figure 6**).

Discussion

Acupuncture is a traditional Chinese medicine and as an important part of treatment, it has been used to treat different types of diseases. In many countries it is now being accepted as a complementary and alternative therapy. Animal and human experiments indicated that acupuncture could be related to the release of opioids for pain relief, immune regulation, the release of neurotransmitters and hormones, as well as the dilation of blood vessels [11]. Many articles internationally had reported acupuncture had an undoubted efficacy in the treatment of dry eye [12-16]. EA is a new therapy, referring to the method of the pulse current applied to acupuncture needles and thus uniform stimulation of acupuncture points, in order to ensure quantitative and qualitative unity [17]. EA at some acupoints can stimulate the cholinergic nerves [18, 19]. The experiments selected ching ming (BL1), cuan zu (BL2), sizukong temple (TE23), temporal (Extra-1), and tongziliao (GB1). Among them, ching ming (BL1), an acupoint of meridian of foot tai yang and the convergence of five meridians, can clear stagnated heat and is a key acupuncture point of treating ocular diseases. Cuan zu (BL2) an acupoint of the bladder meridian of foot tai yang, adjusts the qi and blood round the eye, and nourishing yin and clear away heat. Temporal (Extra-1) is an extraordinary point for attending eye diseases; tongziliao (GB1) is the first orifice of head and facial of gallbladder meridian of foot shao yang. It also can dispel wind, purge heat and improve eyesight. Sizukong temple (TE23) is an acupoint of the meridian of hand shao yang, attending diseases-congestion, swelling and pain of the eye. An orbital zygomatic branch of facial nerve and auriculotemporal nerve branches are around sizukong temple (TE23), where the cloth zygomatic branch of the facial nerve and ear temporal nerve branches. The lacrimal gland secretion fibers are from the parasympathetic nerve of the facial nerve and sympathetic fibers of intracranial arterial plexus. So in the study, selecting these points stimulated the parasympathetic to release neurotransmitters, in order to promote lacrimal gland secretion. From the meridian and collateral circulation paths, the twelve meridians are related to the eye directly or indirectly, and surround the eye. So the theoretical base of acupuncture for the treatment of dry eye is to make better flow ‘qi’ and blood in the meridians around the eye, to balance Yin and Yang so as to open the lacrimal orifice [20].

Dry eye has long been considered a complicated condition and its incidence rate has

increased yearly. Abnormal neural regulation is one common cause, which can reduce tear secretion, eventually forming aqueous-deficiency dry eye. In this study, Comparing with Day 0, CON and EA groups were significantly decreased in tears flow and tear film break-up time, and significantly increased in corneal staining score ($P < 0.05$, respectively). Those results showed that the dry eye model was success. EA group was given electro-acupuncture and acupuncture treatment. From Day 7 to Day 17, Compared with MOD group, EA group was significantly higher in tears flow and tear film break-up time ($P < 0.05$, respectively), and was significantly lower in corneal staining score ($P < 0.05$, respectively). This result showed the electro-acupuncture and acupuncture treatment had effects to improve dry eye. However, the pathway was not clear.

The lacrimal gland is controlled by innervation, and the nerve terminal mainly contains neurotransmitter acetylcholine [21]. The cholinergic receptors on lacrimal gland cells are the muscarinic M3 (glandular) subtype (M3AchR) [22]. M3AchR for neurotransmitter Ach and lacrimal M3 cholinergic receptors are formed as the starting protein secretion pathway. The M3AchR is coupled to the Gq/11a subtype of G protein that is then connected to activate the enzyme phospholipase Cb, which breaks down the membrane phospholipids phosphatidylinositol bisphosphate into 1, 4, 5-inositol trisphosphate (InsP3) and diacylglycerol (DAG) [23]. InsP3 is water-soluble and binds to its receptor on the endoplasmic reticulum. There are 3 distinct subtypes of InsP3 receptors: types 1-3 that are expressed to varying degrees on in different cell subtypes. InsP3 interaction with its receptor causes a rapid, immediate release of intracellular Ca^{2+} represented by a spike in Ca^{2+} response that quickly peaks [24]. Experiments confirmed IP3 may play a role in the connection gap that can be reduced to some extent in apoptosis [25]. Surprisingly, cholinergic agonists not only activate pathways that stimulate secretion, they also activate those that attenuate secretion. Cholinergic agonists activate p44-p42 mitogen-activated protein kinase (MAPK), also known as extracellular regulated kinase (ERK) 1/2, which decreases cholinergic agonist-activated protein secretion [26]. In this reach Day 17, Ach contents of EA group were significantly higher than MOD group ($P < 0.05$); In EA group, M3AchR Protein and mRNA expression were significantly increased and MAPK protein and mRNA expression were significantly decreased compared with MOD group ($P < 0.05$), However, the IP3 protein and mRNA expression were no significant between these two groups. Depend on these results; we inferred the Ach contents increase by inhibiting MAPK and stimulating M3AchR.

In this research, the rabbits were treated with 1% atropine sulfate eye drops to make dry eye models [4], the results showed that the model was success. From Day 7, the symptoms of EA group was significantly improved. The results of this study suggested electro-acupuncture and acupuncture treatment (EA group used treatment methods) improve dry eye by improving Ach contents, and Ach contents were increased by stimulating M3AchR and inhibiting MAPK.

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Disclosure of conflict of interest

None.

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