Effect of electroacupuncture on arginine vasopressin-induced endolymphatic hydrops

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Abstract

OBJECTIVE: To investigate the influence of electroacupuncture (EA) on experimentally induced endolymphatic hydrops (EH) in guinea pigs, and eluciate the association between the dehydrating effect of EA and changes in stria vascularis ultrastructure and expression of vasopressin type 2 receptor (V2R), cyclic adenosine monophosphate (cAMP), and aquaporin 2 (AQP2) in the endolymphatic sac (ES).

METHODS: The EH model was established by intraperitoneal injection of arginine vasopressin (AVP). As a treatment, EA was delivered to Baihui (GV 20) and Tinggong (SI 19) acupoints, once daily for 10 consecutive days. For histomorphological studies, degree of cochlear hydrops was evaluated by hematoxylin-eosin staining, and the ratio of scala media (SM) area to SM + scala vestibuli area was calculated. In mechanical studies, ultrastructural changes in stria vascularis tissue were examined by transmission electron microscopy. In addition, cAMP levels and mRNA expression levels of V2R and AQP2 in the ES were compared among groups.

RESULTS: EA treatment significantly reduced cochlear hydrops compared with hydropic guinea pigs (P = 0.015). Furthermore, EA attenuated ultrastructural changes in the stria vascularis tissue following EH, significantly upregulated the expression of V2R (P = 0.016), and attenuated AVP-induced up-regulation of both cAMP (P = 0.038) and AQP2 expression (P = 0.017) in the ES.

CONCLUSION: Collectively, the results of the present study suggest that the dehydrating effect of EA is associated with improvement of stria vascularis ultrastructure and V2R-cAMP-AQP2 signaling pathway regulation in the ES.

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Keywords: Meniere disease; Electroacupuncture; Endolymphatic hydrops; Arginine vasopressin; Receptors, vasopressin; Cyclic AMP; Aquaporin 2

INTRODUCTION

Meniere’s disease (MD) is a chronic inner ear disorder that mainly presents with intermittent episodes of vertigo, sensorineural hearing loss, and tinnitus. Currently, the mechanism of MD onset is not fully understood.
However, endolymphatic hydrops (EH) has been shown to be the histopathological basis of MD. One line of evidence has suggested that arginine vasopressin (AVP) may be involved in the regulation of inner ear fluid, and may participate in the generation of EH. Mori et al. reported that the stria vasculosa (SV) in the cochlea might be the main action site of AVP. Ultrastructural studies showed that acutely administered AVP caused vacuole formation in strial cells and an enlargement of intrastrial space. Progressive damage in SV tissue could increase water influx from the perilymph to the endolymph, ultimately resulting in the formation of EH following AVP application. Recently, the existence of aquaporins (AQPs) and their function in water homeostasis of the inner ear have become well elucidated. In the case of AQPs, it is reportedly expressed in the endolymphatic sac (ES), which is the main site regulating endolymph volume by means of absorption. Many lines of evidence suggest that AQPs expression and the inner ear water regulation system are partly regulated by AVP. As in the kidney collecting duct, AVP acts on vasopressin type 2 receptor (V2R) to cause a rise in AQPs abundance in the ES via cyclic adenosine monophosphate (cAMP)-mediated phosphorylation, thus greatly promoting fluid influx into the endolymph space. Dysregulation of the V2R-cAMP-AQP2 signaling pathway in ES contributes to excess water retention in the endolymph compartment, namely EH. The present study hypothesized that morphological changes in the SV and dysregulation of the V2R-cAMP-AQP2 signaling pathway in ES are closely associated with EH.

According to Traditional Chinese Medicine (TCM), MD belongs to the vertigo family. Acupuncture has been accepted as a primary strategy for treating the symptoms of MD in complementary and alternative medicine. Electroacupuncture (EA), the application of electrical stimulation to acupuncture needles, has proven more adjustable and repeatable than traditional acupuncture. Accumulating evidence from clinical studies demonstrates that EA is safe and effective for management of vertigo in patients with MD. Our previous work indicated that EA at Baihui (GV 20) and Tinggong (SI 19) can suppress the development of cochlear hydrops in aldosterone-induced EH model guinea pigs. However, the underlying mechanism of action for EA on MD remains undefined. In the current study, the influence of EA at Baihui (GV 20) and Tinggong (SI 19) acupoints on AVP-induced EH, as well as mechanisms underlying the dehydrating effect of EA, were investigated.

**MATERIALS AND METHODS**

**Ethics statement**

All procedures were conducted with the approval of the Animal Care Committee of Zhejiang Chinese Medical University and in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA). All efforts were made to minimize the number of animals employed and their suffering.

**Guinea pig model of EH**

Healthy adult male albino guinea pigs of clean grade (six-week-old, weighing 350-400 g and with a positive Preyer’s reflex) were supplied by Rabbit Farm in Dashiju Town, Xinchang county (Certificate of quality No. SCXK [zhe] 2015-0004) and fed in the Animal Center of Zhejiang Chinese Medical University (24 ± 1 °C with a 12-h light/dark cycle). All animals were supplied with standard food and water. Guinea pig model of EH was induced with [Arg] -VP (V9879, Sigma-Aldrich, St. Louis, MO) via intraperitoneal injection (4 µg·kg⁻¹·d⁻¹) for 7 d, as previously described.

**Experimental groups and treatments**

Guinea pigs were randomly divided into 3 groups by random number table method (n = 18 per group): (a) blank group, no treatment; (b) hydrops group, AVP injection and immobilization without other treatment; (c) hydrops + EA group, AVP injection and immobilization with EA stimulation.

**EA stimulation**

Following 7-d AVP injection, guinea pigs in the hydrops + EA group were administered EA treatment for 10 d. Disposable acupuncture needles (0.25-mm diameter, 25-mm length; Suzhou Medical Apparatus, Jiangsu, China) were inserted to Baihui (GV 20) (in the depression anterior to the tragus) about 10 mm - VP (V2R) to cause a rise in AQPs abundance in the ES via cyclic adenosine monophosphate (cAMP)-mediated phosphorylation, thus greatly promoting fluid influx into the endolymph space. Dysregulation of the V2R-cAMP-AQP2 signaling pathway in ES contributes to excess water retention in the endolymph compartment, namely EH. The present study hypothesized that morphological changes in the SV and dysregulation of the V2R-cAMP-AQP2 signaling pathway in ES are closely associated with EH.

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and imaged using a light microscope (Olympus BX60, Tokyo, Japan) and digital camera (Nikon Cool PIX990, Tokyo, Japan).

**Degree of EH**

The second turn in the mid-modiolar section of each cochlea was selected for assessment because of its good stability during section preparation. The cross-sectional area of the scala media (SM) and scala vestibuli (SV) for the second turn of each cochlea on both the left and right sides of the mid-modiolar axis were examined by a blinded observer using image analysis software (Image Pro Plus 6.0, Media Cybernetics, Rockville, MD), as previously reported. The ratio of SM cross-sectional area to SM + SV cross-sectional area (R value) for the second turn was calculated and the degree of EH was assessed according to the average R value from the second turn of cochlear modiolus from both sides.

**Transmission electron microscopy (TEM) of the stria vascularis**

Following treatment, left cochleae were obtained from guinea pigs (n = 5 per group) under intraperitoneal anesthesia with 10% chloral hydrate (300 mg/kg). The membranous cochlea was immersed in 2.5% glutaraldehyde (pH = 7.4) for 2 d at 4 °C. Specimens were washed in 0.01 M PBS for 30 min at 4 °C. All tissue samples were decalcified with 10% EDTA for 4 weeks, and further fixed with 1% osmic acid for 2.5 h. Subsequently, specimens were dehydrated using a graded series of ethanol, embedded in Spion 812 epoxy resin mixture (SPI Supplies, West Chester, PA, USA), and cut into 80-nm ultrathin sections. After staining with toluidine blue and uranyl acetate-lead citrate, sections were examined and photographed under a H7100 transmission electron microscope (Hitachi, Tokyo, Japan). The SV at the second turn in the mid-modiolar section of each cochlea was selected for evaluation.

**Immunohistochemical staining procedures**

Temporal bone specimens were prepared as described above for HE staining (n = 8 per group). Specimens containing the ES were sectioned at a thickness of 4 µm from the proximal end towards the distal part, stained with HE, and observed by light microscopy. Morphological identification was confirmed as previously reported. Expression of V2R, cAMP, and AQP2 in the ES was further examined by the diaminobenzidine tetrahydrochloride method. Immunohistochemical staining was performed on ES sections using routine procedures. Briefly, sections were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity and subsequently incubated with primary rabbit anti-rat polyclonal antibodies against V2R (1: 100; Lot No. 2601716; Millipore, Burlington, MA), cAMP (1: 75; Lot No. 2102; Proteintech, Chicago, IL) and AQP2 (1: 60; Lot No. Y-B1-07C17B; Boster, Wuhan, China) followed by incubation with a biotin-conjugated secondary antibody (goat anti-rabbit IgG, 1: 400) and a diaminobenzidine (DAB) substrate kit (Lot No. K142415E; ZSGB-Bio, Beijing, China). Sections were counterstained with Harris hematoxylin. Photographs were digitized at 200× original magnification with a digital camera (Nikon Cool PIX990) and analyzed using Image Pro plus 6.0 for semi-quantitative evaluation of the integrated optical density (IOD) of V2R/cAMP/AQP2 labeling in the ES. All sections were coded and measured under blinded conditions.

**Statistical analyses**

All quantitative data are expressed as mean ± standard deviation (x ± s) and analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni test using Statistical Package for the Social Sciences version 19.0 (SPSS, Chicago, IL, USA). Statistical significance was assumed when P < 0.05.

**RESULTS**

**Guinea pig inner ear**

Figure 1A-C shows typical light microscopy images of cochlea. EH was not evident in ears of the blank group. Reissner’s membrane was almost straight with 45°. Conversely, displacement of Reissner’s membrane with a bulge to the scala vestibuli was noted in ears of the hydrops group, which also exhibited distinct hydrops of the scala media. Cochlear hydrops was found in the hydrops + EA group, but the distension of Reissner’s membrane was less obvious compared with the hy-
Ultrastructural changes in the SV
TEM indicated that AVP exposure produced significant structural damage in the SV. Electron density in cytoplasm and organelles of SV cells were inhomogeneous in hydrops (Figure 2B) and blank (Figure 2A) groups, with relative higher density in the hydrops group. Morphological damage such as mitochondrial swelling and vacuolar degeneration, and fragmentation and disappearance of mitochondrial cristae were also observed in guinea pigs with EH. After EA intervention, ultramorphological changes in the SV were relatively milder (Figure 2C). The representative manifestation was a remarkably reduced degree of mitochondrial swelling and vacuolar degeneration compared with the hydrops group.

V2R protein expression in the ES
Figure 3A-C shows typical pictures of immunohistochemical staining for V2R in the ES epithelium. Nuclei of cells were visible in sections counterstained with hematoxylin. In the blank group, immunolabeling for V2R was evident in the ES in counterstained sections. In contrast, very little V2R immunoreactivity was found in the ES epithelium of the hydrops group. However, compared with the hydrops group, strong V2R immunolabeling was found in cells comprising the ES epithelium in the hydrops + EA group.

Table 1 presents IOD values of V2R-immunoreactive cells in the ES epithelium. One-way analysis of variance indicated statistically significant differences among the three groups ($F = 8.515, P = 0.002$). Immunohistochemical analysis demonstrated high basal expression of V2R in the blank group. AVP injection induced a decrease in IOD values of V2R-positive cells compared with the blank group ($P = 0.002$). However, EA stimulation significantly increased V2R expression in response to AVP compared with the hydrops group ($P = 0.016$).

cAMP expression in the ES
Figure 4A-C shows representative immunohistochemical staining for cAMP in the ES epithelium. Nuclei of cells were visible in sections counterstained with hematoxylin. In the blank group, very little cAMP immunostaining was evident in the ES in counterstained sections. In contrast, in the hydrops group, strong cAMP immunolabeling was found in the ES epithelium. Compared with the hydrops group, CAMP-specific antibody immunoreactivity in cells comprising the ES epithelium was relatively weak in the hydrops + EA group.

Table 1 presents IOD values of cAMP-immunoreactive cells in the ES epithelium. One-way ANOVA indicated statistically significant differences among the three groups ($F = 9.582, P = 0.001$). Immunohistochemical
Table 1 Comparison of V2R, cAMP and AQP2 IOD values in the ES of guinea pigs ( x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>V2R</th>
<th>cAMP</th>
<th>AQP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>8</td>
<td>5.1±2.3</td>
<td>2.5±1.3</td>
<td>4.0±2.2</td>
</tr>
<tr>
<td>hydrops</td>
<td>8</td>
<td>1.0±0.9*</td>
<td>5.3±1.3*</td>
<td>8.9±4.5*</td>
</tr>
<tr>
<td>hydrops+EA</td>
<td>8</td>
<td>4.3±2.5*</td>
<td>3.5±1.2*</td>
<td>4.1±1.9*</td>
</tr>
</tbody>
</table>

Notes: blank group: no treatment; hydrops group: AVP injection and immobilization without other treatment; hydrops + EA group: AVP injection and immobilization with EA stimulation. V2R: vasopressin type 2 receptor; cAMP: cyclic adenosine monophosphate; AQP2: aquaporin 2; IOD: integrated optical density; ES: endolymphatic sac; AVP: arginine vasopressin; EA: Electroacupuncture. Compared with the blank group, *P < 0.01; compared with the hydrops group, *P < 0.05; compared with the blank group, *P < 0.05.

Figure 4 cAMP immunoreactivity in endolymphatic sac epithelium (diamionbenzidine, × 200)
A: blank group: no treatment; B: hydrops group: AVP injection and immobilization without other treatment; C: hydrops + EA group: AVP injection and immobilization with EA stimulation. Arrows indicate cAMP expression. Bar = 50 µm. cAMP: cyclic adenosine monophosphate; AVP: arginine vasopressin; EA: electroacupuncture.

Figure 5 AQP2 immunoreactivity in endolymphatic sac epithelium (diamionbenzidine, × 200)
A: blank group: no treatment; B: hydrops group: AVP injection and immobilization without other treatment; C: hydrops + EA group: AVP injection and immobilization with EA stimulation. Arrows indicate AQP2 protein expression. Bar = 50 µm. AQP2: aquaporin 2; AVP: arginine vasopressin; EA: electroacupuncture.

analysis demonstrated low basal expression of cAMP immunoreactivity in the blank group. AVP injection induced an increase in IOD values of cAMP-positive cells compared with the blank group (P = 0.001). EA treatment significantly attenuated the AVP-induced increase in cAMP expression (P = 0.038).

**AQP2 protein expression in the ES**
Figure 5A-C shows representative images of immunohistochemical staining for AQP2 in the ES epithelium. Cell nuclei were visible in sections counterstained with hematoxylin. In the blank group, very little AQP2 immunostaining was evident in the ES in counterstained sections. However, in the hydrops group, strong AQP2 immunolabeling was observed in the ES epithelium. Compared with the hydrops group, AQP2 immunoreactivity in cells comprising the ES epithelium was relatively weak in the hydrops + EA group.

Table 1 presents IOD values of AQP2-immunoreactive cells in the ES epithelium. One-way ANOVA indicated statistically significant differences among the three groups (F = 6.526, P = 0.006). Immunohistochncal analysis demonstrated low basal expression of AQP2-immunoreactivity in the blank group. AVP injection induced an increase in IOD values of AQP2-positive cells compared with the blank group (P = 0.014). EA treatment significantly attenuated the AVP-induced increase in AQP2 expression (P = 0.017).

**DISCUSSION**
The current investigation demonstrates that 10 d of EA treatment can significantly inhibit the development of EH and improve SV ultrastructure. As such, this study may provide a basis for the development of EA-based treatments for MD. Moreover, we found that EA significantly upregulated V2R protein expression and attenuated AVP-induced upregulation of cAMP and AQP2 expression in the ES. Thus, these observations indicated that water homeostasis in the guinea pig inner ear might be partially regulated by EA via improved ultramorphological changes of SV, regulation of the V2R-cAMP-AQP2 signaling pathway, and downstream decreases in AQP2 expression in the ES. The EH model induced by AVP administration in guinea pigs has frequently been used to study the pathophysiology of MD. There is considerable evidence that dysregulation of the V2R-cAMP-AQP2 signaling pathway contributes to AVP-induced EH. Consistent with previous studies by Takeda and Ma HZ, we found a drastic increase in the volume of the endolymph compartment. Recent studies confirmed a thera-
peutic effect of EA on edematous conditions, such as brain edema, myocardial ischemia, and acute lung injury. In the current study, we found that EA suppressed cochlear hydrops induced by intraperitoneal injection of AVP. TCM theory holds that Baihui (GV 20) belongs to the governing vessel and functions to collect the Yang Qi, while Tinggong (SI 19) is utilized as a point for treating inner ear diseases. Baihui (GV 20) and Tinggong (SI 19) have been widely applied in combination for acupuncture treatment of MD. Experimentally, repetitive EA stimulation of Baihui (GV 20) and Tinggong (SI 19) reportedly produces beneficial therapeutic effects on cochlear hydrops.

V2R-cAMP-AQP2-mediated water transport might actively work both in the SV and in the ES. The SV lines the external wall of the cochlear duct. V2R is expressed on the apical (intrastrial) side, while AQP2 is localized to the basolateral (perilymph) side of SV basal cells, thus forming a boundary between perilymph and endolymph compartments. Through interactions with V2R, chronic AVP application results in a rise in AQP2 abundance. Subsequent upregulation of AQP2 expression in the basolateral side of basal cells greatly increases the directional flow of fluid from the perilymph into the SV and endolymph. In the current study, obvious morphological damage of strial cells was observed in the form of absent organelles and vacuolar degeneration in the cytoplasm following administration of exogenous AVP. These results indicated that AVP enhanced water influx into the SV, eventually leading to the formation of EH. Our findings are consistent with previous studies. The SV is responsible for energy metabolism. Vacuolar degeneration in the cytoplasm may disrupt oxygen diffusion in strial cells, leading to functional disturbances of Na\(^+\)-K\(^+-\)ATPase and Na\(^-\)K\(^-\)2Cl\(^-\)-cotransporter-1 (NKCC1), which are abundantly expressed in the SV. It was previously reported that Na\(^-\)K\(^+-\)ATPase and NKCC1 mediate osmotic pressure gradients across the cell membrane via energy-dependent ion transport. As water transport via AQP2 is driven by osmosis, disturbances of Na\(^-\)K\(^+-\)ATPase and NKCC1 might cause an osmotic imbalance and AQP redistribution that ultimately aggravates EH.

The presence of V2R, cAMP, and AQP2 was recently verified in the ES, whereby they play a pivotal role in fluid transport in the inner ear. V2R mRNA and protein are abundantly expressed in the ES. Clinical evidence has shown significantly higher V2R levels in the ES of Meniere’s patients, whereby a significant negative co-relationship between plasma AVP concentration and V2R mRNA expression was observed. In addition, molecular biological studies demonstrated that the V2R mRNA expression in the rat inner ear is downregulated by chronic AVP application, similar to kidney. These previous studies established that AVP regulates V2R mRNA expression in both human and rat inner ear. In this paper, we used immunohistochemistry to broaden these observations. Consistently, our studies revealed that chronically-administered AVP produces downregulation of V2R protein expression in the ES of guinea pig. By interacting with V2R, AVP induces a rise in AQP2 abundance via cAMP-triggered phosphorylation. cAMP activity was reported to be basically upregulated in patients with MD and in vitro, and cAMP sensitivity was largely elevated at the time of AVP exposure. Accumulating evidence has shown that AQP2 plays a crucial role in fluid homeostasis of the inner ear. Maekawa et al. showed that both mRNA and protein expression of AQP2 were significantly higher in the ES of Meniere’s patients. Moreover, similar results were found in the EH model. Sawada et al. reported significantly upregulated levels of AQP2 mRNA in the ES of AVP-treated rats. Consistent with these previous studies, we observed cAMP-hypersensitivity and AQP2-overexpression in the ES after AVP injection. Taking these results together, it appears that AVP-induced V2R-cAMP-AQP2 signaling pathway dysregulation and subsequent upregulation of AQP2 expression in the ES may be important mechanisms for the formation and development of EH. EA reportedly regulated ion and energy metabolism to relieve cellular edema. In a middle cerebral artery occlusion model, EA stimulation at Baihui (GV 20) and Shenmen (GV 24) acupoints attenuated ultramicrostructural alterations of impaired nerve cells. Consistently, TEM results of the present investigation indicated a marked improvement of morphological damage to strial cells in the hydrops + EA group compared with the hydrops group. These findings suggest that repeated EA at Baihui (GV 20) and Tinggong (SI 19) acupoints may exert aquaretic effects on AVP-induced EH via improvement of SV ultrastructure. Acupuncture has been suggested to inhibit vasopressin receptor activation in uterine tissue to produce analgesic effects. However, in the current study, we observed that V2R protein levels in the ES of guinea pigs with hydrops was increased by EA treatment. This result reveals that acupuncture stimulation may exhibit differential effects on vasopressin receptor expression in different parts of the body under different pathological conditions; notably, this is consistent with the holistic view, a basic tenet of TCM theory. As an intracellular second messenger, cAMP is a universal regulator of metabolism and cellular functions. A previous study reported that pretreatment with EA significantly decreased cAMP concentration in the myocardium of rats suffering from myocardial ischemia and reperfusion. Our present investigation demonstrated for the first time that AVP-induced ES expression of cAMP was significantly downregulated by repeated EA at Baihui (GV 20) and Tinggong (SI 19) acupoints. Previous work revealed that acupuncture can regulate the expression of AQP2s in colonic tissues, spinal cord, and...
brain" to produce aquaretic effects. According to the results of our present study, EA at Baihui (GV 20) and Tinggong (SI 19) with alternating-frequency stimulation resulted in decreased AQP2 expression in the ES of EH guinea pigs. Thus, water influx via AQP2 decreases from the hypo-osmotic capillary into the hyper-osmotic endolymph compartment. Foldings of Reissner’s membrane in the EA-treated group are suspected to reflect a reduction of endolymph volume. Therefore, it is satisfactory to conclude that EA exhibits a dehydrating effect via the V2R-cAMP-AQP2 signaling pathway.

In conclusion, EA stimulation at Baihui (GV 20) and Tinggong (SI 19) could reduce cochlear hydrops of guinea pigs following EH. The mechanism underlying therapeutic efficacy of EA treatment might be, at least in part, associated with improved ultramorphological changes and cellular function of SV, regulation of the signaling pathway, and subsequent changes and cellular function of SV, regulation of the in part, associated with improved ultramorphological changes and cellular function of SV, regulation of the

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