Chinese Journal of Integrative Medicine
Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
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Acupuncture Research

Purines Change at Acupoints along the Pericardium Meridian in Healthy and Myocardial Ischemic Rats*

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ABSTRACT **Objective:** To quantify the purine concentrations of the acupoints along the pericardium and nonpericardium meridians under healthy and myocardial ischemia conditions to investigate the relationship between acupoint purine change and body functional status in rats. **Methods:** A total of 70 rats underwent an operation for myocardial ischemia, while 40 of them survived. They were randomly assigned to the following 5 subgroups: Neiguan (PC 6), Quze (PC 3), Tianquan (PC 2), Quchi (LI 11), and Jianyu (LI 15). Simultaneously, another 40 healthy rats were also randomized into the same 5 subgroups as the control group. The tissue fluids at the acupoints were collected by microdialysis for 30 min. Subsequently, the concentration of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine (ADO) were quantified using the high-performance liquid chromatography method. **Results:** Compared with the healthy group, the ADO at PC 6 (P=0.012), PC 3 (P=0.038), PC 2 (P=0.024), and LI 15 (P=0.042) obviously increased in the model group, while no significant difference was observed at LI 11 (P=0.201). However, ATP, ADP, and AMP manifested no significant changes in these areas, except for ATP at LI 15 (P=0.036). **Conclusions:** Myocardial ischemia could induce an increase in ADO at acupoints of the upper arm and shoulder area, suggesting that the body functional status could affect the responsiveness of acupoints. The status of these acupoints could be pathogenically activated by disease, and distribution following some specific courses.

KEYWORDS acupoint, myocardial ischemia, pericardium meridian, purine, adenosine, microdialysis, high-performance liquid chromatography

Acupoints, which are located along the skin line of the meridians, are the special areas for transporting qi-blood of Zang-Fu organs bidirectionally. Recently, some scientists revealed that acupoints are dynamic.(1-3) In other words, the function of acupoints could transform from physiologically "silent" to pathologically "active" when some organs are affected by diseases. This phenomenon is well known as acupoint sensitization. For instance, the Evans Blue (EB) points from exudative plasma could be observed on the skin of rats with an acute gastric mucosal injury. These points were distributed mainly at Pishu (BL 20, 88.23%), Weishu (BL 21, 82.35%), and Zhongwan (CV 12, 17.64%).(4) Additionally, the local area of sensitized acupoints was found to highly express mast cells and algogenic bioactivators, such as 5-hydroxytryptamine (5-HT), substance P (SP), calcitonin gene-related peptide (CGRP), transient receptor potential vanniloid-1 (TRPV-1), histamine (HA), and bradykinin (BK), than nonsensitized areas.(5) This theory has explained the acupoint specificity problem in part. Nevertheless, the biological basis and the characteristics of dynamic changes for acupoint sensitization still deserve further study.

Besides the aforementioned substances and factors, the purines, including adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine (ADO), are one kind of interesting and well-known extracellular signal transmitters, which have been popularly studied in recent years in the field of acupuncture.(6-8) In 2009,

*Supported by National Natural Science Foundation of China (No. 81590951, 81373559, 81373561 and 81573885), the State Key Program for Basic Research of China (No. 2012CB518501), and the Project of the Second Clinical Medical College of Nanjing University of Chinese Medicine (No. RLZZ201605)
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DOI: https://doi.org/10.1007/s11655-018-2932-8

some raised that purines and their receptors are vital in acupuncture. Some studies demonstrated that the ATP, ADP, AMP, and ADO levels in the acupoint area obviously increased in mice and humans.(6,7) If the A1 receptor was blocked, the acupuncture analgesia effect vanished. Moreover, the extracellular purines could also be regulated by moxibustion and low-level laser therapy.(9,10) All these results strongly suggested that purines may play crucial roles in the peripheral mechanism of acupuncture analgesia. Some other studies also revealed that purinergic signaling takes part in acupoint initiating effect,(11) indicating they could be used to measure the responding ability of acupoints. Therefore, purines may possibly illustrate the potential complicated relationship between acupoints, meridians, and diseases themselves, while further revealing the essence and nature of acupoints.

In the present study, the purines changes in the 5 acupoints area, including Neiguan (PC 6), Quze (PC 3), Tianquan (PC 2) at pericardium meridian, Quchi (LI 11) and Jianyu (LI 15) at Large Intestine meridian, were observed in healthy and myocardial ischemic conditions. Hence, the present study aimed to observe whether myocardial ischemic could induce the purines change, and compare the concentration difference of purines at the acupoints in different meridians, in order to confirm the phenomenon of acupoint sensitization and analyze the characteristics of acupoint sensitization.

METHODS

Animals and Grouping

A total of 110 adult Sprague-Dawley rats (55 males and 55 females, weighing 250–300 g), supplied by the Experimental Animal Center of Sichuan Provincial People's Hospital [Certificate No. SCXK (Chuan) 2013-15], were used for this study. They were housed in cages at a temperature of 23–26 ℃ and humidity of 40%–70%, under a 12-h light/dark cycle, with free access to food and water. All operations and procedures were performed in the laboratory of Chengdu University of Traditional Chinese Medicine (CDTCM). The study was approved by the Institutional Animal Care and Use Committee of CDTCM, China, and all the experimental procedures were directed by the Guide for the Care and Use of Laboratory Animals.

Among all the rats, 70 rats were attributed to the model group and underwent an operation for myocardial ischemia, while 40 of them survived. They were randomly assigned into 5 subgroups: PC 6, PC 3, PC 2, LI 11, and LI 15 by a random number table. At the same time, another 40 healthy rats were also randomized into same 5 subgroups as the control group. Therefore, eight rats were present in each subgroup.

Reagents

The standard substances of ATP (specification: A3377-5G; batch No. 1001420716), AMP (specification: A2753-1G; batch No. 1001447709), ADP (specification: A1752-5G; batch No. 1001428843) and ADO (specification: A1752-1G; batch No. 101183447) were purchased from Sigma-Aldrich Ltd., Chengdu, China, while the methyl alcohol [high-performance liquid chromatography (HPLC) grade] was bought from Fisher scientific Ltd., Chengdu, China.

Myocardial Ischemia Model Replication

The myocardial ischemia model was created by performing permanent ligation of the left anterior descending coronary artery (LAD) as described in a previous study.(12) The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate at a dose of 0.4 mL/100 g, and placed in a supine position in the laboratory. A tracheal cannula was inserted via the mouth, with one end connected to a mini rodent ventilator (Kent PhysioSuite, Torrington, USA). The relevant parameters were conversed automatically with animal's weight. That is, the tidal volume ratio was set at 1 mL/100 g, and the inspiration and expiration ratio was 1:1. Thoracotomy was performed using an ophthalmic operating set to expose the heart, and the LAD was ligated with a 4-0 nylon suture to create a permanent coronary occlusion.

Electrocardiographic Examination

Electrocardiography (ECG) of the rats were monitored throughout the whole operation by AD instruments (Australia). Three needles, which punctured in the two foot and right palm, were connected with electrodes to record the lead Ⅱ of the ECG. Furthermore, this information was amplified, recognized and analyzed by PowerLab system. Thus, the S-T segment elevated over 0.2 mV from the baseline was recruited as an index of myocardial ischemia.

Locations of Acupoints

Some specific acupoints at the pericardium meridian, such as PC 6, PC 3, and PC 2, were selected to observe the changes of purines along the meridians. These points were frequently used for cardiac diseases. Furthermore, two acupoints at Large intestine meridian was chosen as a comparison, such as LI 11 and LI 15, whose locations were close to the pericardium meridian in the elbow and shoulder but were irrelevant to cardiac diseases. According to the textbook of experimental acupuncture,(13) PC 6 is at the inner side of the left forelimb, 3 mm distance above the wrist joint and between the ulnar and radial side. LI 11 is at the lateral side of the elbow joint, which is nearer to the radial bone. However, PC 3, PC 2, and LI 15 are not recorded in the textbook. Therefore, these acupoints were considered to be located according to the real sites of human in the present study: PC 3 at the cubital crease, in the ulnar side of bicipital tendon; PC 2 at the inner side of the left upper arm, 3 mm distance below the anterior axillary fold; and LI 15 at the lateral side of the shoulder, between the acromion and the greater tuberosity of the humerus.

Microdialysis Method and Sample Collection

At the 3rd day of the model replication and regular binding, each rat was anesthetized and fixed as described earlier at 37 ℃ using an animal constant temperature controller (CMA/450, Holliston, USA). All the acupoints in the present study were well positioned according to the textbook of experimental acupuncture and focused on the left forelimb of rats.(13) A microdialysis probe (CMA20, Kista, Sweden) was implanted at about 1–2 mm depth in the skeletal muscle of required acupoints, with one end linked to the microdialysis pump (CMA402, Holliston, USA) and the other attached to the microscale cooling collector (MAB85, Holliston, USA). The probe was perfused with 0.9% sodium chloride solution at a constant rate of 1.5 μL/min for balancing 1 h (the microdialysis sample not collected during the 1 h). After the balancing, the sample collection time lasted for 30 min. These samples were stored at –20 ℃ for analyzing the concentration of purines substances (ATP, ADP, AMP and ADO) with HPLC method.

HPLC Analysis of Purines

The purine analysis was carried out using the Shimadzu LC-10AD system Chromatographic separation was achieved using a reverse-phase column (Shimadzu Intertsustain C_{18}, 4.6 mm × 150 mm, 5 μm). The mobile phase consisted of 50 mmol/L KH_2PO_4 buffer (pH 6.5) and methyl alcohol (HPLC grade), which were pumped by A and B pumps, respectively. The flow rate was maintained at 1.0 mmol/L/min. The whole retention time was 30 min, while the injected volume of methyl alcohol turned into 0.02%, 0.02%, 15%, 15%, 32%, 32%, 0.02%, and 0.02%, respectively, at times 0.01, 8, 11, 17, 18, 24, 25, and 30 min. Each sample injection volume was 20 μL (loop injection volume). The temperature of the column was 25 ℃ and purine concentration was detected at an ultraviolet wavelength 254 nm.

Peak identification and quantification were assessed using standard solutions of purines (ATP, AMP, ADP, and ADO) dissolved in distilled water (Watsons, Chengdu, China, Figure 1A). Fresh standard solutions were prepared every week, while working standard solutions were prepared with the appropriate dilutions of stock solution. A standard curve was prepared prior to the measurement of each batch of collected samples. The concentrations (μg/mL) of ATP, ADP, AMP, and ADO were determined from the chromatogram peak areas. The chromatograms of standard substances and collected samples were manifested as follows (Figure 1B).

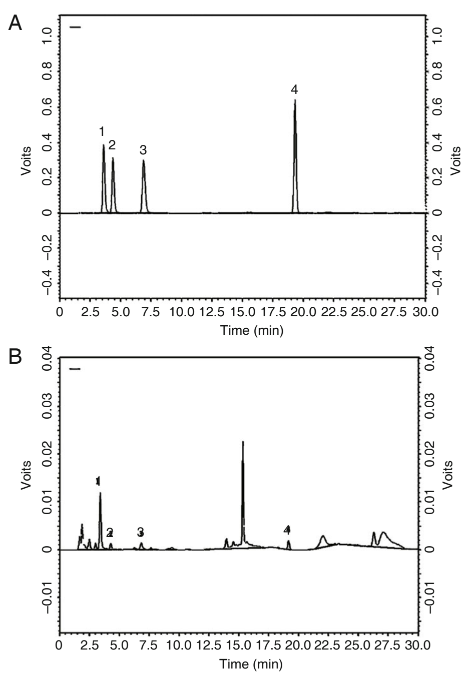

Figure 1. Peaks Identification and Quantification by HPLC

Notes: (A) is the chromatogram of 4 standard purines solutions(ATP, AMP, ADP, ADO) dissolved in distilled water (Watsons). Peak 1 is the retention time of ATP, at 3.35 min. Peak 2 is the retention time of ADP, at 4.36 min. Peak 3 is the retention time of AMP, at 6.45 min. Peak 4 is the retention time of ADO, at 18.96 min. Simultaneously, (B) is the chromatogram of 4 purines in collected samples. The retention times of 4 purines are identified well and almost in coincidence.

The linearity of HPLC measurement results was examined with a calibration curve specifically prepared for the linearity test. This calibration curve was prepared with the concentrations of 2, 1, 0.5, 0.25, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, and 0.0001 mg/L. Purines (ATP, AMP, ADP, and ADO) of these standards were measured on the same day using a 20 μL injection into HPLC. Peak areas versus concentrations were regressed to a linear function from which slope of the fitted line and the goodness of fit R2 were determined. The concentration curves are illustrated in Figure 2.

Statistical Analysis

All tests of sample for purines (ATP, AMP, ADP, ADO) were performed twice. Data were presented as means ± standard deviation ($\bar{x} \pm s$). Statistics analysis was carried out with software SPSS 19.0; and the comparisons between groups were performed using *t*-test analyses. P<0.05 was considered statistically significant.

RESULTS

Baseline Data of Rats in Different Groups

In total, 110 rats were launched in this study. Because of the high modeling mortality, 8 rats survived in each subgroup and underwent the microdialysis procedure. Finally, 5 valid data in each subgroup were included in the statistical analysis on account of accidental death and data loss.

ECG Changes in Different States

ST segments were noticeably elevated after ligation in the model group, indicating that the model replication was successful. Additionally, no significant difference was observed about the changes of ST segment in these 5 subgroups of the model group. The ECG results of the two groups are presented in Figure 3.

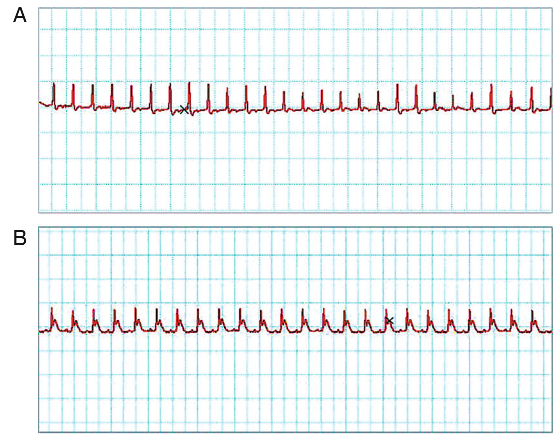

Figure 3. Changes of ECG in Model Group and Healthy Group

Notes: (A) is the ECG in the healthy group, while (B) is the typical ECG in the model group that the ST segment was elevated significantly during the myocardial ischemia.

Purine Changes of Acupoints in Different States

The alteration of purines at different acupoints between myocardial ischemic and healthy rats was studied. PC 6, PC 3, and PC 2 belonged to the pericardium meridian, which was greatly relevant to the Heart (Xin) organ. However, LI 11 and LI 15 were the acupoints located on the Large Intestine meridian.

Compared with the control group, the ADO concentration in the PC 6 (P=0.012), PC 3 (P=0.038), PC 2 (P=0.024), and LI 15 (P=0.042) areas obviously increased in the model group; however, no significant difference was observed in the LI 11 area (P=0.201). Moreover, no significant difference was observed in the five acupoint areas involving ATP, ADP, and AMP concentrations, except for the obvious difference of ATP in the LI15 area (P=0.036). The purine substances of the two groups are illustrated in Figure 4.

DISCUSSION

Previous studies revealed that acupuncture stimulation could change the extracellular purine concentration of acupoints in pain-related diseases.[6,7] The present study attempted to observe the changes in purine concentration in the dialysate of acupoint areas by the same method of microdialysis, which could partly explain the hypothesis in this study. Findings of

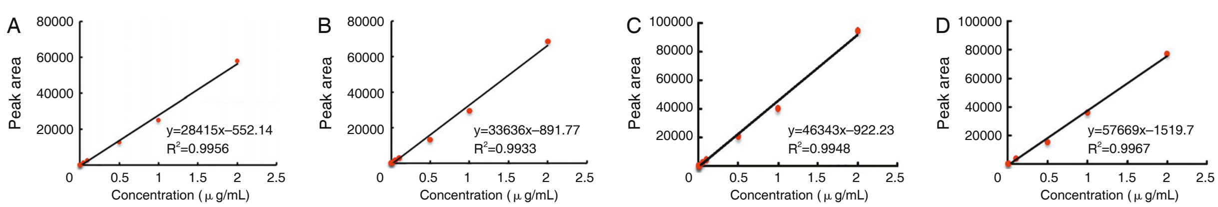

Figure 2. Concentration Curves of Four Standard Purines

Notes: (A)–(D) are the concentration curves and regression equation of ATP, ADP, AMP, and ADO, respectively.

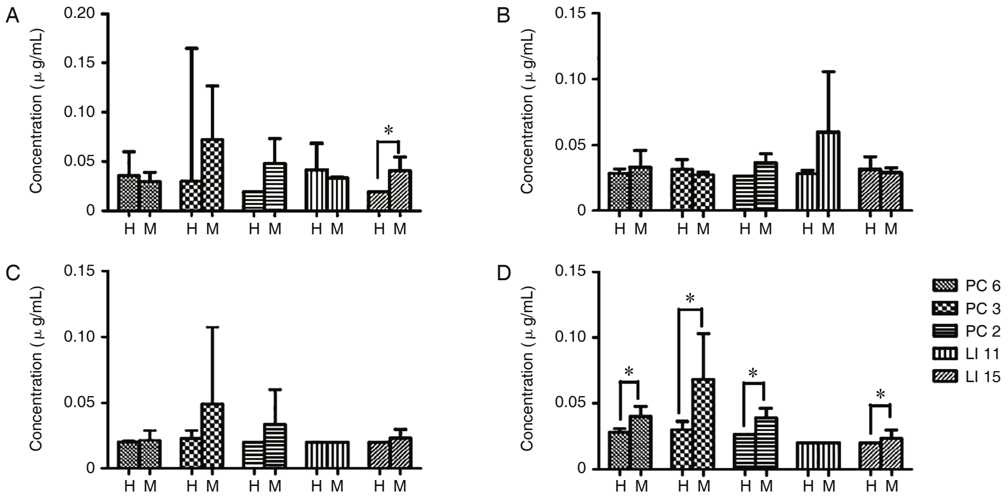

Figure 4. Purines Changes of Acupoints in Different States

Notes: The concentration of purines of different acupoints were quantified by HPLC. (A) reveals the comparison of ATP at PC 6, PC 3, PC 2 and LI 15 in the healthy and model groups, while (B), (C) and (D) are respectively representing the changes of ADP, AMP and ADO in the healthy and model groups, respectively. H: healthy group; M: myocardial ischemic group. $^{*}P<0.05$, compared with the myocardial ischemic group.

the present study suggested that the ADO levels in the PC 6, PC 3, PC 2, and LI 15 areas in the model group significantly increased, as well as the ATP level in the LI 15 area. In contrast, no changes were observed in ATP, ADP, and ADO concentrations in the acupoint areas.

PC 6, PC 3 and PC 2 are all in the pericardium meridian. Previously, multiple kinds of pathological changes occurred along line routes of Xin and Pericardium meridians during some cardiac diseases. For instance, some study demonstrated that the infrared spectra of PC 6 and Laogong (PC 8) in the coronary heart disease condition were different from those of healthy people.(14) Additionally, the transcutaneous oxygen pressure and carbon dioxide emission along the pericardium meridian varied significantly from the nonmeridian area under some specific pathological conditions.(15,16) Apart from these changes of multiple physical phenomena, some biochemical molecules have also been detected, such as, cyclic 3',5'-guanosine monophosphate (cGMP),(17) nitric oxide,(18) and nitrotyrosine.(19,20) In the present study, we found that ADO presented significant increase especially at PC 6, PC 3, and PC 2 points in the pathological situation. However, no obvious changes were noted in the control point LI 11. The findings of the present study provided one kind of possible biological basis for visceral-meridian theory to some extent.

In recent decades, scientists have endeavored to illuminate the nature of visceral-meridian theory. Some researchers were inclined to explain the phenomenon with acupoint sensitization as mentioned earlier. Studies have found some materials, such as 5-HT, SP, CGRP, TRPV-1, HA, and BK,(5) are highly expressed in the acupoint and meridian areas, causing the peripheral acupoints and meridian to be in a state of inflammatory soup.(2) The present study tried to observe the variation in 4 kinds of extracellular purines in the local acupoint area, which were proved to be novel kinds of molecules in acupuncture analgesia.(21) In generally, the intracellular concentration of ATP is higher than that of extracellular concentration. Once the body undergoes anoxia, ischemia, mechanical stretch, or some other stimulation, normal cells and nerve endings release the intracellular ATP. ATP is degraded into ADP, AMP, and ADO by dephosphorylation, resulting in the accumulation of extracellular purines and activation of cell-membrane purine receptors, while the ADO could further degraded into other metabolic products, such as trioxypurine and inosine.(22) In the present study, the acupoint microdialysis was enforced on the third day after the operation for myocardial ischemia; the rats were just recovered from the acute injury and were in a relatively stable period, which also conformed to previous studies that sensitization reached the peak at the first and second days and then declined gradually. (23) Therefore, only the content of ADO change was observed with the injury duration visibly. However, no changes were observed in the other three purines. This result was similar to the findings of Goldman, et al[6,7] that ADO levels in the acupoint area were obviously increased in mice and humans. Unfortunately, these three molecules failed to respond to diseases at all or had already declined to the normal level after acute raising, leaving the content of ADO to remain high in the sensitized acupoint. Therefore, ADO was important in the peripheral activation of acupoint sensitization.

In addition, we also found the concentrations of ADO in myocardial ischemic rats significantly raised miraculously at LI 15 point on the shoulder joint and inner side of the upper arm, which was greatly similar to referred pain caused by angina pectoris. Anatomically, the median nerve in the inner side of the upper arm was just the afferent nerve of the pericardium meridian.[24] Besides, studies also demonstrated that the afferent nerve segment of Pericardium meridian coincided with the cardiac afferent nerve segment, which partly provided the morphological basis to the close connection of Pericardium meridian and cardiovascular diseases.[25,26] Therefore, when the rats were myocardial ischemic, the Pericardium meridian could exert some specific change, especially the rising level of ADO in the present study. In other words, the LI 15 point was also sensitized and activated, which might be due to the visceral referred pain of myocardial ischemia. In clinical practice, many patients could suffer from the referred pain in the shoulder, back, and upper arm when angina pectoris attacked. In the laboratory, some scientists reached a consensus that either the cardiac nociception of rats could induce different cardiac-somatic and cardiovascular reflexes or the electromyography of trapezius muscle could be a common index for qualitative and quantitative analyses.[27,28] All of these findings confirmed the close relationship between myocardial ischemia and peripheral referred pain, giving a reasonable account for the pathological change of LI 15 point. However, future studies should uncover the role of purines in acupoint sensitization, such as downstream of extracellular purine. Some previous studies have already revealed that the purine receptor could mediate actin cytoskeleton remodeling of human fibroblasts,[29] and other studies have noted that extracellular ATP and ADP could increase the skeletal muscle blood flow after manual acupuncture stimulation in rats.[30]

In conclusion, myocardial ischemia could induce the increase in the concentration of ADO at acupoints along the pericardium meridian and shoulder joint. This finding suggested that the body functional status could affect the responsiveness of acupoints. The status of acupoints could be pathogenically activated by diseases, while the distribution of these sensitized acupoints follows some specific courses and regulations. Therefore, the sensitive acupoints can help in the diagnosis and treatment in acupuncture clinical practice.

Conflicts of Interest

All authods have no conflict of interests to disclose.

Author Contributions

Zhou YM performed the whole experiments, Zhou YM and Zhuang Y wrote the manuscript. Lv PR, Zhou J and Wan M did some preliminary experiments to establish the methodology of HPLC. Ren YL analyzed the data. Cai DJ contributed to the conceptualization and design of the experiments. Liang FR made the comments on the experiments and manuscript.

Acknowledgement

We are grateful to all the participants and staffs in the trial.

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(Received December 12, 2016; First Online September 28, 2018)
Edited by WANG Wei-xia