Electroacupuncture at Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU 20) regulate the aging-related changes in gene expression profile of the hippocampus in sub-acutely aging rats

Jianmin Liu1,2☯, Jing Liu1,2☯, Guang’an Wang3, Guanya Liu1,2, Huanjiao Zhou1, Yun Fan1, Fengxia Liang1,2, Hua Wang1,2*  

1 Acupuncture-moxibustion and Orthopedic College, Hubei University of Chinese Medicine, Wuhan, China, 2 Hubei Provincial Collaborative Innovation Center of Preventive Treatment by Acupuncture & Moxibustion, Wuhan, China, 3 The Third Clinical Medicine College, Henan University of Chinese Medicine, Zhengzhou, China  

☯ These authors contributed equally to this work.  
* huang.edu@hotmail.com

Abstract

To investigate the molecular mechanisms of sub-acutely aging and demonstrate the effect of electroacupuncture (EA) at the Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU 20) acupoint on the sub-acutely aging brain, cDNA microarrays and bioinformatics analyses were carried out. Thirty Sprague-Dawley (SD) male rats were selected and randomly divided into three groups: the control group (C), the sub-acutely aging model group (M) and the electroacupuncture group (M+EA). Sub-acutely aging model rats were obtained by D-galactose s.c. injection continuously for 40 days. Total RNA was extracted from the hippocampus area of brains in three groups for cDNA microarrays. The data of different groups were compared and analyzed by differential expression analysis, Gene ontology (GO) term enrichment, Kyoto Encyclopedia of Genes Genomes (KEGG) pathway enrichment and quantitative real-time PCR. According to the results, 4052 DE genes were identified in our study. Among them, there were 3079 differently expressed (DE) genes between group M and the control group (C), the sub-acutely aging model group (M), and the electroacupuncture group (M+EA). Sub-acutely aging model rats were obtained by D-galactose s.c. injection continuously for 40 days. Total RNA was extracted from the hippocampus area of brains in three groups for cDNA microarrays. The data of different groups were compared and analyzed by differential expression analysis, Gene ontology (GO) term enrichment, Kyoto Encyclopedia of Genes Genomes (KEGG) pathway enrichment and quantitative real-time PCR. According to the results, 4052 DE genes were identified in our study. Among them, there were 3079 differently expressed (DE) genes between group M and group C, and these genes are associated with the aging of rats. Moreover, 983 genes were expressed differently in group M+EA compared with group M, revealing that points stimuli could regulate gene expression in brain with aging. Gene ontology (GO) term enrichment and KEGG enrichment were performed to further classify the differential expression genes. Important GO terms and KEGG pathways connected with sub-acutely aging EA effects were identified. At last, 3 significant differentially expressed genes were selected for real-time quantitative PCR to clarify the cDNA microarray results. In conclusion, the cDNA microarray data first compared and analyzed the differences of gene expression profile in the hippocampus of rats in different groups, which contribute to our knowledge on the molecular mechanisms of EA towards sub-acutely aging.
Introduction

In recent years, the number of older persons is increasing rapidly. Under this circumstance, the problem of aging is drawing more and more social attention. Aging is accompanied by cognitive decline in a major segment of the population and is the primary risk factor for Alzheimer’s disease and other prevalent neurodegenerative disorders. As people become older, the age-related changes will occur, such as changes in the functions and composition of the human body [1, 2]. Coupled with physiologic changes, there are also medical conditions that rise more commonly with advancing age. These changes and conditions increase an older adult’s vulnerability to and injuries from abuse or neglect [3]. According to formal research, aging is associated with deterioration of the immune system (immunosenescence), an increased susceptibility to infection, autoimmune disease and cancer and reduced responsiveness to vaccination [4, 5]. A key feature of the aged human immune system is the accumulation of highly differentiated CD8(+)CD28(-) T cells, a phenomenon that negatively influences immune function in the elderly. As a result, the mechanisms that regulate survival or death of CD8(+)CD28(-) T cells has become the focus of many researchers [6]. To combat immunosenescence, a lot of strategies are emerging, focusing on cellular and genetic therapies, which include bone marrow transplantation and genetic reprogramming [7]. Except for immunosenescence, brain aging processes are also enormously complex phenomena that can include cognitive decline and increase the risk of Alzheimer’s disease (AD) [8]. There are different kinds of methods to combat age-related brain diseases or delay brain aging, including drug therapy and non-drug therapy [9]. For instance, Mannosylated liposomal flavonoid is useful in combating age-related ischemia-reperfusion induced oxidative damage in rat brain [10]. Moreover, Caloric restriction (CR) and transgenic over expression of endogenous antioxidants therapy were proven to delay or inhibit a number of age-associated pathologic and biologic changes in the brain, thus to have life-extending function [11, 12].

Acupuncture is a traditional therapy applied for thousands of years, and it was also a powerful non-drug therapy which is used extensively in Oriental Medicine and has emerged as an important modality of complementary and alternative therapy to Western Medicine [13]. In the theory of traditional Chinese medicine, it is proposed that acupuncture can strengthen the human body to resist diseases by puncturing needles at certain points [14]. It has also been proved that acupuncture theory could apply to rats and that the results of acupuncture experiments based on rats agree with the results based on human. Until now, rats’ models have been applied to the acupuncture research of various kinds of diseases, such as hypertension, Alzheimer disease, diabetes, aging, and have achieved great success [15]. For example, previous research demonstrated that acupuncture at “Taichong” (LR3) has immediate effect on patients with hypertension of 1 or 2 degree, and the lowering extent is positively related with the blood pressure before acupuncture [16]. Then other research groups proved that moderate-stimulation of “Tai chong” (LR 3) can lower blood pressure and plasma EA-1 level in hypertension rats. So the spontaneously hypertensive rats (SHR) model was used by many researchers to seek a better acupuncture parameter for clinical treatment of hypertension [17, 18]. Double reinforcing-one unblocking acupuncture was proved to have a significantly effect on senile immunologic function [19]. Other researches demonstrated that double reinforcing-one unblocking acupuncture could also improve the spatial learning and memory in aging model rats [20, 21].

So far, the function of acupuncture in regulating the immune system has been revealed in many researches. For instance, acupuncture can enhance anticancer and antistress immune function and exert antiinflammation effects. The acupuncture point ST36 (Zusanli) is an important acupoint which is widely applied in immune-related diseases. M. Zhu et al. reported
that EA preconditioning at ST36 obviously ameliorated CLP-induced intestinal injury and high permeability and reduced the mortality of CLP-induced sepsis rats through increasing the concentration of sIgA and the percentage of CD3+, γ/δ, and CD4+ T cells and the ratio of CD4+/CD8+ T cells [22]. Another research group discovered a novel cholinergic anti-inflammatory pathway activated by acupuncture as well as a chemokine-mediated proliferation of opioid-containing macrophages in inflamed tissues in response to acupuncture [23]. Acupuncture was proved to play a role in delaying brain aging and treating age-related brain diseases. “YiQi-TiaoXue, FuBenPeiYuan” acupuncture method has been proved to improve cognition ability of dementia rat [24]. Moreover, acupuncture can induce different cell proliferation in different brain regions of SAMP8, which brings forth the need to explore further for the mechanism of cognitive deficits and acupuncture intervention in this field [25].

Based on a long clinic experience and studying of the traditional Chinese medicine theory, the electroacupuncture treatment which insert points at Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU20) with needle, also named “Shuanggu Yitong” (double-reinforcing and one-unblocking) acupuncture therapy has been found the special effect to regulate the immune system for rats [26–28]. Some mechanisms of how the “Shuanggu Yitong” electroacupuncture therapy regulates the immunosenescence have been studied in our previous research. It turns out that EA at Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU 20) could delay aging by regulating T cells in proliferation, secretion of IL-2 and its receptor and improve the expression of CD8+, CD28+ [22, 23]. Further study reveals that EA at Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU 20) could decrease the content of IL-1β and IL-6 of serum [28]. Based on the previous researches and in order to gain a better understanding of aging-related changes and how “Shuanggu Yitong” EA affects the brains of sub-acutely aging rats, DNA microarray analysis was explored. The microarray data demonstrate differences between control group (C), the electroacupuncture group (M+EA) and the sub-acutely aging model group (M). The differences existed in gene expression level, GO categories and pathway categories, which contribute to our knowledge on the effect of EA to the brain of sub-acutely aging rats in the molecular level. Important genes related with sub-acutely aging and EA effects were selected for further study.

Materials and methods

Animal treatment

Thirty SD male rats in 3-month-old were obtained from the Animal Experimental Center of Hebei Medical University, China [Experimental animal production license number: SCXK (Hebei)2008-1-003]. All the rats were raised in the individually ventilated cages with the temperature between 20°C to 25°C. The humidity was between 45% and 55%. Light was provided from 8 am to 8 pm to simulate the circadian rhythms while food and water was offered sufficiently. All animal treatments were approved by the Animal Ethics Committee of the Hebei University of Chinese Medicine, No. [2016] IEC (010).

After 1 week of adaptation, the rats were randomly divided into three groups as following: the control group (C), the sub-acutely aging model group (M) and the electroacupuncture group (M+EA), 10 rats for each group. Rats in group C were raised normally without any treatments. While the sub-acutely aging model rats were obtained by D-galactose s.c. injection continuously for 40 days since the second week. The concentration of D-galactose s.c. was 350mg. kg-1.d-1, one time for each day [29]. Rats in group M were raised without any treatments after modeling. While the Rats of M+EA group started EA treatment at Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU 20) once a day after modeling for 27 days (the procedures are shown as following). The process of the experiment was shown in S1 Fig. The body temperature, tongue
temperature and the weight of rats in different groups were measured every three days during the model making and electroacupuncture procedures to make sure that the physiological indicators of rats are normal. All efforts were made to minimize suffering.

**Electroacupuncture (EA) treatment**

Sterile acupuncture needles [size: φ0.30×25 mm (diameter: 0.30 millimeter, length: 25 millimeter); made by Suzhou Acupuncture & Moxibustion Appliance Co., Ltd, Suzhou, P.R. China] and the pulse generator (6805-II, Shanghai Taicheng technology development co., LTD) were used during the EA treatment. Needles were inserted perpendicularly into the muscle layer at Guanyuan (CV 4), Zusanli (ST 36) and inserted horizontally into subgaleal tissue at the point of Baihui (DU 20) to a depth of 2 mm. The Guanyuan (CV 4) acupoint was connected to the negative charge of the pulse generator and the Zusanli (the bilateral Zusanli were used alternately in the EA treatment) was connected to the positive charge (continuous wave: 2 Hz, 1 mA, lasted 15 min). Baihui (DU 20) was stimulated with needles at the same time: After the needle has reached its desired location, to twirl and rotate the needle backward and forward continuously with the frequency 2–3 times per second for 30 seconds. The same manipulation was done after every 5 minutes for three times until withdrawing the needle. The EA treatment was performed every day except Sunday and lasted for 27 days. This electroacupuncture (EA) treatment protocol has been deposited in the protocols.io. The digital object identifier (DOI) link is: [http://dx.doi.org/10.17504/protocols.io.ky8czw](http://dx.doi.org/10.17504/protocols.io.ky8czw).

**Tissue sampling**

At the end of the EA treatment, rats from three groups were fasted overnight. Then all the rats were killed by dislocation of cervical vertebra and the brains were quickly excised after intraperitoneal anesthesia using pentobarbital natrium. The collected samples were washed by the cold normal saline. Subsequently, the hippocampus area was divided, frozen in the liquid nitrogen and then kept under -80˚C for RNA extraction. The hippocampus area of ten rats in each group makes up one sample, and then the three samples from group C, group M and group M+EA were prepared for the RNA extraction.

**RNA preparation and quantitative real-time PCR (q-PCR)**

RNAiso Plus (TaKaRa Biotech. Co., Dalian, China) was used to extract the total RNA according to the manufacturer’s protocol. All RNA samples were treated with DNase I (TaKaRa Biotech. Co., Dalian, China) and frozen at −80˚C before DNA microarray experiment. RNA quantity and purity was assessed using NanoDrop ND-1000 (Pass criteria for absorbance ratios are established at A260/A280 ≥ 1.8 and A260/A230 ≥ 1.6). RIN values are ascertained using Agilent RNA 6000 Nano assay to determine RNA integrity (Pass criteria for RIN value is established at ≥7 indicating acceptable RNA integrity). gDNA contamination was evaluated by gel electrophoresis.

First-strand cDNA was prepared by All-in-one First strand cDNA Synthesis Kit (Genecopeia, Guangzhou, China) following the manufacturer’s protocol. Then the BIO-RAD CFX96 q-PCR system (SYBR Green I fluorescent dye detection) was used to perform the qRT-PCR. The mRNA abundance was normalized with the housekeeping gene β-actin, and the relative expression levels were calculated using the 2-ΔΔCt method [30].

**Microarray**

Microarray analysis was carried out to investigate gene expression patterns in the hippocampus of rats in different groups. 6-OHDA-targeted transcripts were analyzed with Whole Rat
Genome 4 × 44 K microarrays (Gene expression hybridization kit, Agilent) according to the manufacturer’s instruction. Rosetta Resolver SystemR (Rosetta Biosoftware) was used to process data analysis: 1. Rosetta profile error model calculation: the error due to random factors and systematic biases are estimated by Rosetta error model which can capture the predictable behavior of the variance in microarray measurement [31]; 2. Squeeze replicated probes: the repeated probes within one chip are averaged; 3. Normalize intensities: Median scaling performed on data set without flagged and control data; 4. Pearson’s correlation coefficient: statistical analysis calculated on three technical replicates to assess reproducibility; The replicated analysis of the RNA from the same sample can decrease the technical variation and the false positive, making the results more accurately. 5. Merge technical replicate data: Average intensity values calculated on technical replicates; 6. Pairwise ratio calculation: Probe filtering, normalization, pair-wise comparison and error-weighted modeling are performed based on customers’ designated sample groups; 7. Differentially expressed gene lists: Standard selection criteria to identify differentially expressed genes are established at |Fold change| ≥ 1 and \( P < 0.05 \). Student’s t-test (two-tailed) was used for data analysis this study. 8. The heat map was obtained using the software HemI [32], while the Venn diagram was obtained using VENNY [33]. We applied KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways.

**Results**

**RNA preparation**

Total RNA was extracted from the hippocampus of rats in different groups and treated with DNase I. The absorbance ratios of A260/A280 in group C, M and M+EA were 1.99, 1.99 and 1.88, respectively. While the absorbance ratios of A260/A230 were 1.98, 2.16 and 1.96. RIN values in three groups were all above the pass criteria (8.5, 9.0 and 8.3 respectively) (Table 1). The results indicating that the RNA purity and integrity are suitable for cDNA microarray experiment.

**Identification of differentially expressed genes between different groups**

To demonstrate the molecular mechanisms of the sub-acutely aging and EA effects, the differentially expressed (DE) genes between different groups were identified and analyzed. Firstly, the heat map was made to compare the expression patterns of DE genes in three groups. There are 4052 DE genes identified in our study (S1 Table). As can be seen in Fig 1, the gene expression pattern of group M was quite different from that of group C, revealing that the sub-acutely aging was associated with the expression changing of a large sum of genes in the hippocampus. Meanwhile, the expression level of DE genes in group M+EA was different from that in group M, which suggested that EA has changed the gene expression pattern of brain in sub-acutely aging rats.

According to the results, there were 3079 DE genes between group M and group C, among them 1750 genes were up-regulated and 1329 were down-regulated. These genes are related with the aging of brain in rats. When comparing group M+EA and group M, 983 DE genes

| Sample       | A260/A280 | A260/A230 | RIN  | Conc(ng/ul) |
|--------------|-----------|-----------|------|-------------|
| Group C      | 1.99      | 1.98      | 8.5  | 1042.7      |
| Group M      | 1.99      | 2.16      | 9.0  | 734.0       |
| Group M+EA   | 1.88      | 1.96      | 8.3  | 705.8       |

Table 1. Quality control of RNA sample in three groups.
were identified. The number of up-regulated genes was 620 while the number of down-regulated genes was 363, as shown in Table 2. Further analysis was made to compare the number and overlapping relationships of DE genes between different groups. According to Fig 2A, the comparing groups M vs. C and M+EA vs M shared 445 DE genes. 2634 DE genes were peculiar to comparing group M vs. C and 538 DE genes were peculiar to M+EA vs M. Then the shared 445 DE genes were analyzed to figure out important genes related with sub-acutely aging and EA effects. By comparing the up-regulated genes between M vs. C and M vs. M+EA, we identified 348 overlapping DE genes, which mean EA can reverse the down-regulate of these genes in the sub-acutely aging group. Besides, 1404 DE genes were peculiar to M vs. C comparing group and 274 were peculiar to M vs. M+EA (Fig 2B). Likewise, by comparing the down-regulated genes of M vs. C and M vs. M+EA, 78 shared DE genes were identified. 1251 DE genes were peculiar to M vs. C down-regulated genes and 285 were peculiar to comparing group M vs. M+EA (Fig 2C). The result reveals that EA treatment can up-regulate the expression of 78 genes which were down-regulated in group M. Common elements in “M vs C-UP” and “M vs M+EA-UP” and in “M vs C-DOWN” and “M vs M+EA-DOWN” were shown in S2 Table.

### Functional distribution of differentially expressed genes

The identified differentially expressed genes were further analyzed by Gene ontology (GO) term enrichment and Kyoto Encyclopedia of Genes Genomes (KEGG) pathway enrichment. Comparing groups M vs C and M+EA vs M were chosen for GO and KEGG analysis. According to the GO categories, the identified DE genes were categorized into three major functional groups: cellular component, molecular function, and biological process. In comparing group M vs C, the abundant genes were categorized into 20 major functional groups based on the GO categories, and protein import into nucleus translocation, trans golgi network transport vesicle and kinase activoter acvitity are the top three functional categories, as can been seen in Table 3. Likewise, in comparing group M+EA vs M, the abundant DE genes were categorized into 20 major functional groups, and functional mategories are positive regulation of transport, voltage gated potassium channel complex and vitamin binding are the most abundant (Table 4).

The KEGG pathway enrichment were then performed to category the DE genes in comparing group M vs C and M+EA vs M. Compared with group C, 5 KEGG pathway were identified up-regulated and 9 were identified down-regulated in group M. The up-regulated terms were as following: rno04080: Neuroactive ligand-receptor interaction, rno04916: Melanogenesis, rno04270: Vascular smooth muscle contraction, rno04020: Calcium signaling pathway and rno04912: GnRH signaling pathway; the down-regulated terms were: rno04070: Phosphatidylinositol signaling system, rno04670: Leukocyte transendothelial migration, rno04960: Aldosterone-regulated sodium reabsorption, rno05223: Non-small cell lung cancer, rno05214: Glioma, rno05200: Pathways in cancer, rno04662: B cell receptor signaling pathway and

### Table 2. The number of DE genes between different samples.

| NO | Comparison   | Up-regulated | Down-regulated |
|----|--------------|--------------|----------------|
| 1  | C vs. M+EA   | 1164         | 945            |
| 2  | M vs. M+EA   | 363          | 620            |
| 3  | M vs. C      | 1750         | 1329           |

https://doi.org/10.1371/journal.pone.0191623.t002
Small cell lung cancer (Fig 3A). DE genes involved in these pathways were listed in Table 5. In comparing group M+EA vs M, 18 KEGG pathway terms were up-regulated, and the most abundant five terms were rno04070: Phosphatidylinositol signaling system, rno04720: Long-term potentiation, rno04010: MAPK signaling pathway, rno04310: Wnt signaling pathway and rno04020: Calcium signaling pathway. Compared with group M, 5 KEGG pathway terms were down-regulated in group M+EA, including rno04080: Neuroactive ligand-receptor interaction, rno04270: Vascular smooth muscle contraction, rno04916: Melanogenesis, rno0420: Calcium signaling pathway and rno00350: Tyrosine metabolism, as shown in Fig 3B and Table 5. Notably, while the gene expression in phosphatidylinositol signaling system pathway was down-regulated in group M compared with group C, it was up-regulated after the EA treatment. Besides, while KEGG pathways neuroactive ligand-receptor interaction, vascular smooth muscle contraction, melanogenesis and calcium signaling pathway were up-regulated in the sub-acutely model group, they were all down-regulated after the EA treatment. The results reveal that EA treatment can affect the expression of genes in these KEGG pathways, and reversing the gene expression changes in these pathways can be regarded
Table 3. The most enriched GO terms between group M and group C.

| Biological Process                                      | Cellular Component                          | Molecular Function                              |
|---------------------------------------------------------|---------------------------------------------|-------------------------------------------------|
| PROTEIN_IMPORT INTO NUCLEUS_TRANSLOCATION               | TRANS_GOLGI_NETWORK_TRANSPORT_VESICLE       | KINASE_ACTIVATOR_ACTIVITY                       |
| INTERACTION_WITH_HOST                                   | TRANSPORT_VESICLE                           | SERINE_TYPE_ENDOPEPTIDASE_ACTIVITY               |
| HEMOSTASIS                                              | ENDOCYTIC_VESICLE                           | RAS_GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY   |
| ACUTE_INFLAMMATORY_RESPONSE                            | NUCLEAR_CHROMATIN                           | VITAMIN_BINDING                                 |
| REGULATION_OF_RA_D_PROTEIN_SIGNAL_TRANSDUCTION          | BASOLATERAL_PLASMA_MEMBRANE                 | SERINE_TYPEPEATIDASE_ACTIVITY                    |
| CDCA4_PROTEIN_SIGNAL_TRANSDUCTION                       | LAMELLIPODIUM                               | SERINE_HYDROLASE_ACTIVITY                       |
| NLS_BEARING_SUBSTRATE_IMPORT_INTO_NUCLEUS              | OUTER_MEMBRANE                              | GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY       |
| SMOOTH_MUSCLE_CONTRACTION_GO_0006939                    | GOLGI_ASSOCIATED_VESICLE                    | METABOTROPIC_GLUTAMATIGABA_B_LIKE_RECEPTOR_ACTIVITY |
| BLOOD_COAGULATION                                       | EARLY_ENDOSOME                              | CARBON_CARBON_LYASE_ACTIVITY                     |
| COAGULATION                                              | SECRETORY_GRANULE                           | GABA_RECEPTOR_ACTIVITY                           |
| WOUND_HEALING                                           | NUCLEAR_MEMBRANE_PART                       | OXIDOREDUCTASE_ACTIVITY_ACTIVATING_PEROXIDE_AS_ACCEPTOR |
| PROTEIN_IMPORT INTO NUCLEUS                             | TIGHT_JUNCTION                              | RHO_GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY   |
| REGULATION_OF_SMALL_GTPASE_MEDIATED_SIGNAL_TRANSDUCTION | CONDENSED_CHROMOSOME                        | INWARD_RECTIFIER_POTASSIUM_CHANNEL_ACTIVITY      |
| NUCLEAR_IMPORT                                          | CENTROSOME                                  | NEUROPEPTIDE_HORMONE_ACTIVITY                    |
| REGULATION_OF_BODY_FLUID_LEVELS                         | APICAL_JUNCTION_COMPLEX                     | PHOSPHATE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY     |
| POSITIVE_REGULATION_OF_TRANSPORT                        | APICALOCLATE_categorical_PLASMA_MEMBRANE     | MRNA_BINDING                                    |
| NEGATIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS | PORE_COMPLEX                                | NEUROPEPTIDE_RECEPTOR_ACTIVITY                   |
| NEGATIVE_REGULATION_OF_CELL_CYCLE                       | CHROMATIN                                   | CHROMATIN_BINDING                               |
| FEEDING_BEHAVIOR                                        | CLATHRIN_COATED_VESICLE                     | NEUROPEPTIDE_BINDING                            |
| MITOTIC_SISTER_CHROMATIDSEGREGATION                     | ORGANELLE_OUTER_MEMBRANE                    | SMALL_GTPASE_BINDING                            |

https://doi.org/10.1371/journal.pone.0191623.t003
Table 4. The most enriched GO terms between group M and group C.

| Biological Process                                      | Cellular Component                     | Molecular Function                          |
|---------------------------------------------------------|----------------------------------------|---------------------------------------------|
| POSITIVE_REGULATION_OF_TRANSPORT                        | VOLTAGE_GATED_POTASSIUM_CHANNEL_COMPLEX| VITAMIN_BINDING                             |
| INTERACTION_WITH_HOST                                   | TRANS_GOLGI_NETWORK_TRANSPORT_VESICLE  | AUXILIARY_TRANSPORT_PROTEIN_ACTIVITY        |
| ACUTE_INFLAMMATORY_RESPONSE                             | ENDOCYTIC_VESICLE                      | VOLTAGE_GATED_POTASSIUM_CHANNEL_ACTIVITY    |
| POTASSIUM_ION_TRANSPORT                                 | ENDOosome                               | METABOTROPIC_GLUTAMATE_GABA_ReL_RECEPTOR_ACTIVITY |
| CELL_FATE_COMMITMENT                                    | EARLY_ENDOSOME                          | POTASSIUM_CHANNEL_ACTIVITY                  |
| NEGATIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS | VESICULAR_FRACTION                     | GUANYL_NUCLEOTIDE_EXCHANGE_FACTOR_ACTIVITY   |
| REGULATION_OF_TRANSFORMING_GROWTH_FACTOR_BETA_RECEPTOR_Signaling_PATHWAY | COLLAGEN                              | INWARD_RECTIFIER_POTASSIUM_CHANNEL_ACTIVITY |
| DIGESTION                                                | MICROSOME                              | KINASE_ACTIVATOR_ACTIVITY                   |
| FEEDING_BEHAVIOR                                         | CENTROSOME                             | LOW_DENSITY_LIPOPROTEIN_BINDING             |
| ORGANELLE_LOCALIZATION                                  | GOLGI_ASSOCIATED_VESICLE               | NEUROPEPTIDE_HORMONE_ACTIVITY               |
| MONOVALENT_INORGANIC_CATION_TRANSPORT                  | TRANSPORT_VESICLE                      | SMAD_BINDING                                |
| ESTABLISHMENT_OF_ORGANELLELOCALIZATION                  | TIGHT_JUNCTION                         | PHOSPHOLIPASE_A2_ACTIVITY                   |
| REGULATION_OF_RAS_PROTEIN_SIGNAL_TRANSDUCTION           | CELL_SURFACE                           | POTASSIUM_CHANNEL_REGULATOR_ACTIVITY        |
| CELLULAR_RESPONSE_TO_NUTRIENT_LEVELS                    | BASOLATERAL_PLASMA_MEMBRANE            | SOLUTE_SODIUM_SYMPORTER_ACTIVITY            |
| CELLULAR_RESPONSE_TO_STRESS                             | MICROTUBULE_ORGANIZING_CENTER          | RAS_GTPASE_BINDING                          |
| TRIOACYLGLYCEROL_METABOLIC_PROCESS                      | APICAL_JUNCTION_COMPLEX                | COPPER_ION_BINDING                          |
| COFACTOR_TRANSPORT                                      | APICOLATERAL_PLASMA_MEMBRANE           | PEPTIDE_RECEPTOR_ACTIVITY                   |
| RESPONSE_TO_EXTRACELLULAR_STIMULUS                      | CLATHRIN_COATED_VESICLE                | LIPID_TRANSPORTER_ACTIVITY                  |
| REGULATION_OF_SMALL_GTPASE_MEDIATED_SIGNAL_TRANSDUCTION| EXTRACELLULAR_SPACE                    | SERINE_TYPE_ENDOPROTEASE_ACTIVITY           |
| CDC42_PROTEIN_SIGNAL_TRANSDUCTION                       | COATED_VESICLE                         | SODIUM_CHANNEL_ACTIVITY                     |

https://doi.org/10.1371/journal.pone.0191623.t004
as one of the mechanisms of EA against brain sub-acutely aging. Important genes involved in these five pathways will be further studied.

**Real-time PCR validation of DE genes which change the most in comparing group M vs C and M+EA vs M**

The expression levels of genes that changed the most in comparing groups M vs C and M+EA vs M were validated by Real-time PCR. In comparing group M vs C, genes *pth2r* (RefSeq: NM_031089.1) and *pmch* (RefSeq: NM_012625.1) were up-regulated and down-regulated for
| Comparing group | Term                                      | Count | %   | Genes                                                                 |
|-----------------|-------------------------------------------|-------|-----|----------------------------------------------------------------------|
| M vs C-Up       | Neuroactive ligand-receptor interaction   | 11    | 6.58| CALCR, HCRTR2, EDNRB, GABBR1, PTH2R, AGTR1A, GLRA2, P2RX2, GPR50, MC3R, TSHR |
| M vs C-Up       | Melanogenesis                             | 7     | 4.19| DCT, EDNRB, WNT3, PLCB4, ADCY7, GNAS, POMC                            |
| M vs C-Up       | Vascular smooth muscle contraction         | 5     | 2.99| RAMP3, PLCB4, ADCY7, AGTR1A, GNAS                                     |
| M vs C-Up       | Calcium signaling pathway                 | 6     | 3.59| EDNRB, PLCB4, ADCY7, AGTR1A, P2RX2, GNAS                              |
| M vs C-Up       | GnRH signaling pathway                    | 4     | 2.59| PLCB4, GNRH1, ADCY7, GNAS                                            |
| M vs C-Down     | Phosphatidylinositol signaling system      | 6     | 0.32| PIK3CD, SYNJ2, INPP5D, ITPKA, PIK3R2, PRKCB                           |
| M vs C-Down     | Leukocyte transendothelial migration       | 6     | 0.32| PTK2B, MMP9, PIK3CD, CXCL12, PIK3R2, PRKCB                           |
| M vs C-Down     | Aldosterone-regulated sodium reabsorption | 4     | 0.21| PIK3CD, NEUD4L, PIK3R2, PRKCB                                        |
| M vs C-Down     | Non-small cell lung cancer                | 4     | 0.21| E2F3, PIK3CD, PIK3R2, PRKCB                                          |
| M vs C-Down     | Glioma                                    | 4     | 0.21| E2F3, PIK3CD, PIK3R2, PRKCB                                          |
| M vs C-Down     | Pathways in cancer                        | 8     | 0.42| WNT2, WNT10A, E2F3, MMP9, PIK3CD, LAMC2, PIK3R2, PRKCB               |
| M vs C-Down     | B cell receptor signaling pathway          | 4     | 0.21| PIK3CD, INPP5D, PIK3R2, PRKCB                                        |
| M vs C-Down     | Small cell lung cancer                    | 4     | 0.21| E2F3, PIK3CD, LAMC2, PIK3R2                                          |
| M+EA vs M-Up    | Phosphatidylinositol signaling system      | 11    | 2.10| PIK3CD, PIP5KI, SYNJ2, DGKZ, PRKCG, INPP5D, PLCB1, ITPKA, PIP4K2C, ITPR1, PRKCB |
| M+EA vs M-Up    | rno04720: Long-term potentiation           | 9     | 1.72| GRIA2, CAMK4, GRIA1, PPP1R1A, GRIN1, PRKCG, PLCB1, ITPR1, PRKCB     |
| M+EA vs M-Up    | MAPK signaling pathway                    | 18    | 3.44| MEF2C, NLR, MRAS, CACNB1, MAP4K1, NR4A1, PRKCG, CACNG3, MAPK10, HSPA1B, PRKCB, DUSP5, FOS, BDNF, RASGRF2, DUSP1, DUSP9, DUSP6 |
| M+EA vs M-Up    | Wnt signaling pathway                     | 12    | 2.29| WNT2, TBL1XR1, WNT10A, WNT16, PRICKLE1, CCND2, NLR, PRKCG, MAPK10, WNT9A, PLCB1, PRKCB |
| M+EA vs M-Up    | Calcium signaling pathway                 | 13    | 2.49| GNA14, GRIN1, PRKCG, ITPKA, ITPR1, PRKCB, ADB3, CAMK4, PTK2B, PDE1A, PLCB1, ADRA1D, HTR5B |
| M+EA vs M-Up    | Endocytosis                               | 13    | 2.49| GIT1, PIP5KI, VPS37B, HSPA1B, SRC, ADB3, ACP3, PSD, NEDD4L, AGAP2, DNAM1, COI20A1, SH3GL1 |
| M+EA vs M-Up    | Glycosphingolipid biosynthesis             | 4     | 0.76| ST3GAL5, ST8SIA5, SIAT7E, B4GALNT1                                    |
| M+EA vs M-Up    | Fcga mediated phagocytosis                | 8     | 1.53| WASF1, PIK3CD, PIP5KI, PRKCG, INPP5D, PRKCE, DNAM1, PRKCB            |
| M+EA vs M-Up    | Long-term depression                      | 7     | 1.34| GRIA2, GRIA1, GRIA3, PRKCG, PLCB1, ITPR1, PRKCB                     |
| M+EA vs M-Up    | Inositol phosphate metabolism             | 6     | 1.14| PIK3CD, PIP5KI, SYNJ2, PLCB1, ITPKA, PIP4K2C                        |
| M+EA vs M-Up    | Neuroactive ligand-receptor interaction    | 14    | 2.6 | GRIN1, GRIA3, VIPR1, SSTR4, ADBR3, SSTR2, CHRM4, GRIA2, SSTR1, GRIA1, MAS1, ADRA2C, ADRA1D, HTR5B |
| M+EA vs M-Up    | Axon guidance                             | 9     | 1.72| EPHB6, EPHA6, SEMA7A, SEMA3E, EFNA3, ROBO2, CXCL12, SLIT1, SLIT3   |
| M+EA vs M-Up    | Melanogenesis                             | 7     | 1.34| WNT2, WNT10A, WNT16, PRKCG, WNT9A, PLCB1, PRKCB                    |

(Continued)
the most with the normalized expression level 6.643 and -6.64 respectively. In comparing group M+EA vs M, the expression level variation of genes kcnj2 (RefSeq: NM_017296.1) and pth2r (RefSeq: NM_031089.1) were up-regulated and down-regulated the most significant and the normalized expression levels were 6.193 and -6.643. It is noteworthy that gene pth2r was up-regulated significantly in the model group compared with control group, but the expression of this gene back to normal after EA treatment. Description of pth2r showed that it encodes parathyroid hormone 2 receptor. Since the accumulation of parathyroid hormone is associated with aging, the regulation of parathyroid hormone maybe another mechanism of EA towards sub-acutely aging. The real-time PCR results reveals that expression trend of pth2r, pmch (RefSeq: NM_012625.1) and kcnj2 (RefSeq: NM_017296.1) were consistent with the cDNA microarray data, as can be seen in Fig 4.

**Discussion**

Acupuncture is an effective alternative to drugs for the treatment of various kinds of disease by inserting thin needles deeply into the skin at specific points on the body. EA is a type of acupuncture wherein needles are attached to an apparatus that produces continuous electric pulses. As a traditional therapy, acupuncture has been used in China for thousands of years and these years began to gain increasing worldwide attention also. It is no doubt that acupuncture can improve or cure diseases [34]. However, the molecular mechanisms of how acupuncture effect have not been totally illustrated yet and need to be further studied.

According to previous report, acupuncture showed beneficial effect on anti-aging and age-related diseases [9]. As known, aging is an enormously complex phenomenon which involves immunosenescence, cognitive decline and so on [8]. In the field of brain, several genes have

### Table 5. (Continued)

| Comparing group | Term                              | Count | %    | Genes                                                                 |
|-----------------|-----------------------------------|-------|------|----------------------------------------------------------------------|
| M+EA vs M-Up    | Regulation of actin cytoskeleton   | 11    | 2.10 | GIT1, CHRM4, ARHGGEF7, MRAS, BAIAP2, WASF1, PIK3CD, ITGA11, PIP5K1C, ACTN2, PIP4K2C |
| M+EA vs M-Up    | Adherens junction                  | 6     | 1.14 | BAIAP2, NLK, WASF1, LMO7, ACTN2, SRC                                 |
| M+EA vs M-Up    | Tight junction                     | 8     | 1.53 | MRAS, MYH3, MYH2, PRKCG, ACTN2, PKRCE, SRC, PKRCB                    |
| M+EA vs M-Up    | Circadian rhythm                   | 3     | 0.57 | Npas2, PER2, PER1                                                    |
| M+EA vs M-Up    | Focal adhesion                     | 10    | 1.91 | CCND2, PIK3CD, ITGA11, RELN, LAMC2, PRKCG, ACTN2, MAPK10, SRC, PKRCB |
| M+EA vs M-Down  | Neuroactive ligand-receptor interaction | 19 | 4.13 | CALCRL, PTH2R, DRD2, GLRA2, NTSR1, GRM1, HCRTR2, EDNRB, GRM4, P2RX6, PRLR, AGTR1A, HTR7, P2RY1, P2RX2, AVPR1A, GPR50, MC3R, TSHR |
| M+EA vs M-Down  | Vascular smooth muscle contraction | 11    | 2.39 | RAMP3, PRKQ, PLCB4, ADCY7, AGTR1A, GNA12, MRVI1, AVPR1A, PKRCH, GNAS, PKRC |
| M+EA vs M-Down  | Melanogenesis                      | 9     | 1.96 | DCT, EDNRB, WNT3, PLCB4, ADCY7, WNT9B, GNAS, POMC, ASIP              |
| M+EA vs M-Down  | Calcium signaling pathway          | 12    | 2.61 | GNAL, EDNRB, P2RX6, PLCB4, ADCY7, AGTR1A, HTR7, P2RX2, AVPR1A, GNAS, NTSR1, GRM1 |
| M+EA vs M-Down  | Tyrosine metabolism                | 4     | 0.87 | DCT, DDC, TH, AOX1                                                  |

[https://doi.org/10.1371/journal.pone.0191623.t005](https://doi.org/10.1371/journal.pone.0191623.t005)
been proved to be related with aging. For example, the down-regulate of gene Mdm-2, a kind of apoptosis depressed gene can place a premium on apoptosis indirectly and causes neuron loss [35]. Besides, Kinesin family member 5C (Kif5C), expressed almost exclusively in murine brain, was also related with axonal elongation the maintenance of motor neurons [36, 37]. Our previous study demonstrate that EA at Guanyuan (CV 4), Zusanli (ST 36) and Baihui (DU 20) could delay aging by regulating T cells in proliferation, secretion of IL-2 and its receptor and improve the expression of CD8\(^+\),CD28\(^+\)[26–27]. Moreover, content of IL-1\(\beta\) and IL-6 of serum and the expression of apoptosis genes can also regulated by EA [28]. According to the basic theory of traditional Chinese medicine, we should take the body as a whole in curing a disease and that is actually the essence of acupuncture therapy. Thus it is possible that acupuncture therapy may cure disease by bringing the expression change of a large sum of genes.

D-galactose induced aging model was established in 1991 by a Chinese scholar named Gong Guoqing. This aging model can be obtained by D-Gal s.c. (D-Gal) intraperitoneal injection continuously, which is convenient, cheap and stable so that it has been used widespread [29]. The physiological changes of multiple organizations, multiple organ and multiple levels brought by this model were similar to those brought by natural aging. For example, the weight of D-Gal aging model rats decreased significantly (P<0.01) compared with the control group. The serum content of SOD decreased while that of MDA increased (P<0.05). Besides, the atrophy of thymus and the gain of weight of spleen were also brought by this model. The changing levels were similar to natural aging rats [29, 38–39]. In this study, in order to investigate the molecular mechanisms of sub-acutely aging and demonstrate the effect of electroacupuncture (EA) on the sub-acutely aging brain, the microarray analysis was explored to research the molecular mechanisms of EA in improving sub-acutely aging from the whole gene expression aspect.

![Real-time PCR analysis of genes pmch, pth2r and kcnj2](https://doi.org/10.1371/journal.pone.0191623.g004)

**Fig 4.** Real-time PCR analysis of genes pmch, pth2r and kcnj2. A: the expression of pmch and pth2r in group C and group M; B: the expression of kcnj2 and pth2r in group M+EA and group M. The mRNA abundance was normalized using the housekeeping gene β-actin, and the relative expression levels were calculated using the \(2^{ΔΔCt}\) method. Three biological replicates were performed.
gene expression levels. In future researches, we may measure the gene expression levels for each of 10 samples and then use average gene expression level as the estimate of the expression level for this gene. In this way, we could not only get accurate estimate of gene expression level, but also get an estimate of between sample variation.

4052 DE genes were identified among three samples. As can be seen in Fig 1, the gene expression profile of the sub-acutely model group changed significantly compared with the control group, and the changes were brought by the effect of aging. Meanwhile, the gene expression also changed after the EA treatment, the gene expression profile of M+EA group was more similar to that of control group comparing with the model group. This result demonstrates that EA can bring the gene expression change in the hippocampus of sub-acutely rats in the whole aspect.

Subsequently, more analyses were carried out to classify DE genes and select important genes related with EA treatment. According to Fig 2A, comparing groups M vs C and M+EA vs M have 445 common DE genes. Further study reveals that among these 445 genes, 346 were down-regulated and 78 were up-regulated in group C and group M+EA compared with group M. This means EA treatment can reverse the expression change of 424 DE genes brought by the sub-acutely aging. Besides, 538 DE genes were specific to comparing group M+EA vs M, and these change were brought by the EA treatment (Fig 3B and 3C). The GO and KEGG pathway enrichment was then performed to category the DE genes in comparing groups M vs C and M+EA vs M. In comparing group M vs C, 5 KEGG pathways were up-regulated and 9 were down-regulated. While in comparing group M+EA vs M, 18 KEGG pathways were up-regulated and 5 were down-regulated. Further analysis reveals that EA treatment can reverse the change of five KEGG pathways caused by the sub-acutely aging, that are: phosphatidylinositol signaling system pathway, neuroactive ligand-receptor interaction, vascular smooth muscle contraction, melanogenesis and calcium signaling pathway. DE genes involved in these pathways were listed in Table 5. Changing of these pathways can be regarded as another mechanism of EA towards sub-acutely aging.

In order to validate the correction of microarray data, the gene expression levels of ptth2r (RefSeq: NM_031089.1), pmch (RefSeq: NM_012625.1) and kcnj2 (RefSeq: NM_017296.1) were measured by real-time PCR. In comparing groups M vs C and M+EA vs M, these genes were up-regulated and down-regulated for the most. Noteworthy, ptth2r was the most significant up-regulated gene in comparing group M vs C; it was also the most significant down-regulated gene in comparing group M+EA vs M, which means the expression of ptth2r back to normal after EA treatment. Gene annotation showed that this gene is the parathyroid hormone 2 receptor encoding gene. Since the accumulation of parathyroid hormone is associated with aging, ptth2r should be regarded as an important gene related with the sub-acutely aging. In group M+EA, the expression level of ptth2r down regulated significantly to the level of that in group C, meaning that EA treatment has reverse the expression change of gene ptth2r, which was another mechanism of EA against sub-acutely aging in rat’s brain.

To sum up, this study first compared and analyzed the differences of gene expression profile in the hippocampus of sub-acutely aging rats before and after the EA treatment. Important genes associated with sub-acutely aging and EA effects were identified, which contribute to our knowledge on the molecular mechanisms of EA towards sub-acutely aging and pave the way for the future study.

Supporting information

S1 Table. The comparison of gene expression profile between different samples.
(XLS)
S2 Table. Common elements in "M vs C-UP" and "M vs M+EA-UP"; Common elements in "M vs C-DOWN" and "M vs M+EA-DOWN".

(XLSX)

S1 Fig. The process of the experiment.

(TIF)

Acknowledgments

This work was supported by 2016 scientific research guidance projects of Hubei province (NO. B2016111).

Author Contributions

Conceptualization: Jianmin Liu.
Data curation: Jianmin Liu.
Formal analysis: Jing Liu.
Funding acquisition: Jianmin Liu.
Investigation: Guang’an Wang.
Methodology: Guangya Liu.
Project administration: Hua Wang.
Resources: Huanjiao Zhou.
Supervision: Hua Wang.
Validation: Fengxia Liang.
Visualization: Yun Fan.
Writing – original draft: Jing Liu.
Writing – review & editing: Jing Liu.

References

1. Yankner B A, Tao L, Loerch P. Annual Review of Pathology Mechanisms of Disease. The aging brain. 2008; 3:41–66.
2. Turnheim K. When drug therapy gets old: pharmacokinetics and pharmacy odynamics in the elderly. Experimental Gerontology. 2003; 38:843–853. PMID: 12915206
3. Homeier D. Aging: Physiology, Disease, and Abuse. Clinics in Geriatric Medicine. 2014; 30: 671–686. https://doi.org/10.1016/j.cger.2014.08.001 PMID: 25439635
4. Corinne S, Rafał W, Yaroslav I, Gerlinde M, Igor K, Olga K. Immunosenescence is associated with altered gene expression and epigenetic regulation in primary and secondary immune organs. Frontiers in Genetics, 2013; 4:211. https://doi.org/10.3389/fgene.2013.00211 PMID: 24151501
5. Pawelec G, Larbi A. Immunity and ageing in man: Annual Review 2006/2007. Experimental Gerontology. 2008; 43:34–8. https://doi.org/10.1016/j.exger.2007.09.009 PMID: 17977683
6. APAArnold CR, Pritz T, Brunner S, Knabb C, Salvenmoser W, Holzwarth B, et al. T cell receptor-mediated activation is a potent inducer of macroautophagy in human CD8 + CD28 +, T cells but not in CD8 + CD28 −, T cells. Experimental Gerontology. 2014; 54:75–83. https://doi.org/10.1016/j.exger.2014.01.018 PMID: 24468331
7. Stahl E C, Brown B N. Cell Therapy Strategies to Combat Immunosenescence. Organogenesis. 2015; 11:00–00.
8. Tanzi R E, Bertram L. New Frontiers in Alzheimer's Disease Genetics. Neuron. 2001; 32: 181–184. PMID: 11689889
9. Ding X, Yu J, Tao Y, Yu F, Han J. Acupuncture regulates the aging-related changes in gene profile expression of the hippocampus in senescence-accelerated mouse (SAMP10). Neuroscience Letters. 2006; 399: 11–16. https://doi.org/10.1016/j.neulet.2006.01.067 PMID: 16516385

10. Sarkar S, Das N. Mannosylated liposomal flavonoid in combating age-related ischemia–reperfusion induced oxidative damage in rat brain. Mechanisms of Ageing & Development. 2006; 127: 391–397.

11. Brasnjevic I, Rutten BPF, Kooten IAJV, Korr H, Steinbusch HWJ, Schmitz C. P4-347: combating age-related accumulation of (n) dna damage in the brain by caloric restriction or transgenic overexpression of endogenous antioxidants: which approach is more effective?. Alzheimers & Dementia. 2006; 2: S619.

12. Roberts SB, Pi-Sunyer X, Kuller L, Lane M A, Ellison P, Prior J C, et al. Physiologic effects of lowering caloric intake in nonhuman primates and nonobese humans. Journals of Gerontology. 2001; 56 Spec No 1(Supplement 1): 66–75.

13. Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, et al. Trends in alternative medicine use in the United States, 1990–1997: results of a follow-up national survey. JAMA the Journal of the American Medical Association. 1998; 280: 1569–75. PMID: 9820257

14. Liang F, Cooper EL, Wang H, Jing X, Quispe-Caballlas JG, Kondo T. Acupuncture and Immunity. Evidence-based Complementary and Alternative Medicine. 2015; 2015: 530–537.

15. Jiang QQ, Wu HJ, Zhang P, Zhao C, Liu HR, Zhang LS. Emplay and prepare of model of animal test in the research of acupuncture. Modern Rehabilitation. 2000; 4: 1690–1691.

16. Wu HL, Li XQ, Wang X. The immediate effect on blood pressure of acupuncture at Taichong (LR 3) in 65 cases of hypertension patient with hyperactivity of Liver-yang. Zhong Yi Za Zhi (J Trad Chin Med, Chin). 2008; 49:622–624.

17. Yang HY, Zhong XH, Liu TY, Kuai L, Gao M. Impact of different emulated acupuncture-needle manipulations on blood pressure and myocardial angiotensin II content in spontaneous hypertensin rats. Zhen Ci Yan Ju (Acupunct Res, Chin). 2008; 33: 186–190.

18. Wang JY, Tang CZ, He ZQ, Zhang J, Hao MF, Ma CM, et al. Effect of Moderate Acupuncture-stimulation of “Taichong”(LR 3) on Blood Pressure and Plasma Endothelin-1 Levels in Spontaneous Hypertension Rats. Zhen Ci Yan Jiu (Acupunct Res, Chin). 2011; 36: 36–39.

19. Tang HT, Wang H. Effect of Double Reinforcing-one Unblocking Acupuncture on Immunologic Function in Senile Patients with Yang Deficiency. Shanghai J Acu-mox. 2010; 29: 573–575.

20. Wang H, Mao HF, Liu JM, Hao Q, Tang HT, Li J. Effect of Electroacupuncture on Spleen Lymphocyte Apoptosis-related Gene Expression in Aging Rats with Yang Deficiency. Shanghai J Acu-mox. 2012; 37: 266–270.

21. Liu JM, Wang H, Hao MF, Hao Q, Hong YQ, Peng R, et al. Effect of “Double Reinforcing and One Unblocking” Electroacupuncture on Lymphocyte Apoptosis and Inflammatory Factors IL-6 and IL-1p in a Rat Model of Senile Yang Deficiency. Shanghai J Acu-mox. 2014; 33: 66–69.

22. Zhu MF, Xing X, Lei S, Wu JN, Wang LC, Huang LQ. Electroacupuncture at Bilateral Zusanli Points (ST36) Protects Intestinal Mucosal Immune Barrier in Sepsis. Evidence-based Complementary and Alternative Medicine. 2015; 2015.

23. Mcdonald J L, Cripps A W, Smith P K. Mediators, Receptors, and Signalling Pathways in the Anti-Inflammatory and Antihyperalgesic Effects of Acupuncture. Evidence-based complementary and alternative medicine: eCAM. 2015; 2015.

24. Yu J, Liu C, Zhang X, Han J. Acupuncture improved cognitive impairment caused by multi-infarct dementia in rats. Physiology & Behavior. 2005; 86: 434–441.

25. Cheng H, Yu J, Jiang Z, Zhang X, Liu C, Peng Y. Acupuncture improves cognitive deficits and regulates the brain cell proliferation of SAMP8 mice. Neuroscience Letters. 2008; 432:111–116. https://doi.org/10.1016/j.neulet.2007.12.009 PMID: 18215464

26. Liu JM, Liang FX, Li J, Liu XQ, Tang HT, Wu S, et al. Influence of Electroacupuncture of Guanyuan (GV 4) and Zusanli (ST 36) on the Immune Function of T Cells in Aging Rats. Acupuncture Research. 2009; 34: 242–247. PMID: 19916287

27. Xiao L, Wang GA, Wang H. Effects of electroacupuncture of “Shuanggu Yitong” prescription on the T lymphocyte subset proportions in aging rats. Chinese Acupuncture & Moxibustion. 2012; 32: 435–439.

28. Liu JM, Wang H, Mao HF, Hao Q, Hong YQ, Peng R, et al. Effect of “Double Reinforcing and One Unblocking” Electroacupuncture on Lymphocyte Apoptosis and Inflammatory Factors IL-6 and IL-1β in a Rat Model of Senile Yang Deficiency. Shanghai Journal of Acupuncture-Moxibustion. 2014; 33: 66–69.

29. Zhu YZ, Zhu HG. Establishment and measurement of D-galactose induced aging model. Fudan University Journal Of Medical Sciences. 2007; 34: 617–619.
30. Marioni JC, Mason CE, Mane SM, Stephens M, Gilad Y. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome Res. 2008; 18:1509–1517. https://doi.org/10.1101/gr.079558.108 PMID: 18550803

31. Weng L, Dai H, Zhan Y, He Y, Stepaniants SB, Bassett DE. Rosetta error model for gene expression analysis. Bioinformatics. 2006; 22: 1111–1121. https://doi.org/10.1093/bioinformatics/btl045 PMID: 16522673

32. Deng W, Wang Y, Liu Z, Cheng H, Xue Y. Hemt: a toolkit for illustrating heatmaps. Plos One. 2014; 9: e111988–e111988. https://doi.org/10.1371/journal.pone.0111988 PMID: 25372567

33. Oliveros J.C. VENNY. An interactive tool for comparing lists with Venn Diagrams. 2007. Available from: http://bioinfogp.cnb.csic.es/tools/venny/index.html.

34. Organization WH. Acupuncture: review and analysis of reports on controlled clinical trials. Parkinsonism & Related Disorders. 2002;(Suppl 2): S163.

35. Mendrysa SM, Mcelwee MK, Michalowski J, OLeary KA, Young KM, Perry ME. mdm2 is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Molecular & Cellular Biology. 2003; 23:462–72.

36. Aizawa H, Sekine Y, Takemura R, Zhang Z, Nangaku M, Hirokawa N. Kinesin family in murine central nervous system. Journal of Cell Biology. 1992; 119: 1287–1296. PMID: 1447303

37. Kanai Y, Okada Y, Tanaka Y, Harada A, Terada S, Hirokawa N. KIF5C, a novel neuronal kinesin enriched in motor neurons. Journal of Neuroscience the Official Journal of the Society for Neuroscience. 2000; 20: 6374–6384.

38. Hai F, Qiu J, Song. Behavioural study of the D-galactose induced aging model in C57BL/6J mice. Behavioural Brain Research. 2005; 157:245–251. https://doi.org/10.1016/j.bbr.2004.07.003 PMID: 15639175

39. Zhang XH, Yang J, Zhao JM. The effect of replenishing qi to invigorate the spleen recipe on free radical metabolism and immune function of aging mice induced by D-gal. Chinese Journal of Gerontology. 2005; 8: 933–934.