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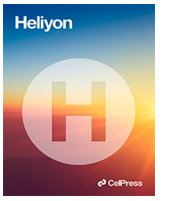

Effect of acupuncture therapy on vaccine-induced immune response in D-galactose-induced aging rats☆,☆☆

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ABSTRACT

Objective: This study aimed to explore whether acupuncture and moxibustion can enhance the immune response by increasing the expression of the endogenous adjuvant HSP70 mRNA.
Method: Forty Wistar rats were divided into four groups: model immune acupuncture group (A), model immune control group (B), normal immune acupuncture group (C), and normal immune control group (D). Model immune groups A and B were induced by injecting D-galactose for 6 weeks. Rats in groups A and C were then treated with low-frequency electroacupuncture (EA) at Zusanli (ST36), Guanyuan (CV4), and Baihui (GV20) and moxibustion for 3 weeks. Subsequently, all rats were observed for 2 more weeks. At the 12th week, diphtheria antitoxin titers were determined using the Vero cell trace neutralization method, $CD4^+T/CD8^+T$ cell ratios in peripheral blood were examined by flow cytometry, and the relative expression of spleen cell HSP70 mRNA was measured by RT-PCR.
Results: Compared with the normal immune control, the diphtheria antitoxin titer, $CD4^+T/CD8^+T$ cell ratio, and expression of spleen cell HSP70 mRNA significantly decreased in the model immune control group ($P < 0.01$). However, the model immune acupuncture group showed a significant increase in antitoxin titer ($P < 0.01$) and elevated $CD4^+T/CD8^+T$ cell ratio and HSP70 mRNA expression ($P < 0.05$) after EA and moxibustion intervention.
Conclusion: Acupuncture and moxibustion may enhance the humoral immune response (diphtheria antitoxin titer) and cellular immune response (peripheral blood $CD4^+T/CD8^+T$ cell ratio) by increasing the expression of the endogenous adjuvant HSP70 mRNA, suggesting that acupuncture may serve as a new vaccine adjuvant.

1. Introduction

Immunosenescence refers to the diminished ability of an aging immune system to produce an appropriate and effective response to challenges. This immune dysfunction may manifest as increased susceptibility to infections, cancer, autoimmune diseases, and vaccine

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failure [1]. The efficacy of vaccines primarily depends on the strength of the immune response, and immunocompromised individuals, such as immunosenescent older adults with cellular and humoral immune deficiencies, are at the greatest risk of inadequate protection [2]. To overcome immunosenescence, strategies such as vaccine adjuvants are being developed [3].

For the first time, the author proposes that acupuncture can be used as a novel adjuvant in vaccine research. Firstly, acupuncture is considered one of the essential methods to prevent infectious diseases. Secondly, the mechanism by which acupuncture enhances the effect of antigen-presenting cells and lymphocytes [4] is similar to that of adjuvants. Thirdly, it has been confirmed that moxibustion could enhance the vaccine immune response to S. aureus bacteria and improve antibody titers in rabbits [5]. Preliminary experiments have also shown that acupuncture and moxibustion can improve DTaP vaccine antibody titers in normal rats, with the optimal vaccine concentration being 1/20 of the human dose. Acupuncture is safe and has no side effects. Additionally, DTap vaccination has been reported to be effective in adolescents and adults [6], but antibody levels drop rapidly in vaccinated individuals [7], Furthermore, DTap vaccination may cause gastrointestinal reactions (nausea, vomiting, diarrhea, and stomach pain) [8]. Therefore, acupuncture may potentially improve the body's immune response to the vaccine by acting as an adjuvant.

Based on traditional Chinese medicine theory and previous experiments, acupuncture at Zusanli (ST36), Guanyuan (CV4), and Baihui (GV20), also known as "Double-reinforcing and One-unblocking" acupuncture therapy, has a special effect on regulating the immune system and postponing immunosenescence in rats [9–12]. Mitochondrial damage is a key marker of aging, and damaged mitochondria can regulate innate immunity through REDOX signaling or direct inflammasome activation [13]. Oxidative stress is believed to be a primary factor in neurodegenerative diseases, as well as in the normal aging process [14]. Mice injected with D-galactose have been used as an animal model of oxidative stress [15], and D-galactose-induced rat models have been widely used in aging and other research areas. Elevated levels of D-galactose cause ROS formation and reduce antioxidant enzyme activity in the brain, leading to cognitive dysfunction, brain aging, diminished motor function, and shortened lifespan, mimicking natural aging in rodents [16]. Thus, the model rats were induced by the injection of D-galactose in our research.

The widely used and mature DTaP vaccine (diphtheria, tetanus, and acellular pertussis combined vaccine, adsorbed) was applied in this study to investigate the vaccine adjuvant effect. Diphtheria antitoxin titer detection and $CD4^+T/CD8^+T$ cell ratios are reliable indicators of the body's immune response to the DTaP vaccine. However, the effects of electroacupuncture (EA) and moxibustion on these aspects have not been studied [17,18]. The strength of the immune response induced by the DTaP vaccine was assessed by detecting the diphtheria antitoxin neutralization titer in vaccinated mouse serum, showing a high correlation (+0.98) between in-vivo biological assays and in-vitro Vero cell assays [19]. In our research, D-galactose-induced model rats were vaccinated with the DTaP vaccine and treated with "Double-reinforcing and One-unblocking" acupuncture, aiming to explore whether acupuncture can enhance the humoral and cellular immune response levels by acting as an adjuvant and delay the aging process.

2. Materials and methods

2.1. Animals

Wistar rats, SPF Females, weighing 230g–280g (10 weeks old), were purchased from the Experimental Animal Research Center of Hubei Province SCXK. They were housed at 22 °C in a controlled environment and received 12 h of artificial light per day. All experiments conducted on these rats were approved by the Animal Experimental Committee of Hubei University of Chinese Medicine.

2.2. Experimental design

Forty Wistar rats were divided into four groups: model immune acupuncture group (A), model immune control group (B), normal immune acupuncture group (C), and normal immune control group (D). The D-galactose-induced model rats were subcutaneously injected with D-galactose at 350 mg/kg daily, while control rats received subcutaneous injections of 0.9 % saline daily for 6 weeks. At the 7th week, all rats were injected with DTaP vaccine, with a vaccine concentration in rats at 1/20 times the dose used in humans.

EA was applied at the acupuncture points ST36, CV4, and GV20 for 15 min each time using 0.30 × 25 mm needles (Suzhou Acupuncture & Moxibustion Appliance Co, China). The needles were inserted vertically into the muscle layer of CV4 and ST36 and horizontally into the subgaleal tissue of GV20 at a depth of about 2 mm. Needles at CV4 and ST36 on one side were connected to two electrodes of an electrostimulator (Han's Electric acupuncture therapeutic apparatus, Beijing Medical Instrument Factory, China). The points were electrically stimulated with successive low-frequency waves at 2 Hz and 1 mA. Moxibustion was performed using a handmade mox roll (0.6 cm in diameter) about 2 cm above the points CV4, GV20, and ST36 for 5 min each time, maintaining the skin temperature at 42 ± 1 °C. This treatment was repeated 6 times once a week and lasted for 3 weeks. Rats in the control group were fixed and suspended for 15 min in the same way but without treatment.

2.3. Diphtheria toxoid antibody titer detection

The response to diphtheria toxoid vaccine was measured by the Vero cell trace neutralization method. The diluted immune serum was mixed with 1/10000 LCD diphtheria toxin in 96-well cell culture plates. At the same time, standards for diphtheria antitoxin, diphtheria toxin control, and cell control were set. After incubating the immune serum and toxins for 1 h, Vero cell suspensions were added to the wells, which were then incubated for 6 days at 37 °C and 5 % CO_2. The production of cell lesions was observed, and normal cells were identified using inverted microscopes. The antibody titer was determined as the dilution multiples of half death cell holes, which were the holes in front of all cells' death holes. The geometric mean $\log_2 X$ was used for statistical analysis.

2.4. Flow cytometry detection and T cell subgroup

Three types of antibodies were added to 100 μL anticoagulant whole blood cells: Alexa Fluor-*anti*-CD3 monoclonal antibody 2 μL (concentration of 0.5 μg/μL, BD), FITC-*anti*-CD4 monoclonal antibody 2 μL (concentration of 0.5 μg/μL, BD), and PE-*anti*-CD8 monoclonal antibody 5 μL (concentration of 0.2 μg/μL, BD). The mixture was gently blended and kept in a dark place for 15 min. Then, 1 mL red blood cells were added and cultured for 5 min, followed by the addition of 3 mL PBS, and centrifuged at 1000 RPM/min for 5 min. The filtered cells were tested using the FACS Calibur flow cytometry instrument (Becton Dickinson).

2.5. Real-time reverse transcriptional polymerase chain reaction

The spleen tissues' RNA was extracted using the RNA simple total RNA extraction kit (Beijing Day Root, DP419). The splenic tissue was added with 1 mL lysate and treated with a homogenizer. The homogenized sample was placed at room temperature for 5 min, and then 200 μL chloroform was added, vigorously shaken for 15 s, and left at room temperature for 3 min. After centrifugation at 12000 rpm/min for 10 min at 4 °C, the upper colorless water phase was transferred to a new tube. Then, 0.5 times the volume of anhydrous ethanol was slowly added, mixed well, and transferred the solution and precipitation together to the adsorption column. The column was centrifuged for 30 s under the same conditions, and the waste liquid in the collection tube was discarded. Then, 500 μL of deproteinizing solution was added to the adsorption column, and it was centrifuged again for 30 s to remove the waste solution. Next, 700 μL bleach solution was added to the adsorption column, standing at room temperature for 2 min, and then centrifuged for 30 s, and the waste solution was discarded. The adsorption column was placed into a 2 mL collection tube, centrifuged for 2 min, and the total RNA of spleen cells was obtained after further treatment. RNA concentrations were determined at the 260/280 nm absorbance ratio. 3 μg RNA was reverse transcribed into first-strand complementary DNA (cDNA) using a cDNA synthesis first chain kit (Fermentas K1621) following the 2 steps of reverse transcription instructions provided by the manufacturer. The following thermal cycling protocol was used for reverse transcription: 42 °C for 60 min, 70 °C for 5 min termination reaction, in a 20 μL reaction volume. It was then cooled on ice for 5 min and stored at – 20 °C for later use.

Real-time reverse transcriptional polymerase chain reaction (RT-PCR) was performed with the Ht 7900 Fluorescent Quantitative PCR (ABI PRISM, USA), and the reaction mixture was SYBR Green Master Mix 10 μL (Roche, Company Ltd., Germany). The PCR protocol included an initial denaturation of 10 s at 50 °C, followed by 40 cycles of amplification for 10 min at 95 °C, 15 s at 95 °C, and 1 min at 58 °C. Duplicate samples were run for real-time RT-PCR, and amplification products were qualified using a standard calibration curve. Specific primers used for PCR are listed below:

HSP70 upstream primer: GCTCGAGTCCTACGCCTTCAATA.

HSP70 downstream primers: TCCTGGCACTTGTCCAGCAC;

Internal GAPDH upstream primer: GCAAGTTCAACGGCACAG.

Internal GAPDH downstream primers: GCCAGTAGACTCCACGACAT.

The relative mRNA expression level of HSP70 was analyzed by relative quantitative method, and the mRAN expression level of HSP70 was calculated by $\triangle$Ct value with GAPDH gene as the internal reference. Calculation formula: relative mRNA expression $= 2^{-\triangle Ct}$, $\triangle$Ct = sample mean value - internal reference Ct mean value.

2.6. Statistical analysis

Data were expressed as mean ± SDs. The trapezoidal rule was used to determine the area under the IPGTT curve (AUCg). Analysis of variance (ANOVA) with subsequent Tukey's test was employed to determine the significance of differences in multiple comparisons. A P-value of less than 0.05 was considered statistically significant. SPSS 16.0 statistical software was used for data processing, and analysis of variance was used for comparisons between two groups.

3. Result

3.1. Diphtheria antitoxin titer detection

After being injected with D-galactose for 6 weeks, rats in the model groups exhibited slow weight growth, sluggishness, fatigue, lethargy, poor curly appearance, withered and yellow fur, shiny coat, and aging symptoms. Diphtheria antitoxin titers were detected.

Table 1
Diphtheria antitoxin titer detection. (log_2X) (means ± SDs).

groups	n	Antibody titer
A (model immune acupuncture)	10	$4.4\pm1.51^{1)}$
B (model immune control)	10	$2.7\pm1.42^{2)}$
C (normal immune acupuncture)	10	$5.6\pm1.35^{3)}$
D (normal immune control)	10	4.5 ± 1.27

Compared with the model immune control group $^{1)}P < 0.01$; Compared with the normal immune control group $^{2)}P < 0.01$; Compared with the normal immune control group $^{3)}P < 0.05$.

As shown in Table 1, compared with the normal immune control group, the diphtheria antitoxin titer in the model immune control group significantly decreased ($P < 0.01$). However, the antitoxin titer in the model immune acupuncture group was significantly increased after EA and moxibustion intervention ($P < 0.01$). Moreover, it was also increased in the normal immune acupuncture group ($P < 0.05$).

3.2. $CD4^+T/CD8^+T$ cell ratio in peripheral blood

Fig. 1A shows that $CD3^+$ cells (T cells) account for about 43.3% of peripheral blood lymphocyte, and as shown in Table 2 and Fig. 1B, in $CD3^+$ cells, the $CD4^+T/CD8^+T$ cell ratio in the model immune control group significantly decreased compared to the normal immune control group ($P < 0.01$). However, the ratio increased in the model immune acupuncture group after EA and moxibustion intervention ($P < 0.05$).

Compared with normal immune control in group D, $CD4^+T/CD8^+T$ cells ratio in model immune control in group B was significantly lower. Compared with model immune control in group B, $CD4^+T/CD8^+T$ cells ratio in model immune acupuncture in group A was higher. Compared with normal immune control in group, $CD4^+T/CD8^+T$ cells ratio in normal immune acupuncture increased ($P < 0.05$).

3.3. Real-time quantitative PCR detection of HSP70 mRNA relative expression compared with GAPDH

3.3.1. The spleen cells agarose electrophoresis results HSP70 RT-PCR products

The total RNA concentrations of spleen cells were between 1000 and 3000 ng/μL in all groups, and 4 μL of RNA from each group was used for the reverse transcription experiment. Through agarose electrophoresis and comparison with the DNA marker (no. 70503 r), the PCR products were observed using a multiImager. Clear bands appeared at 105 bp, and the HSP70 gene amplification products were consistent with the results of the preliminary design. GAPDH bands appeared at 139 bp (Fig. 2).

3.3.2. Relative expression of spleen cells HSP70 mRNA (GAPDH)

As shown in Table 3, compared with the normal immune control group, the relative expression of spleen cells HSP70 mRNA in the model immune control group significantly decreased ($P < 0.01$). However, it increased in the model immune acupuncture group after EA and moxibustion treatment ($P < 0.05$). Moreover, compared with the normal immune control group D, the relative expression of

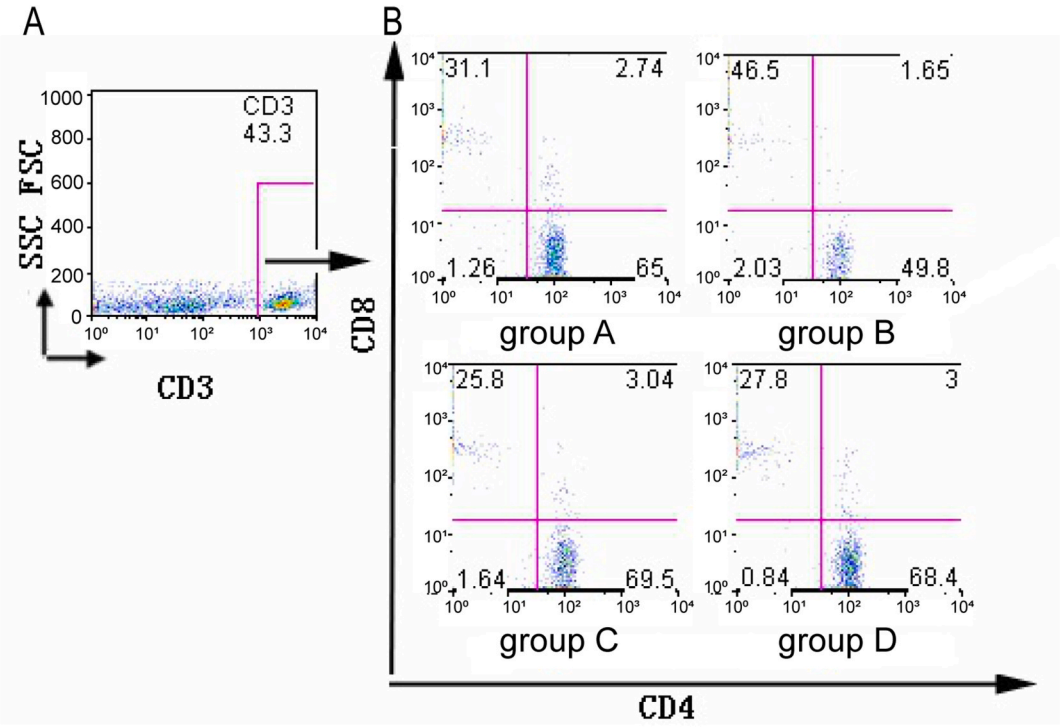

Fig. 1. Peripheral blood $CD4^+T/CD8^+T$ cells ratio, T cell ratio of flow cytometry double parameters a scatter diagram. (A): the red oval cells are the of percentage $CD3^+$ cells (T cells) in peripheral blood lymphocyte. (B): further analysis the proportion of cells in T cells. Left lower quadrant (LL) values is percentage of double negative cell ($CD8^-$, $CD4^-$) T cell, upper left quadrant (UL) values is the percentage of Y positive cells ($CD8^+$, $CD4^-$) T cell, and lower right quadrants (LR) values is the percentage of X axis is positive cell ($CD8^-$, $CD4^+$) T cell, upper right quadrant (UR) values is the percentage of double positive cells ($CD4^+$, $CD8^+$) T cells and $CD4^+T/CD8^+T$ cells ratio, T ratio calculation formula is: LR/+ UL.

Table 2
peripheral blood $CD4^+T/CD8^+T$ cells ratio (means ± SDs).

groups	n	$CD4^+T/CD8^+T$ cells ratio
A (model immune acupuncture)	10	$2.01\pm0.484^{1)}$
B (model immune control)	10	$1.66\pm0.391^{2)}$
C (normal immune acupuncture)	10	$2.33\pm0.443^{3)}$
D (normal immune control)	10	2.18 ± 0.388

Compared with model immune control group, $^{1)}P < 0.05$; Compared with normal immune control group, $^{2)}P < 0.01$; Compared with normal immune control group, $^{3)}P < 0.05$.

spleen cells HSP70 mRNA in the normal immune acupuncture group C increased slightly ($P > 0.05$).

4. Discussion

Immunosenescence decreases vaccine efficacy in older adults (age 65 and over). Previous reports have shown that the ability of the influenza vaccine to induce protection is related to age, with an efficacy between 70 % and 90 % in those under 65 years of age, but only 30–40 % for those over 65 years of age [20]. In our experimental results, the potency of diphtheria antitoxin in the DTaP vaccine was significantly reduced in D-galactose-induced rats compared to the normal control group. This suggests that the D-galactose-induced model rats exhibited a lower immune reaction to the vaccine effect. However, the application of the Double-reinforcing and One-unblocking acupuncture method improved the antibody titer. Acupuncture was shown to act as a vaccine adjuvant, enhancing the humoral immune reaction to the vaccine in D-galactose-induced rats. Furthermore, acupuncture treatment was found to enhance the titer of diphtheria antitoxin in normal rats.

Our previous experiments revealed that the DTaP vaccine liquid source had toxicity to the cells. Therefore, we utilized the proportion of T cell subgroups to indirectly observe the effect of cellular immune responses. T cell apoptosis rate increased in aging bodies, with $CD4^+T$ cells being more susceptible to apoptosis than $CD8^+T$ cells, leading to a significant reduction in their numbers, resulting in an inverse $CD4^+T/CD8^+T$ cells ratio [21].

The decrease of cellular immune responses in the aging body was associated with the inversed $CD4^+T/CD8^+T$ cells ratio. Our experiment found that D-galactose-induced model rats exhibited a lower $CD4^+T/CD8^+T$ cells ratio compared to the normal control group, leading to a decline in both cellular and humoral immune responses to vaccines in these rats. However, the application of the Double-reinforcing and One-unblocking acupuncture method showed potential in improving the cellular immune response to vaccines and postponing immune senescence, indicating its potential as a vaccine adjuvant. Although acupuncture treatment improved the ratio of $CD4^+T/CD8^+T$ cells, the differences observed were not statistically significant. This might be attributed to the immune system reaching a new balance five weeks after vaccination, making changes in the $CD4^+T/CD8^+T$ cells ratio less apparent.

Heat shock proteins (HSP) are molecular chaperones that play a critical role in regulating a variety of immune cells and ensuring protein homeostasis and integrity [22]. HSPs are produced when cells are subjected to sudden changes in temperature or other forms of stress. The release of HSPs can stimulate macrophages, activate dendritic cells, and promote the process of antigen presentation. HSPs have been known to play important roles in antigen presentation [23].

The body's resistance to stress and expression of heat shock proteins decrease with aging. Studies have shown a significant age-related decrease in the induction of Hsp70 after heat shock in both monocytes and lymphocytes [24,25]. Peripheral blood T cells in older individuals produced 66 % less HSP70 than in young individuals under thermal stress (42 °C) [26]. Additionally, the induction of Hsp70 by heat shock (43 °C, 1 h) decreased with age in adherent alveolar macrophages [27]. Furthermore, research suggests that the

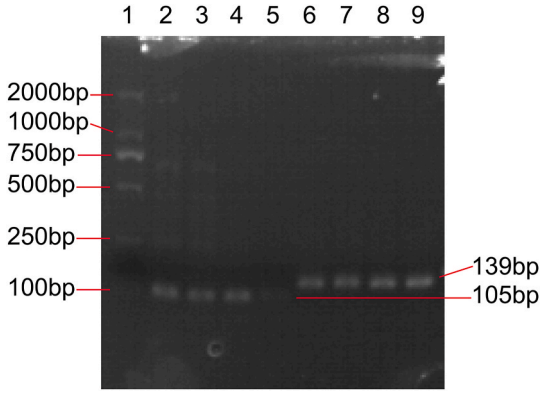

Fig. 2. The spleen cells of HSP70 and electrophoresis RT - PCR products. 1:DNA marker; 2: normal immune acupuncture group HSP70; 3: normal immune control group HSP70; 4: model immune acupuncture group HSP70; 5: model immune control group HSP70; 6: normal immune acupuncture group GAPDH; 7: normal immune control group GAPDH; 8: model immune acupuncture group GAPDH; 9: model immune control group GAPDH.

Table 3
Relative expression of spleen cells HSP70mRNA compared to GAPDH. (means ± SDs).

groups	n	relative expression of HSP70 mRNA
A model immune acupuncture	10	$0.925 \pm 0.448^{1)}$
B model immune control	10	$0.523 \pm 0.230^{2)}$
C normal immune acupuncture	10	$1.133 \pm 0.418^{3)}$
D normal immune control	10	1.082 ± 0.427

Compared with the model immune control group, $^{1)}P < 0.05$; Compared with the normal immune control group, $^{2)}P < 0.01$; Compared with the normal immune control group, $^{3)}P > 0.05$.

expression of HSP70 in 12-month-old rats was significantly lower than in younger 6-month-old rats, and even lower in 19-month-old rats compared to the 6-month-old rats [28]. The release of HSP is triggered by cellular stress and exposure to immune danger signals. Once released into the extracellular fluid or blood, HSP can bind to the cell surface, activate signals, and stimulate the uptake of antigenic peptides [29]. The peptide associated with HSP70 can effectively be absorbed by antigen-presenting cells [30] via CD91, LOX-1 [31,32], resulting in cross-presentation of the peptide by human leukocyte antigen (HLA) class I molecules. Extracellular HSP70 can heavily activate $CD4^+$T cells and transform their phenotype into a more cytotoxic phenotype, while $CD8^+$T cell functional properties are less affected by these proteins [29], potentially contributing to the shift observed in $CD4^+T/CD8^+$T cells ratio after acupuncture treatment.

The regulation of the immune system by acupuncture through HSP70 may be related to oxidative stress. Acupuncture has been shown not only to increase the activity of antioxidant enzymes and down-regulate the production of reactive oxygen species (ROS) but also to repair DNA, lipids, and proteins attacked by ROS and mediate apoptosis downstream of the ROS pathway [33]. HSP70 protein plays a central role in protein balance and is essential in improving resistance to various stress injuries, including oxidative stress. Research has indicated that acupuncture can enhance immune defense function, regulate inflammation, increase HSP70 expression, and protect the liver and heart from ischemia-reperfusion injury [34]. HSP70-peptide vaccines are currently used for the prevention of infectious diseases and tumors, and previous research confirmed that HSP70 is an antigen carrier and adjuvant [35]. Therefore, it is speculated that acupuncture might induce the expression of the endogenous adjuvant substance HSP70 by reducing oxidative stress, promoting the immune effect of the vaccine, and acting as a vaccine adjuvant.

Experimental results showed that HSP70 mRNA expression in spleen cells was lower in D-galactose-induced model rats, indicating that D-galactose may establish an aging body with lower immune stress proteins. However, the application of the Double-reinforcing and One-unblocking acupuncture method increased HSP70 mRNA expression, suggesting that acupuncture enhanced the vaccine immune response and postponed immune senescence by upregulating HSP70 mRNA expression and adjusting the entire body. Compared to other adjuvants, acupuncture offers the advantages of being cost-effective and widely applicable. In this study, we innovatively proposed acupuncture, a traditional Chinese medicine therapy, as a vaccine adjuvant, and confirmed its effect on the immune system, providing a new approach for the prevention and treatment of infectious diseases. Since the DTaP vaccine used in this study contains an aluminum hydroxide adjuvant, our research also proves that acupuncture can be used in combination with an aluminum hydroxide adjuvant. However, due to limitations in research conditions, we did not detect the expression of HSP70 protein, nor did we study the different stages of immune activation. Additionally, whether acupuncture is effective for a variety of vaccines still requires further research. In the future, we should continue to conduct correlation experiments to more clearly elucidate the mechanism by which acupuncture improves the immune aging of D-galactose-induced rats.

5. Conclusion

These experiments investigated the effect of acupuncture on vaccine reactivity in D-galactose-induced rats. The "Double-reinforcing and One-unblocking" acupuncture and moxibustion method were found to enhance both the humoral immune response (diphtheria antitoxin titer) and cellular immune responses (peripheral blood $CD4^+T/CD8^+$T cells ratio) by stimulating the expression of endogenous adjuvant HSP70 mRNA. This suggests that acupuncture may serve as a new vaccine adjuvant.

Many questions remain in the field of vaccine research. For example, the efficacy of some vaccines in a small number of people is uncertain, such as a small number of individuals who cannot produce antibodies after being vaccinated against hepatitis B. In some patients, such as the elderly and those with immunosuppression, the vaccine response is reduced. There are no therapeutic vaccines for most diseases, such as hepatitis B. Considering the versatility of adjuvants, acupuncture may be used as an adjuvant in vaccine research, and our study may promote the mutual development of acupuncture and vaccines, providing a new field for integrative Chinese and Western medicine research.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Jia Li: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources,

Software, Supervision, Writing – original draft. **Fangyuan Liang:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. **Ling Xiao:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing – original draft. **Wei Lu:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization. **Hua Wang:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22645.
Extended Data_Fig. 1: Original image of electrophoretic RT-PCR products.

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