Am J Transl Res 2015;7(9):1487-1498
www.ajtr.org /ISSN:1943-8141/AJTR0012845

Original Article

Adhesion: a confounding bias in murine cervical heterotopic heart transplantation

Jinghui Yang[1*], Qi Chen[2*], Fang Liu[1], Zhiren Fu[1], Quanxing Wang[3]

[1]Department of Organ Transplantation, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, China; [2]Department of Health Statistics, Second Military Medical University, Shanghai, China; [3]National Key Laboratory of Medical Immunology, Second Military Medical University, Shanghai, China. [*]Equal contributors.

Received July 13, 2015; Accepted September 13, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Tissue adhesion is a common postsurgical phenomenon among the human population. This complication also occurs in murine transplant models. In this study, we investigated the impact of adhesion on murine cervical heterotopic heart transplantation by using sodium hyaluronate (SH) as an anti-adhesive agent. Our study revealed that SH administration produced no significant effect on histological change, TNF-α, IFN-γ, MCP-1, IL-2, IL-6 and IL-10 expression, $CD4^+$ T, $CD8^+$ T, or neutrophil and macrophage counts. Our findings suggest that SH was biocompatible and non-immunogenic. Later, we observed that adhesion not only affected the survival of the graft without mediating rejection, but was closely related to the severity of rejection as manifested by larger and more severe adhesion formation in total-allomismatched and MHC class II-allomismatched murine cardiac allografts. Therefore, we inferred that using the murine cervical heterotopic heart transplant model may lead to an exaggerated *p*-value in statistical significance testing which could mislead experimenters in considering that the results are more significant than the fact. To the best of our knowledge, this study is the first demonstration that proves that adhesion was a confounding bias in the murine cervical heterotopic heart transplant model and highlights the possibilities for improvement in future use.

Keywords: Compounding bias, heart transplantation, sodium hyaluronate

Introduction

Mouse models have many advantages over rat models due to the availability of thousands of inbred strains, the ample supplies of monoclonal antibodies, and the clearer immunologic and genetic background. Since Corry and colleagues introduced the technique of the murine abdominal heterotopic heart transplantation model in 1973 [1], it has been widely employed as a useful tool for the study of allograft rejection and dissection of other immunological questions. With the development of the microsuture technique, Chen et al. [2] further introduced a murine cervical heterotopic heart transplantation model in 1991. The Chen model allowed for two sequential cardiac transplantations to be conducted in the same recipient mouse using the abdominal and cervical site to study donor-type organ-specific tolerance. Compared with the cervical heterotopic heart transplantation model, the abdominal heterotopic heart transplantation model has several drawbacks: 1) The abdominal aorta and inferior vena cava (IVC) must be occluded for 30 minutes which may lead to ischemia-reperfusion (I/R) injury, hind limb paralysis, and potential venous thrombosis. 2) It is difficult to monitor the heterotopic abdominal heart and accurately confirm the time of cardiac arrest because they are buried under a large number of abdominal organs. 3) It requires a lengthy surgical procedure which may cause high postoperative mortality [3].

Another disadvantage with the cervical heterotopic heart transplantation model is that, despite excision of the salivary gland and the sternocleidomastoid muscle, the cervical space is limited. Therefore, the formation of adhesions may further narrow the space and limit cardiac throb. Currently, there is speculation on how influential adhesions are to the heterotopic heart and whether elimination of adhesions

could assist in the reduction of extraneous interference in all the studies using this model.

Sodium hyaluronate (SH) is a biopolymer that naturally exists in the vitreous body of the eye, extracellular matrix of the skin, and synovial fluid of all vertebrates. Owing to its biocompatible, biodegradable, and non-immunogenic activities, SH continues to be the most well-known, and probably the most used, adhesion barrier in the departments of orthopedics, general surgery, and ophthalmology [4-6]. Based on the above understanding of SH, this study was intended to evaluate the effect of SH on cervical heterotopic heart transplantation in mice.

Methods

Animals

Wild-type C57BL/6 ($H2^b$, B6), BALB/c ($H2^d$) and B6(C)-$H2^{bm12}$/KhEgJ male mice, aged 8-12 weeks and weighing 22-26 g (Joint Ventures Sipper BK Experimental Animal Company, Shanghai, China), were used for the study. They were housed in a SPF facility at the National Key Laboratory of Medical Immunology (Shanghai, China). All surgical procedures were carried out by a designated surgeon. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Scientific Investigation Board of the Second Military Medical University (Shanghai, China).

Surgical instruments and cardiac transplantation

The GX-3 operating microscope with magnification ranging from 6X to 30X was obtained from Precision Instruments Company (Shanghai, China). The micro-monopole electro-coagulator was originated from Evergreen (Wuhan, China). Microsurgical forceps, scissors, and other surgical instruments were purchased from Jinzhong Medical Instruments (Shanghai, China). The cuffs were made from 24G or 22G intravenous catheters which were 1 mm-long, an external diameter of 0.7 mm, an internal diameter of 0.5 mm for arterial anastomosis, an external diameter of 0.8 mm, and an internal diameter of 0.6 mm for external jugular vein. Intra-abdominal heterotopic cardiac transplantation was performed by anastomosis, as described previously [7], while cervical heterotopic cardiac transplantation was performed by a modified non-suture cuff technique described previously [8]. The mice in FK506 and FK506+SH groups were injected intra-peritoneally with FK506 (Astellas Pharma, Tokyo, Japan) 1 mg/kg/d.

Cardiac graft survival and evaluation of adhesion area and severity

In a separate group of experiments, mice were assigned to appropriate groups which included: [A] acute rejection (AR) group (BALB/c ($H2^d$) to C57BL/6 ($H2^b$, B6)), [B] acute rejection group+SH, [C] chronic rejection (CR) group (B6(C)-$H2^{bm12}$/KhEgJ to C57BL/6 ($H2^b$, B6)). [D] chronic rejection group+SH, [E] no rejection (NR) group (C57BL/6 ($H2^b$, B6) to C57BL/6 ($H2^b$, B6)), and [F] no rejection group+SH . All mice were allowed free access to food and water and were observed daily. The cease of heartbeat was defined as the end of functioning of the grafted heart. We monitored the survival of the graft on a daily basis via cervical or abdominal palpation.

Ten mice from each group were anesthetized, and the severity and area of adhesion were measured by a blinded and independent staff member. Adhesion formation was evaluated according to the following adhesion scoring system: 0 = no adhesion, 1 = filmy adhesion that was easily separable with blunt dissection, 2 = mild to moderate adhesion with free dissection, and 3 = moderate to dense adhesions with difficult dissection or non-dissection [9]. The acute rejection group (A and B) was measured seven days after operation, while the chronic rejection group (C and D) and the no rejection group (E and F) was measured forty days after operation.

Analysis of graft survival and function

Graft viability was assessed by cervical echocardiography. Cardiac graft function was expressed as the beating score and assessed using the Stanford Cardiac Surgery Laboratory Graft Scoring System as: 0 (no contraction), 1 (barely palpable), 2 (obvious decrease in contraction strength), 3 (strong, coordinated beat

but noticeable decrease in strength), or 4 (strong contraction of both ventricles, regular rate).

Echocardiographic measurements

Cervical echocardiography was performed with a Vevo 770 high-resolution ultrasound system (VisualSonics, Inc.) equipped with a 30-MHz, 100-frame-per-second micro-visualization scan head. The echocardiographic measurements were recorded in accordance with the manufacturer's manual (VisualSonics, Inc.; revised 2006) and previously published reports [10]. Generally, a cervical long-axis B-mode image was acquired with appropriate positioning of the scan head to ensure the maximum left ventricular (LV) length could be identified. From this view, the Doppler transducer was positioned for sampling of the mitral flow waveform and the dimensions between LV end-diastolic (LVEDD) and end-systolic (LVESD) were measured as cardiac contractility.

Histological analysis of the grafts

The grafted hearts of acute rejection groups were harvested at Day 7 and those of chronic rejection groups were harvested at Day 40. Hearts were immediately divided longitudinally into two halves with each comprising half of the right and half of the left ventricle. The tissue was formalin fixed and embedded in paraffin. Sections of 3 μm were taken at one third of the distance from the base to the apex of the heart and stained with Hematoxylin and eosin, Masson trichrome, and Elastica van Gieson's dye.

Myocardial injury was scored as: 0 = no lesion, 1 = focal vacuolization (myocytes become vacuolated), 2 = diffuse vacuolization and mild mononuclear infiltration, 3 = focal necrosis of myocytes (irregular border; fragmented sarcoplasm, debris, myocyte dropout), and 4 = extensive myocyte necrosis (interstitial hemorrhage and eosinophil infiltration).

Luminal occlusion of the coronary arteries was calculated to determine transplant vasculopathy. Cross sectional areas were measured through the utilization of tracings made on arteries that had a well-defined, smooth muscle cell layer and internal elastic lamina (IEL). The region encompassed by the lumen and IEL was traced and the area of luminal stenosis was calculated for each section according to the following formula:luminal occlusion (LO) = (IEL area-luminal area)/IEL area. Cardiac fibrosis was measured as the percentage of the fibrotic area in each field [11]. Two observers, who were blinded to the sham, control and experimental groups, independently evaluated the slides. Their scores were pooled and the mean value was used for further statistical analysis.

Tissue level of cytokine expression

Total heart RNA was extracted by using TRIzol (Invitrogen) reagent in accordance with the manufacturer's instructions. The cDNA was synthesized using oligo d(T) (Applied Biosystems) and a SuperScript III Reverse Transcriptase Kit (Invitrogen). A StepOne™ Real-Time PCR System (Applied Biosystems) and a SYBR RT-PCR kit (Takara) were used for quantitative real-time RT-PCR analysis. All reactions were conducted in a 20 μl reaction volume in triplicate. β-actin was used as a normalizing control, and each reaction was performed in triplicate. The primer sequences for qRT-PCR are: β-actin (*5'-CATCCGTAAAGACCTCTATGCCAAC-3'; 5'-ATGGAGCCACCGATCCACA-3'*); TNF-α (*5'-AAGCCTGTAGCCCACGTCGTA-3'*; *5'-GGCACCACTAGTTGGTTGTCTTTG-3'*); IFN-γ (*5'-CGGCACAGTCATTGAAAGCCTA-3'; 5'-GTTGCTGATGGCCTGATTGTC-3'*); MCP-1 (*5'*-GGAGCATCCACGTGTTGGC-*3'; 5'*-GTAGGAGTGACCAGTGTGACAGT-*3'*); IL-2 (*5'-GGAGCAGCTGTTGATGGACCTAC-3'; 5'-AATCCAGAACATGCCGCAGAG-3'*); IL-6 (*5'*-GATGCTACCAAACTGGATATAATC-*3'; 5'*-GGTCCTTAGCCACTCCTTCTGTG-*3'*); IL-10 (*5'-GACCAGCTGGACAACATACTGCTAA-3'; 5'-GATAAGGCTTGGCAACCCAAGTAA-3'*); Data was analyzed using the comparative C_t ($2^{-\Delta\Delta Ct}$) method.

Flow cytometry analysis

Cells in allografts were prepared by digestion with 2 mg/mL Collagenase D (Worthington Bio, Lakewood, NJ) and 10% fecal calf serum in RPMI 1640 media for 2 hours at room temperature. After filtration, cells were stained with the following antibodies: 7-AAD, CD45-PE, CD45-FITC, CD3-FITC, CD3-APC, CD4-PE, CD8-PE, CD11b-PE, F4/80-APC, GR-1-FITC (BD Biosciences, San Diego, CA). After staining, the cells were washed three times and resuspended in 500 μl of PBS. Data acquired by Accuri C6 (BD

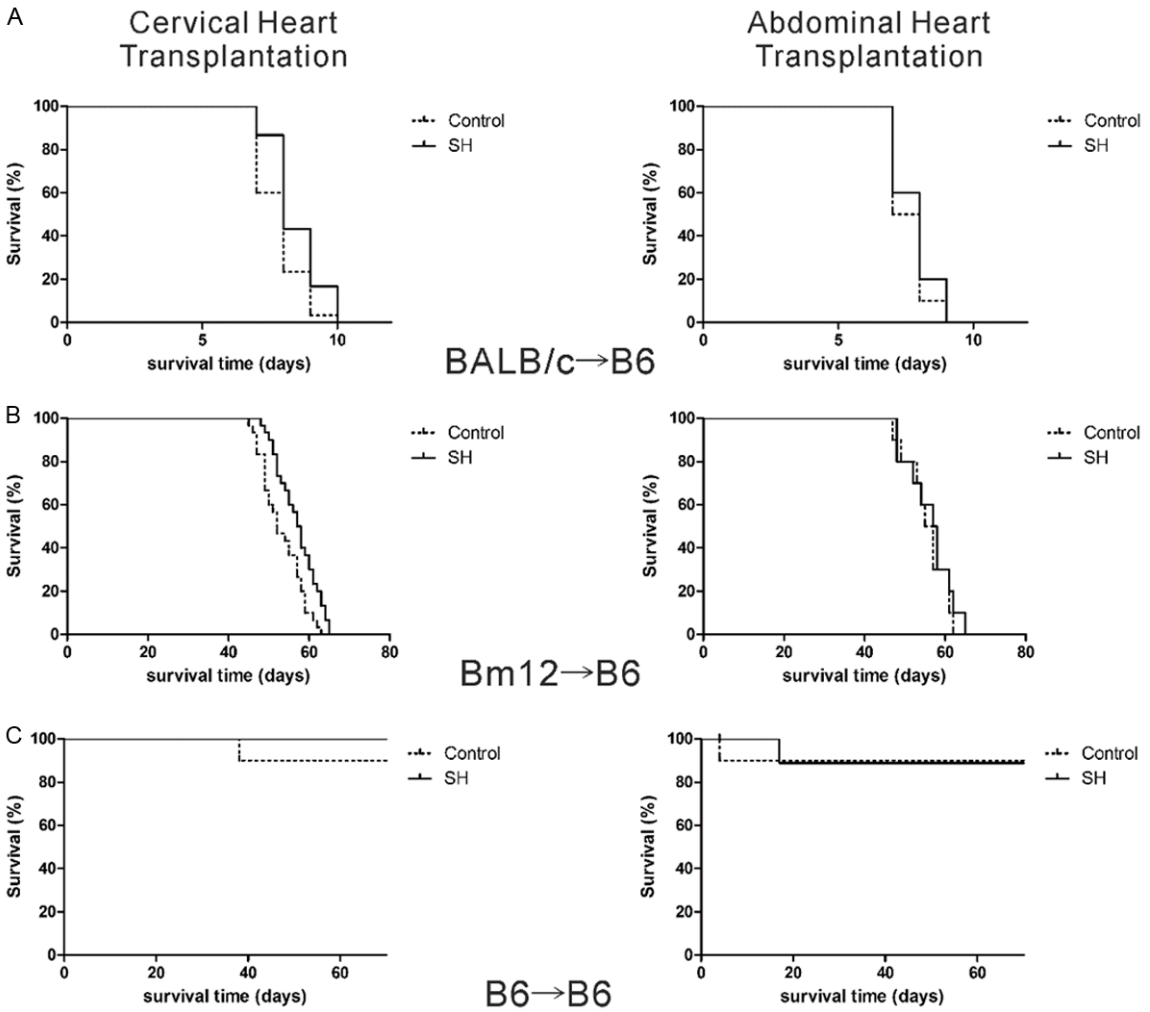

Figure 1. SH prolongs allograft survival following cervical cardiac allograft transplantation. A. Survival time of total-allomismatched allografts after BALB/c ($H2^d$) donor heart transplantation into C57BL/6 ($H2^b$, B6) recipients. B. Survival time of MHC class II-allomismatched allografts after B6(C)-$H2^{bm12}$/KhEgJ donor heart transplantation into C57BL/6 ($H2^b$, B6) recipients. C. Survival time of isografts after C57BL/6 ($H2^b$, B6) donor heart transplantation into C57BL/6 ($H2^b$, B6) recipients.

Biosciences) was analyzed using CFlow software (BD Biosciences).

Statistical analysis

The computer software, GraphPad Prism 5 (GraphPad Software, La Jolla, CA), was used for data analysis. Data, derived from two groups, was analyzed using an unpaired Student's t test or a Mann-Whitney test (two tailed). Data from multiple groups was analyzed using one way ANOVA with post-hoc Bonferroni's correction. The survival rate was analyzed by the Kaplan-Meier method and comparisons were made by log-rank analysis. All data is expressed as mean ± SD. In all cases, $p<0.05$ was considered to be statistically significant.

Results

SH prolongs allograft survival following cervical cardiac allograft transplantation

First, the survival time of mice was monitored in the six groups after cervical heterotopic heart transplantation. It was found that cardiac arrest which occurred in total-allomismatched murine cardiac allografts (BALB/c donor hearts and C57BL/6 recipients) was deferred in the SH treatment group (allograft survival averaged 8.4667±0.937 days) in comparison to the control group (allograft survival averaged 7.867±0.860 days) ($n = 30$, $p = 0.0123$) (**Figure 1A**). In contrast, survival of MHC class II-allomismatched murine cardiac allografts

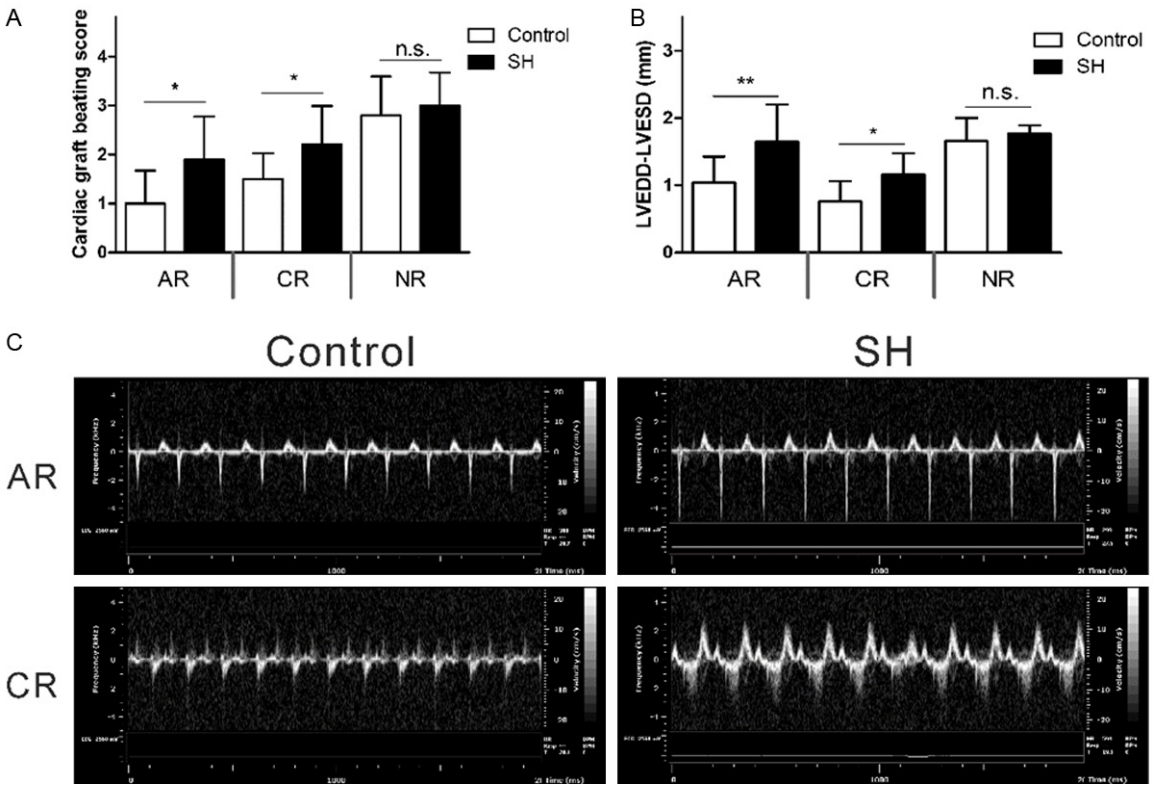

Figure 2. SH enhances transplanted graft viability. A. Beating scores of transplanted cardiac grafts in acute rejection models (AR) at POD7, chronic rejection (CR) and no rejection models (NR) at POD40. B. Cardiac contractility assessed by cervical echocardiography in AR and CR models. C. The waveform of mitral flow waveform in SH treated and control allografts from AR models 7 days after transplantation and CR models 40 days after transplantation. *P<0.05, **P<0.01.

(bm12 donor hearts and C57BL/6 recipients) averaged 57.1±5.101 days in the SH treatment group and 53.3±5.199 in the control group (n = 30, p = 0.006) (**Figure 1B**). However, there was no significant difference in isograft survival between the SH treatment and control groups (**Figure 1C**). Unlike enhanced SH-related survival rate of cervical cardiac allografts, abdominal cardiac allografts did not obtain a benefit from SH administration.

SH enhances transplanted graft viability

Beating scores were assessed by cervical echocardiography at POD7 in AR models (group A and B) and at POD40 in CR models (group C and D) and NR models (group E and F). The mean beating score of the SH treated hearts in the AR models was significantly higher than the corresponding value in the control group (1.9±0.87 *vs* 1.0±0.67 p = 0.0184) at POD7. In the CR models, the mean heart beating was 2.2±0.79 in the SH group vs 1.5±0.53 in the control group (p = 0.0318). There was no significant difference between the two groups in the no rejection models (p = 0.5491) (**Figure 2A**).

Cardiac contractility (LVEDD-LVESD) and mitral flow waveform were observed between the SH treated group and the control group. Echocardiography revealed that cardiac contractility in the SH treated group was significantly enlarged in comparison to the control group (P = 0.0355 in acute rejection group; P<0.001 in chronic rejection group) (**Figure 2B**). In addition, the extent of mitral flow waveform in acute and chronic rejection increased by 70.4% and 57.8%, respectively, after SH treatment. (**Figure 2C**).

SH reduces adhesion severity and area

The animals in the acute rejection group were sacrificed at POD7, and those in the chronic rejection group were sacrificed at POD40 in order to evaluate the adhesion severity and

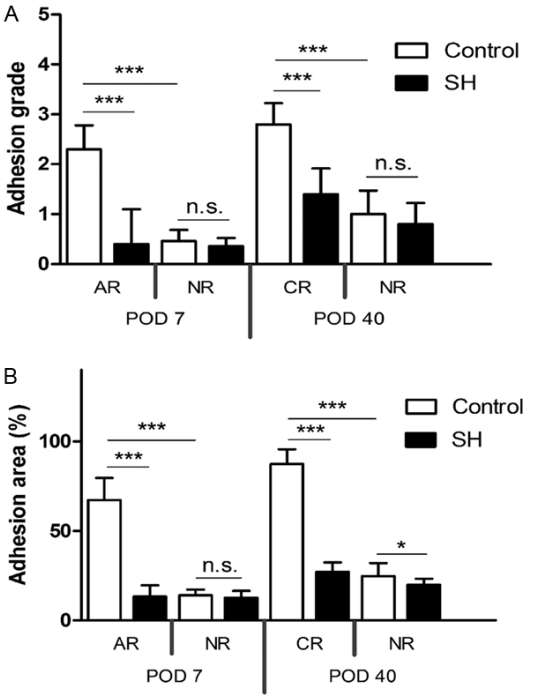

Figure 3. SH ameliorates the severity and area of adhesion. (A) Adhesion grade and (B) adhesion area of the transplanted hearts assessed by a blinded and independent staff. Data are the mean±standard error of the mean. *P<0.05, **P<0.01, ***P<0.001.

area. In the adhesion grade evaluation, administration of SH revealed significant differences in both acute and chronic rejection, 0.45± 0.0.604 vs 2.35±0.489 (n = 20, P<0.001) and 1.35±0.587 vs 2.75±0.550 (n = 20, p<0.001), respectively (**Figure 3A**). Regarding adhesion area, the SH treated group showed 13.0±5.24% and 26.9±5.30% in acute and chronic rejection heart transplantation models, while the control group showed 66.55±10.23% and 87.95±7.76%. These values indicate a significant reduction in adhesion in the SH treated group (P<0.01) (**Figure 3B**).

Additionally, compared with syngenic controls, AR (2.35±0.489 vs 0.45±0.605) [P<0.001] for severity, and 66.55±10.232% vs 13.0±5.24% [P<0.001] for area at POD7, and CR controls (2.75±0.550 vs 1.1±0.447) [P<0.001] for severity, and 87.95±7.76% vs 25.05±6.45% [P<0.001] for area at POD40, displayed larger and more severe adhesions (**Figure 3A** and **3B**).

Results for histological analysis

In order to investigate the possible immune and inflammation response of SH, the effects of SH on the grafts were evaluated with respect to evaluated myocardial injury, cardiac fibrosis, and transplant vasculopathy. H&E staining showed no statistically significant difference in myocardial injury between the SH treated group and the control group (**Figure 4D**). Representative images of myocardial injury are shown in **Figure 4A**. There was no significant difference in transplant vasculopathy and cardiac fibrosis between the SH treated group and the control group (**Figure 4E** and **4F**). Representative images of vasculopathy are shown in **Figure 4B** and those of interstitial fibrosis are shown in **Figure 4C**. Masson staining also showed a thinner layer of adhesion in the SH treated grafts as compared with the control grafts (**Figure 4C**).

SH does not change the cytokine expression in the allografts and the count of cardiac allograft-infiltrating cells

SH administration was evaluated to determine if it was able to modulate cytokine expression in the allografts or the accumulation of graft leukocytes. It was found that mRNA expression of TNF-α, IFN-γ, MCP-1, IL-2, IL-6 and IL-10 did not differ significantly between the SH group and the control group in regards to acute rejection (**Figure 5A**) or chronic rejection (**Figure 5B**). In addition, no significant effect of SH treatment on leukocyte infiltration was observed, including $CD4^+$ T, $CD8^+$ T, neutrophils and macrophages (**Figure 5C**).

FK506 partially abrogated the protective effect of SH on cervically transplanted heart

We evaluated the effect of SH when treated with immunosuppressants in the cervical heterotopic heart transplantation model. In order to ensure a thorough evaluation, we evaluated survival time, graft viability, adhesion severity, and adhesion area between the FK506 group and the FK506+SH group. It was noted that when treated with FK506, both SH treated and control mice showed similar survival time and graft viability (**Figure 6A-C**). As expected, a transplanted heart with FK506+SH exposure showed significantly lower adhesion grade and area at POD7 as compared with those of the FK506 group (**Figure 6D** and **6E**). It should be noted that FK506 treatment alone could also reduce the adhesion grade and area of a transplanted heart as compared with the control group (**Figure 6D** and **6E**).

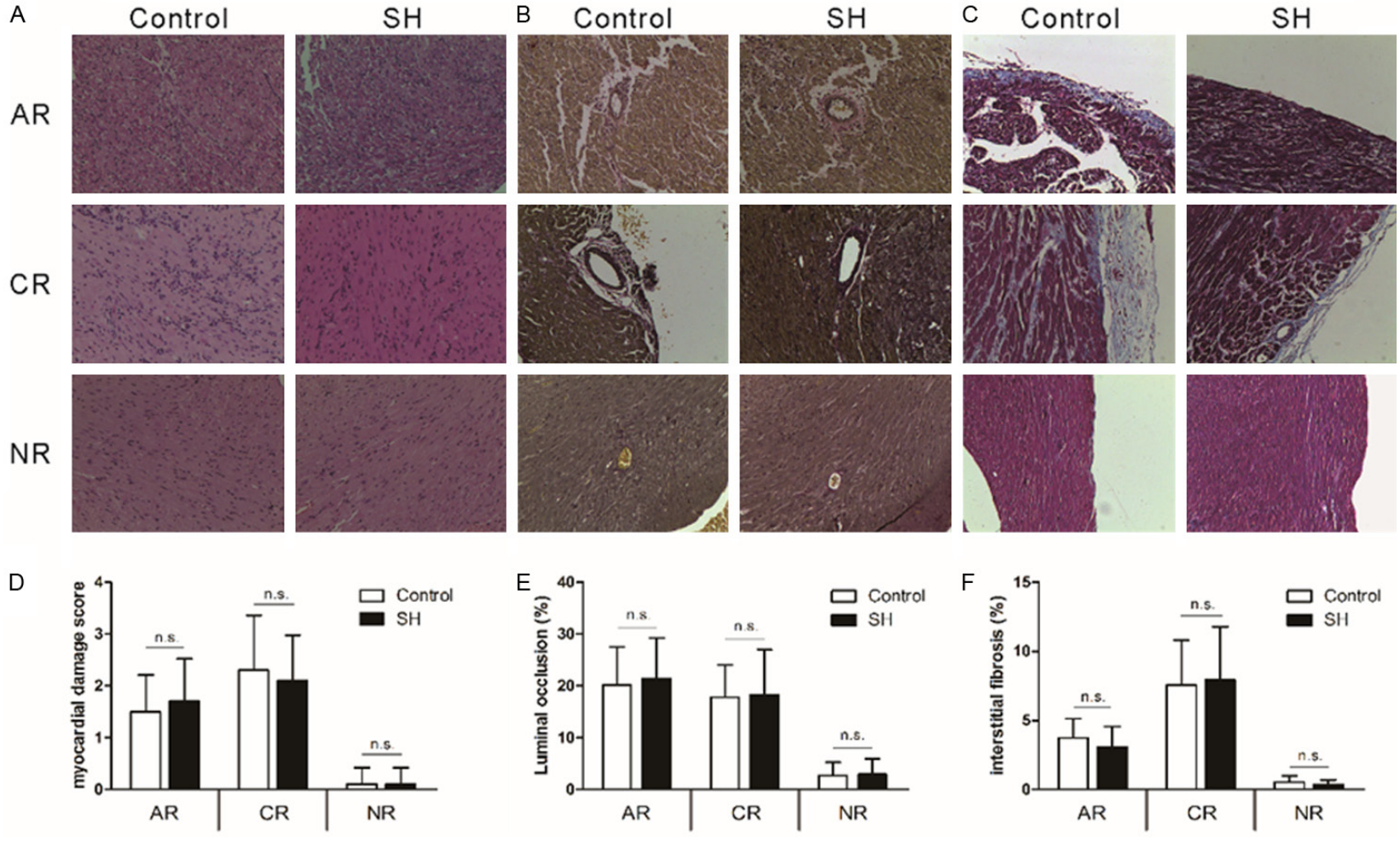

Figure 4. Neither vascular nor myocardial changes in transplant cardiac grafts after SH administration. Representative cardiac grafts in CR and NR models harvested at POD40 and AR models harvested at POD7 showing (A) myocardial damage (Hematoxylin and eosin staining), (B) vasculopathy (Elastica van Gieson staining) and (C) interstitial fibrosis (Masson trichrome staining). Data from morphometric assessment of (D) myocardial damage, (E) luminal occlusion, and (F) interstitial fibrosis in sections like those shown in Panel (A-C). Data are the mean ± standard error of the mean. n.s. $P>0.05$.

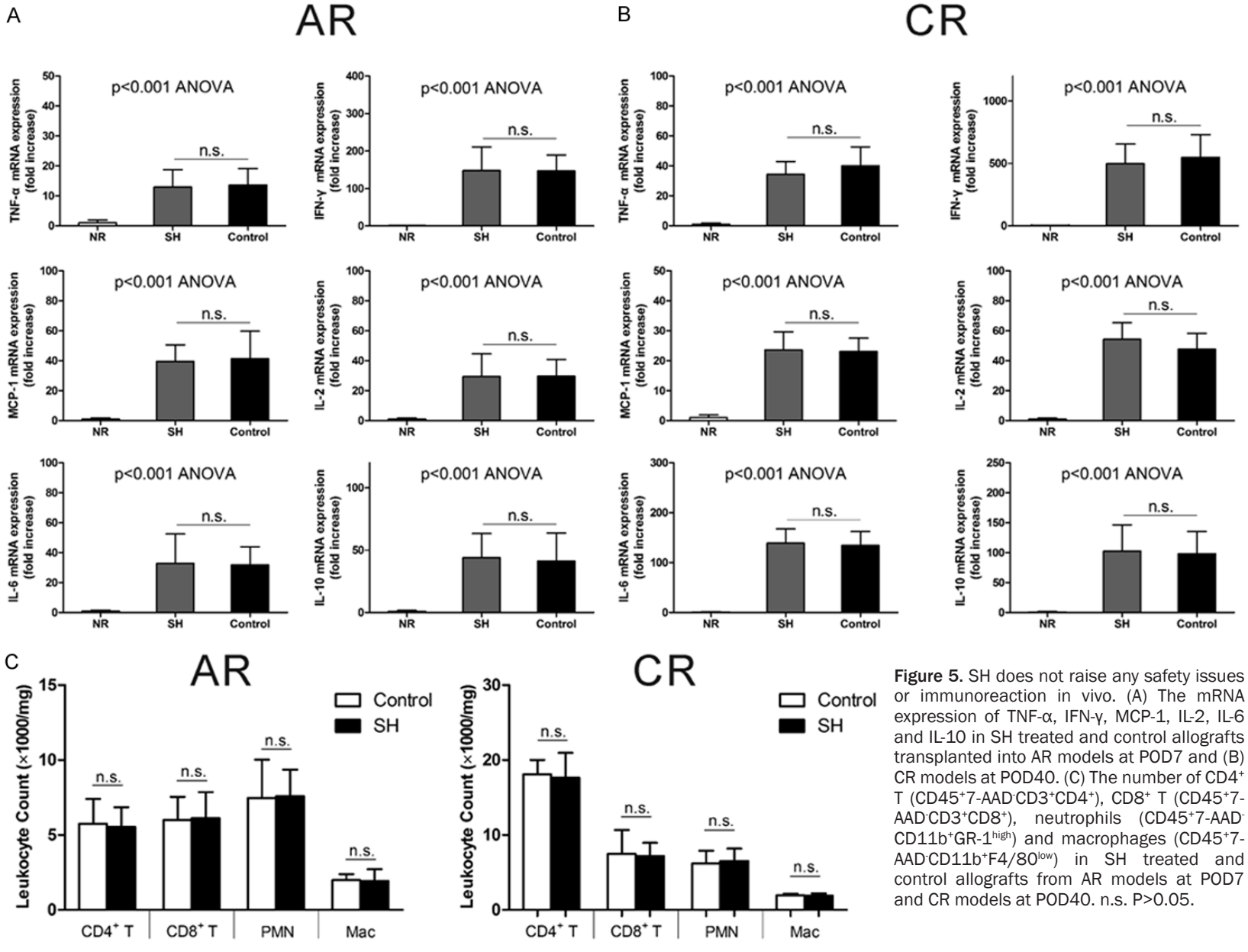

Figure 5. SH does not raise any safety issues or immunoreaction in vivo. (A) The mRNA expression of TNF-α, IFN-γ, MCP-1, IL-2, IL-6 and IL-10 in SH treated and control allografts transplanted into AR models at POD7 and (B) CR models at POD40. (C) The number of $CD4^+$ T ($CD45^+7\text{-}AAD^-CD3^+CD4^+$), $CD8^+$ T ($CD45^+7\text{-}AAD^-CD3^+CD8^+$), neutrophils ($CD45^+7\text{-}AAD^-CD11b^+GR\text{-}1^{high}$) and macrophages ($CD45^+7\text{-}AAD^-CD11b^+F4/80^{low}$) in SH treated and control allografts from AR models at POD7 and CR models at POD40. n.s. P>0.05.

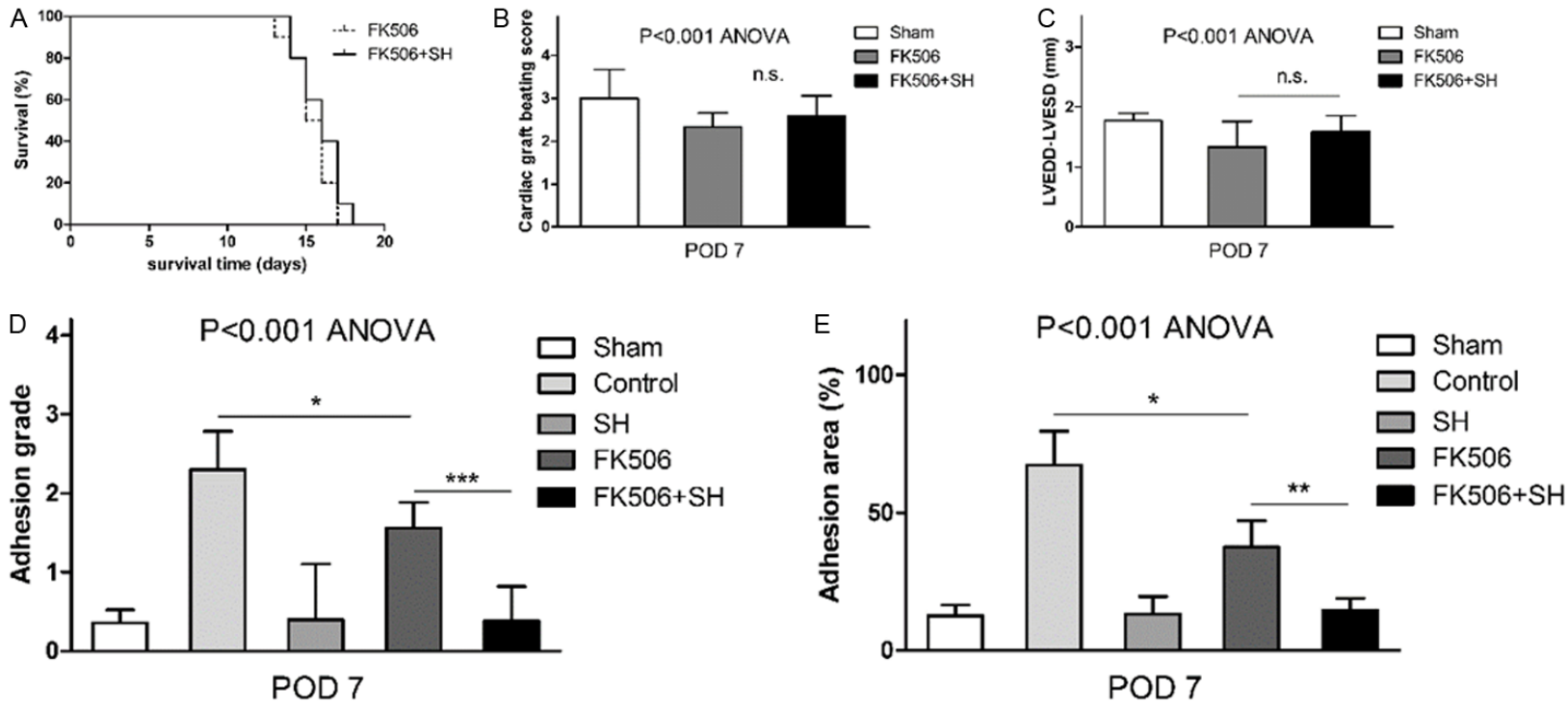

Figure 6. FK506 partially abrogated the protective effect of SH on cervically transplanted heart. (A) Survival time of total-allomismatched allografts (BALB/C→C57BL/6) treated with FK506 (1 mg/kg/d) or FK506+SH. (B) Beating scores of total-allomismatched allografts treated with FK506 (1 mg/kg/d) or FK506+SH at POD7. (C) Cardiac contractility of total-allomismatched allografts treated with FK506 (1 mg/kg/d) or FK506+SH at POD7. (D) Adhesion grade and (E) adhesion area of total-allomismatched allografts treated with FK506 (1 mg/kg/d) or FK506+SH at POD7. Data are the mean ± standard error of the mean. n.s. P>0.05, *P<0.05, **P<0.01, ***P<0.001.

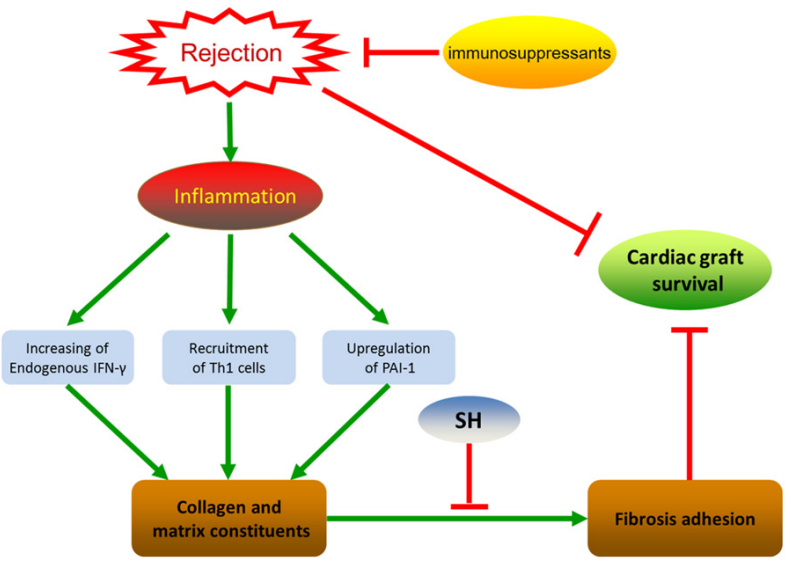

Figure 7. Schematic illustration of the relationship between adhesion formation and cardiac graft survival. The survival time of the transplanted hearts is not only related to the rejection itself, but also to the adhesion formation caused by rejection. After allograft transplantation, rejection-derived IFN-γ, Th1 cells, and PAI-1 *increases the formation of adhesions by activation of inflammatory response. SH administration simply* prevents de novo adhesion formation as a physical barrier without mediating rejection and prolongs the survival of cardiac grafts, thus removing the compounding bias in murine cervical heterotopic heart transplantation model.

Discussion

Compared with abdominal heart transplantation, cervical heterotopic heart transplantation has been widely used as a basic and important mouse model in diverse experimental settings and offering minimal traumatic injury to the circulation of recipient mice. Experimental studies using cervical heterotopic heart transplant have significantly contributed to our understanding of transplantation immunology and have provided the foundations on which much of transplantation biology has been developed and how we practice clinical transplant medicine and surgery today. However, because of the strait cervical region of the recipients, adhesions occurring after the operation may further narrow the space and limit cardiac throb. Therefore, we presented evidence supporting that SH administration on the graft surface could significantly reduce the severity and area of adhesion, thus, prolonging survival.

Sodium hyaluronate is a water-soluble linear polymer built from repeating disaccharide units containing N-acetylglucosamine (GlcNAc) and D-glucuronic acid (GlcUA) [12]. Due to its hydrophilic and viscoelastic properties, it has become an important space filling molecule as evidenced by it applications to the vitreous humor, derma, and joints as a component of the extracellular matrix (ECM) [13]. As an endogenous polysaccharide, SH has good biocompatibility, biodegradability, and intrinsic biological recognition [14]. Therefore, SH-based gels have been widely used in surgical procedures to prevent postoperative de novo adhesion formation as a physical barrier. The prevention of postoperative de novo adhesion formation reduces the health burden associated with infertility, abdominal pain, or bowel obstruction [15]. In line with the above applications, we observed no evidence of SH-induced toxicity or immunoreaction after treatment. Histological evaluation POD7 and POD40 showed neither vascular nor myocardial changes in transplant grafts that were treated with SH. Our study showed no significantly unfavorable effects on cytokines (TNF-α, IFN-γ, MCP-1, IL-2, IL-6 and IL-10) after SH administration. In addition, there were no differences in the numbers of cardiac $CD4^+$ T ($CD45^+7\text{-}AAD^-CD3^+CD4^+$), $CD8^+$ T ($CD45^+7\text{-}AAD^-CD3^+CD8^+$), neutrophils ($CD45^+7\text{-}AAD^-CD11b^+GR\text{-}1^{high}$) or macrophages ($CD45^+7\text{-}AAD^-CD11b^+F4/80^{low}$). Collectively, these data suggest that SH may prove to be a useful therapeutic agent to prevent adhesion formation because it does not raise any safety issues or immunoreaction in vivo.

The present study demonstrated that SH administration, subsequent to murine cervical heterotopic heart transplant, prolonged allograft survival without mediating rejection. After cervical heterotopic heart transplant, SH treated mice displayed significantly preserved viability as characterized by higher beating scores and larger cardiac contractility. In addition, with the prolonged survival, the severity

and area of adhesion were ameliorated as manifested by cervical echocardiography and Masson staining (thinner blue layer). Moreover, we observed that rejection was critically involved in adhesion formation as a significant factor affecting graft survival. As demonstrated in **Figure 3**, AR and CR controls displayed more severe and larger adhesions in comparison to the syngenic controls. In other words, intensified rejection aggravated adhesion formation in the murine cervical heterotopic heart transplant model. Similarly, transplanted hearts treated with FK506 also showed a significant reduction in adhesion severity and area as compared with those of controls. This indicates that the severity of rejection partially contributes to the severity of adhesion formation.

Our data, presented in the current report, is in agreement with previous studies of kidney and aortic transplants [16, 17]. Wang et al. [18] investigated the role of plasminogen activator inhibitor type-1 (PAI-1) and found that it could inhibit tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) in chronic rejection and fibrin deposition. They found that serum levels of PAI-1 were upregulated and persistently expressed during the progressive phase of chronic rejection, which was synchronous with fibrin deposition. Using postsurgical peritoneal tissue, Fometescu et al. [19] demonstrated an increase in the expression of the PAI-1 gene and decrease in the of expression of the tPA gene in patients with increased peritoneal adhesion propensity as compared with patients without this propensity. Th1 cells as well as IFN-γ, both sufficient and required for allograft rejection, are reported to be responsible for the development of adhesions. It has been proven that $CD4^{-/-}$ and $Stat1^{-/-}$ mice experienced a significant reduction in adhesion scores in comparison to WT mice in a cecal abrasion model [20, 21].

Here, we further confirmed that adhesion is a confounding bias in murine cervical heterotopic heart transplantation. **Figure 7** depicts the relationship between adhesion formation and cardiac graft survival. If one were to compare the effects of treatments, such as immunosuppressants, with this model, the survival time of transplanted hearts will not only be affected by mitigated rejection caused by treatment, but also affected by ameliorated adhesion caused by mitigated rejection. Therefore, the use of the murine cervical heterotopic heart transplant model may lead to an exaggerated *p*-value in statistical significance testing. Because of this, scientists are more likely to reject the null hypothesis for a stronger false presumption against a neutral hypothesis caused by confounding bias and are more likely to consider the results more significant than the fact.

In summary, we demonstrated that SH administration following murine cervical heterotopic heart transplant could prolong allograft survival without mediating rejection. In addition, we also revealed that this prolonged survival is adhesion dependent, which is closely related to transplant rejection, and becomes the confounding bias in the murine cervical heterotopic heart transplant model.

Acknowledgements

This study was supported by the National Nature Science Foundation of China (No. 81270550).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Quanxing Wang, National Key Laboratory of Medical Immunology, Second Military Medical University, Xiangyin Road 800#, Shanghai 200433, China. Tel: 0086-1370-1669071; Fax: 0086-02181870404; E-mail: wangqx@immunol.org; Dr. Zhiren Fu, Department of Organ Transplantation, Shanghai ChangZheng Hospital, Second Military Medical University, Fengyang Road 41#, Shanghai 200003, China. Tel: 0086-13901634770; Fax: 0086-02181886999; E-mail: zhirenf@sh163.net

References

[1] Corry RJ, Winn HJ and Russell PS. Heart transplantation in congenic strains of mice. Transplant Proc 1973; 5: 733-735.

[2] Chen ZH. A technique of cervical heterotopic heart transplantation in mice. Transplantation 1991; 52: 1099-1101.

[3] Al-Amran FG and Shahkolahi MM. Total arterial anastomosis heterotopic heart transplantation model. Transplant Proc 2013; 45: 625-629.

[4] Battaglia M, Guaraldi F, Vannini F, Rossi G, Timoncini A, Buda R and Giannini S. Efficacy of ultrasound-guided intra-articular injections of platelet-rich plasma versus hyaluronic acid for hip osteoarthritis. Orthopedics 2013; 36: e1501-1508.

[5] Stawicki SP, Green JM, Martin ND, Green RH, Cipolla J, Seamon MJ, Eiferman DS, Evans DC, Hazelton JP, Cook CH, Steinberg SM; OPUS 12 Foundation, Inc, Multi-Center Trials Group & OASIT Investigators. Results of a prospective, randomized, controlled study of the use of carboxymethylcellulose sodium hyaluronate adhesion barrier in trauma open abdomens. Surgery 2014; 156: 419-430.

[6] Mayordomo-Febrer A, Lopez-Murcia M, Morales-Tatay JM, Monleon-Salvado D and Pinazo-Duran MD. Metabolomics of the aqueous humor in the rat glaucoma model induced by a series of intracamerular sodium hyaluronate injection. Exp Eye Res 2015; 131: 84-92.

[7] Corry RJ, Winn HJ and Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. Transplantation 1973; 16: 343-350.

[8] Gu X, Xiang J, Zhou Y, Qian S and Chen Z. Improved cuff technique for cervical heart transplantation in mice. Microsurgery 2007; 27: 317-319.

[9] Kim JY, Cho WJ, Kim JH, Lim SH, Kim HJ, Lee YW and Kwon SW. Efficacy and safety of hyaluronate membrane in the rabbit cecum-abdominal wall adhesion model. J Korean Surg Soc 2013; 85: 51-57.

[10] Okajima K, Abe Y, Fujimoto K, Fujikura K, Girard EE, Asai T, Kwon SH, Jin Z, Nakamura Y, Yoshiyama M and Homma S. Comparative study of high-resolution microimaging with 30-MHz scanner for evaluating cardiac function in mice. J Am Soc Echocardiogr 2007; 20: 1203-1210.

[11] Hu C, Dandapat A, Chen J, Fujita Y, Inoue N, Kawase Y, Jishage K, Suzuki H, Sawamura T and Mehta JL. LOX-1 deletion alters signals of myocardial remodeling immediately after ischemia-reperfusion. Cardiovasc Res 2007; 76: 292-302.

[12] Vigetti D, Karousou E, Viola M, Deleonibus S, De Luca G and Passi A. Hyaluronan: Biosynthesis and signaling. Biochim Biophys Acta 2014; 1840: 2452-2419.

[13] Duman G, Aslan I, Yekta Ozer A, Inanc I and Taralp A. Liposome, gel and lipogelosome formulations containing sodium hyaluronate. J Liposome Res 2014; 24: 259-269.

[14] Sa MA, Andrade VB, Mendes RM, Caliari MV, Ladeira LO, Silva EE, Silva GA, Correa-Junior JD and Ferreira AJ. Carbon nanotubes functionalized with sodium hyaluronate restore bone repair in diabetic rat sockets. Oral Dis 2013; 19: 484-493.

[15] Arnold PB, Green CW, Foresman PA and Rodeheaver GT. Evaluation of resorbable barriers for preventing surgical adhesions. Fertil Steril 2000; 73: 157-161.

[16] Benza RL, Passineau MJ, Anderson PG, Barchue JP and George JF. The role of fibrinolytic genes and proteins in the development of allograft vascular disease. J Heart Lung Transplant 2011; 30: 935-944.

[17] Perkowska A, Elhasade A, Durlik M, Placha G, Galazka Z, Lao M and Gaciong Z. The effect of chronic allograft rejection on plasma regulators of fibrinolysis. Ann Transplant 2002; 7: 44-51.

[18] Wang Y, Pratt JR, Hartley B, Evans B, Zhang L and Sacks SH. Expression of tissue type plasminogen activator and type 1 plasminogen activator inhibitor, and persistent fibrin deposition in chronic renal allograft failure. Kidney Int 1997; 52: 371-377.

[19] Fometescu SG, Costache M, Coveney A, Oprescu SM, Serban D and Savlovschi C. Peritoneal fibrinolytic activity and adhesiogenesis. Chirurgia (Bucur) 2013; 108: 331-340.

[20] Chung DR, Chitnis T, Panzo RJ, Kasper DL, Sayegh MH and Tzianabos AO. CD4+ T cells regulate surgical and postinfectious adhesion formation. J Exp Med 2002; 195: 1471-1478.

[21] Kosaka H, Yoshimoto T, Yoshimoto T, Fujimoto J and Nakanishi K. Interferon-gamma is a therapeutic target molecule for prevention of postoperative adhesion formation. Nat Med 2008; 14: 437-441.