



Recombinant human secretory leukocyte protease inhibitor ameliorated vessel preservation in experimentally isolated rat arteries

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ABSTRACT

Vessel allograft preservation is essential for vascular reconstruction surgery. The preservation solution is crucial in extending the preservation period while maintaining vascular endothelial function. Currently, a 0.9% normal saline solution (NSS) is widely used as a storage solution although its protective effect on endothelial preservation is limited. In this study, we determine the beneficial effect of recombinant human secretory leukocyte protease inhibitor (rhSLPI), supplemented to 0.9% NSS, for isolated aortic preservation. The thoracic and abdominal aorta were isolated from Wistar rats ($n = 6$), and the aortic rings were subsequently cut and preserved in 0.9% NSS in the presence and absence of 1 $\mu\text{g/ml}$ rhSLPI for 0, 6, 24, and 48 hours. The preservative solution was collected to determine the released lactate dehydrogenase (LDH) activity and pro-inflammatory cytokine levels, including tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6). Furthermore, the appearance and severity of vessel degeneration were subjected to blind histopathological assessment by pathologists. The results indicated that 0.9% NSS, supplemented with rhSLPI, significantly reduced the released LDH activity, TNF- α , and IL-6 levels and could attenuate endothelial detachment, endothelial degeneration, and endothelial necrosis. This study demonstrated for the first time that adding rhSLPI to a saline preservative solution could prevent vascular injury and possibly extend the graft storage duration before clinical intervention.

INTRODUCTION

Vascular reconstruction is a therapeutic technique used in reconstructive arterial surgery for several pathological conditions including organ substitute, coronary artery bypass grafting in an ischemic heart (Ben Ali *et al.*, 2018; Fahner *et al.*, 2006; Kazemi *et al.*, 2018; Wille *et al.*, 2008), and infrarenal aortic replacement (Kieffer *et al.*, 2004; Zatschler *et al.*, 2009). A vascular allograft is the most attractive option for revascularization. However, the major challenge in transplantation is reducing vascular endothelial injury, which can promote inflammation following thrombosis-

mediated vascular graft failure (Ben Ali *et al.*, 2018; Wille *et al.*, 2008). To minimize the vessel injury, the protective strategies should be implemented immediately after meticulous graft harvesting with an appropriate preservation solution. The latter is a critical factor that can influence the clinical outcomes of long-term graft storage (Ben Ali *et al.*, 2018).

Several organ preservative solutions such as the University of Wisconsin (UW) solution, histidine-tryptophan-ketoglutarate (HTK) solution, and Celsior solution have been used in vascular transplantations (Kazemi *et al.*, 2018). Cold 0.9% normal saline solution (NSS) is another preservative solution, which is used in some resource-limited (developing) countries (Kazemi *et al.*, 2018). However, several studies have shown that NSS has a lower preservative efficacy when compared to other preservative solutions (Kazemi *et al.*, 2018; Zatschler *et al.*, 2009). Therefore, the addition or supplementation of a solution that could

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possibly enhance the preservative efficiency of NSS and prolong the vessel graft storage should be considered.

The secretory leukocyte protease inhibitor (SLPI) is a serine protease selective inhibitor that counteracts with an excessive inflammatory response (Majchrzak-Gorecka *et al.*, 2016). The previous studies showed that adding recombinant human SLPI (rhSLPI) in a heart preservative solution could restore myocardial contraction (Schneeberger *et al.*, 2008). Besides, the previous studies showed that the treatment of rhSLPI in human umbilical vein endothelial cells could reduce cell damage and inflammation, prevent death, and preserve cellular cytoskeleton integrity from *in vitro* I/R injury (Nernpermpisooth *et al.*, 2017; Paiyabhroma *et al.*, 2018; Prompunt *et al.*, 2018a, 2018b). This suggests the efficacious cytoprotective effect of rhSLPI on vascular endothelial cells.

The previous study has also demonstrated that endothelial-derived rhSLPI not only reduces endothelial cell injury but also attenuates cardiomyocyte death in an *in vitro* ischemia/reperfusion model (Kongpol *et al.*, 2019). Therefore, the *in vitro* endothelial protective effect of rhSLPI could provide basic information for the implementation of vascular tissue. However, the effect of rhSLPI supplemented to 0.9% NSS on vessel graft preservation has never been investigated. Therefore, we hypothesized that 0.9% NSS preservative solution, supplemented with rhSLPI, could prevent isolated rat aorta injury and prolong storage duration.

MATERIALS AND METHODS

Experiment animals

Adult male Wistar rats weighing 250–300 g ($n = 6$) were purchased from Nomura Siam International, Bangkok, Thailand. All animals were maintained under temperature ($22 \pm 1^\circ\text{C}$) with 12-hours light:dark cycle at the Center for Animal Research, Naresuan University, Phitsanulok, Thailand. All protocols used in this study were approved by the Animal Use and Care Committee at Naresuan University (NU-AE601129) and conformed to the guidelines set by the American Physiological Society and Animal Welfare Act.

Isolation of rat aorta

Rats were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and heparin (150 units). The deep anesthesia was closely observed and confirmed by a lack of both toe pinch and corneal reflexes. After that, the hearts were rapidly isolated. The thoracic and abdominal aorta were then excised quickly and placed in ice-cold NSS. The aorta was carefully cleaned from adjacent fatty tissue and cut into ring segments with 3–5 mm in length. Therefore, a total of 16 aortic rings per animal were incubated in 0.9% NSS in the presence and absence of 1 $\mu\text{g}/\text{ml}$ rhSLPI and stored at 4°C for various preservative periods (0, 6, 24, and 48 hours). One set of aortic rings, which preserved with and without rhSLPI at each period, was used for histopathological study. Another set of aortic rings was homogenized to collect protein and determined for the inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA).

Vascular histopathology

The histopathology technique used in this study was modified from Howat WJ and Wilson BA (Howat and Wilson, 2014). At the end of each preservation period, the aortic rings were fixed with 2.5% (v/v) glutaraldehyde for 24 hours. Then, the rings were further fixed with 10% (v/v) formalin pending a histopathological process.

Briefly, the rings were dehydrated, embedded into paraffin, and cut into 5–10- μm -thick sections (PFM Rotary, Cologne, Germany). Then, the sections were stained with hematoxylin and eosin (H&E) for histopathological analysis. All histopathological examinations were performed by an experienced pathologist. The pathologist was blind toward the experiment group, and the examination and scoring of vascular pathology were performed under a light microscope (Olympus). The identification of vascular endothelial cells was based on the information that the endothelial cells at tunica intima showed as a single layer of squamous-to-fusiform shape cells close to the lumen of the blood vessel. In H&E staining, the endothelial cells reveal as a flat layer of squamous-to-fusiform shape cells characterized by basophilic oval-shaped nucleus, which may protrude into the lumen of the blood vessel, and very few of eosinophilic cytoplasm. The vascular histopathology scoring criteria consisted of endothelial detachment, elastic membrane disruption, necrosis, endothelial degeneration/edema, and complete denudation (Kazemi *et al.*, 2018). The severity score was graded as follows: grade 0 = no lesion, grade 1 = lesion less than 25% of tissue, and grade 2 = lesion more than 25% of tissue.

Determination of released lactate dehydrogenase (LDH) activity

At the end of each preservative period, the preservation solution was collected and stored at -20°C until analysis (Prompunt *et al.*, 2018a). The released LDH activity was determined from the collected solutions using the LDH activity assay kit, which is a modified method based on the recommendations of the Scandinavian Committee on Enzymes (LDH SCE mod.). The kit was purchased from HUMAN (Wiesbaden, Germany). About 10 μl of collected preservative at each period was mixed with 1 ml of reaction buffer and incubated at 37°C for 5 minutes. Then, 250 μl of substrate reagent was added. The solution was mixed, and the absorbance was read at $\lambda 340$ nm. The mean absorbance change per minute ($\Delta A/\text{minute}$) was used for calculating LDH activity with the following formula:

$$\text{LDH activity (U/l)} = \Delta A/\text{minute} \times 20,000$$

Tissue homogenization and protein extraction

Aortic rings, which preserved in NSS in the presence and absence of rhSLPI at each period, were snapped in liquid nitrogen and transferred to -20°C freezer until analysis. About 50 mg of aortic ring sample was homogenized in 500 μl of homogenization buffer [20 mM Tris HCl (pH 6.8), 1 mM Na_3VO_4 , and 5 mM NaF] using a mortar and pestle (Mongkolpathumrat *et al.*, 2019). Then, the tissue homogenate was centrifuged at 14,000 rpm for 10 minutes at 4°C . The supernatant was collected for further experiments.

Determination of protein concentration by Bradford assay

The total protein concentration of tissue homogenate was measured by Bradford protein assay reagent (BIO-RAD, Hercules, CA) (Mongkolpathumrat *et al.*, 2019). About 50 μl of the protein samples were added into 2.5 ml of Bradford reagent (BIO-RAD, Hercules, CA) and incubated at room temperature for at least 5 minutes. The absorbance was measured using a spectrophotometer at $\lambda 595$ nm. The relative protein concentration was calculated by the equation for the line generated in the Bovine Serum Albumin (BSA) standard curve. The protein concentration

was used to adjust the amount of protein used for the determination of inflammatory cytokine by ELISA.

Determination of inflammatory cytokines by ELISA

ELISA was performed using a 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) ELISA Buffer Kit, Preprotech® (Mongkolpathumrat *et al.*, 2019). The ELISA reagents were prepared at room temperature by gentle mixing. First, the precoated step was performed in 96-well plate overnight. On the following day, the plate was inverted to remove the liquid and blot the paper towel. After that, the plate was washed four times using 200 µl of washing buffer solution. About 200 µl of the blocking solution was added into the well and then incubated for 1 hour. Tissue homogenates (10 µg of protein) were added into the wells and incubated at room temperature for at least 2 hours followed by washing 4 times again. Then, the detection antibody was added and incubated at room temperature for 2 hours. The plate was then washed again, and 100 µl of avidin-horseradish peroxidase conjugated 1:2,000 was added and incubated at room temperature for 30 minutes. After that, the ABTS liquid substrate was added and incubated at room temperature until the color was developed. The absorbance was measured using a spectrophotometer at 4405 nm.

Statistical analysis

Statistical analysis was performed using commercially available software (GraphPad Prism version 7). All data were expressed as mean ± S.E.M. All comparisons were assessed for significance using an unpaired *t*-test or analysis of variance (ANOVA) followed by the Tukey-Kramer test or Chi-square test when appropriate. A *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

Protective effect of rhSLPI decrease tissue injury by reduced LDH activity

The aortic rings were preserved in cold NSS in the presence and absences of 1 µg/ml of rhSLPI for 0, 6, 24, and 48 hours. Then, the preservative solution was collected to determine the released LDH activity. The results showed that there was a significant increase in released LDH activity in preservative solution at 6–48 hours when compared to control 0 hour (6 hours: 32.25 ± 1.80 IU/ml, 24 hours: 42.37 ± 2.40 IU/ml, and 48 hours: 77.58 ± 1.60 IU/ml vs. 0.5 ± 0.29 IU/ml, *p* < 0.05). Moreover, the results showed that the released LDH activity in NSS with rhSLPI for 48 hours was significantly lower than that of NSS (61.75 ± 1.58 IU/ml vs. 77.58 ± 1.60 IU/ml, *p* < 0.05). However, there was a trend of the reduction of released LDH activity in NSS with rhSLPI at 6 and 24 hours but was not statistically significant when compared to NSS group (6 hours: 32.25 ± 1.80 IU/ml and 6 hours with rhSLPI: 25.86 ± 2.33 IU/ml, *p* > 0.05, and 24 hours: 42.37 ± 2.40 IU/ml and 24 hours with rhSLPI: 33.59 ± 2.50 IU/ml, *p* > 0.05) (Fig. 1).

Protective effect of rhSLPI decrease inflammatory cytokines

The protein extract from aortic rings was used to determine the inflammatory cytokines by ELISA, including TNF-α and IL-6. The results showed that vessels preserved for 48 hours in both the groups significantly increased TNF-α level when compared to control (NSS: 338.9 ± 50.15 pg/ml, NSS with rhSLPI:

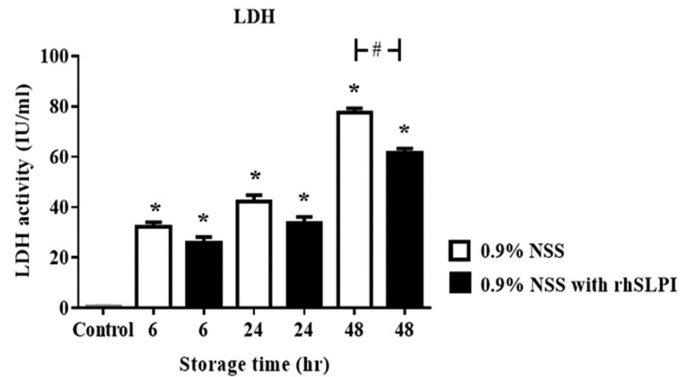


Figure 1. Determination of vascular injury by a released LDH activity in the preservative solution. Aortic rings were preserved in 0.9% NSS or 0.9% NSS with rhSLPI preservative solution for 0, 6, 24, and 48 hours, after which the solution was collected and assessed for LDH activity. Each bar graph represents the means ± S.E.M. **p* < 0.05 versus storage at 0 hour (control) (ANOVA), #*p* < 0.05 versus among storage in 0.9 % NSS, with or without rhSLPI.

237 ± 21.98 pg/ml vs. control: 178 ± 19.03 pg/ml, *p* < 0.05). On the contrary, vascular preserved for 24 hours did not appear to be different when compared to control (NSS: 181.4 ± 6.66 pg/ml and NSS with rhSLPI: 150.3 ± 5.53 pg/ml vs. control: 178 ± 19.03 pg/ml, *p* > 0.05). However, the TNF-α level in the vessel preserved in NSS with rhSLPI for 24 hours was significantly lower than vascular preserved in NSS (150.3 ± 5.53 pg/ml vs. 181.4 ± 6.66 pg/ml, *p* < 0.05) (Fig. 2A). Besides, the level of TNF-α in NSS with rhSLPI for 48 hours was lower than 0.9% NSS without rhSLPI but could not reach statistical significance (237 ± 21.98 pg/ml vs. 338.9 ± 50.15 pg/ml, *p* > 0.05) (Fig. 2A).

Similarly, the vessels preserved for only 48 hours in both the groups showed a significant increase in IL-6 level when compared to control (NSS: 401.6 ± 31.81 pg/ml and NSS with rhSLPI: 298.6 ± 21.88 pg/ml vs. control: 193.7 ± 8.75 pg/ml, *p* < 0.05). Furthermore, the vessel preserved in NSS supplemented with rhSLPI for 24 hours was shown to be significantly lower when compared to control (139.7 ± 11.13 pg/ml vs. 193.7 ± 8.75 pg/ml, *p* < 0.05). The IL-6 level vessels preserved in NSS supplemented with rhSLPI for 24 and 48 hours were shown to be significantly lower than that of NSS (24 hours: 139.7 ± 11.13 pg/ml vs. 180.7 ± 16.17 pg/ml, *p* < 0.05, and 48 hours: 298.6 ± 21.88 pg/ml vs. 401.6 ± 31.81, *p* < 0.05) (Fig. 2B).

Protective effect of rhSLPI on vascular histopathology

Vascular histopathology was performed in both aortic tissues preserved in NSS and NSS supplemented with rhSLPI. The endothelial detachment, elastic membrane disruption, necrosis, endothelial degeneration/edema, and complete denudation were divided into no change or change. The results showed that there was no significant difference in the number of vessels with the presence of histopathological changes during different durations of preservation (Table 1).

To determine the severity of vascular histopathology as well as the effect of rhSLPI to preserve vascular integrity, the severity of endothelial detachment, elastic membrane disruption, necrosis, endothelial degeneration/edema, and complete denudation were graded on a scale ranging from 0 to 2 (Fig. 3).

For endothelial detachment, the results found that the vessels preserved in NSS with rhSLPI for 6 and 24 hours showed a

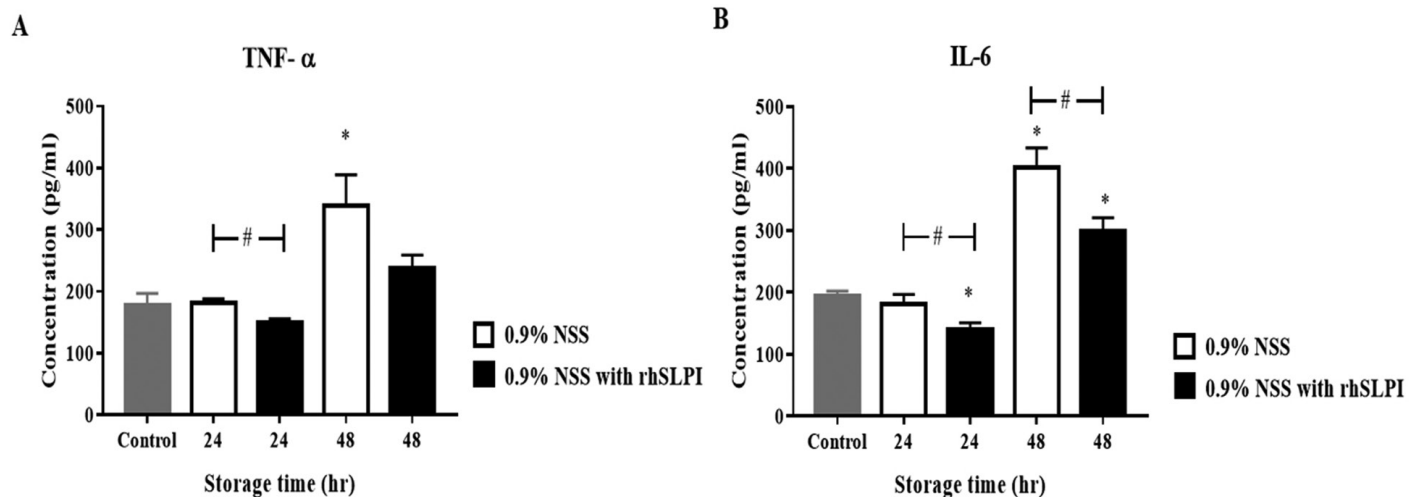


Figure 2. Determination of inflammatory cytokines in vascular tissue. Aortic rings were preserved in 0.9% NSS in the presence and absence of rhSLPI for 0, 24, and 48 hours. The vessel protein was extracted and measured for inflammatory cytokines including (A) TNF- α and (B) IL-6. Each bar graph represents the means \pm SEM. * $p < 0.05$ versus storage at 0 hour (ANOVA), # $p < 0.05$ versus among storage in 0.9%NSS, with or without rhSLPI (ANOVA, $n = 6$).

significantly lower endothelial detachment score when compared to the other groups (Fig. 4A). On the contrary, the vessels preserved in NSS with rhSLPI for 48 hours did not show any significant difference (Fig. 4A).

Similarly, the vessels preserved in NSS with rhSLPI for 6 and 24 hours showed a significantly lower elastic membrane disruption score when compared to the vessels preserved in NSS (Fig. 4B). Although the elastic membrane disruption score of the vessels preserved in NSS with rhSLPI for 48 hours was not significantly lower than other groups, the score was shown to have a lower trend in the vessels preserved in NSS with rhSLPI.

The necrosis scores for the vessels preserved in both groups of preservatives for 6 hours were not shown to be different. On the contrary, the vessels preserved in NSS with rhSLPI for 24 hours showed a significantly lower necrosis score when compared to the other groups (Fig. 4C). There were no significant differences in necrosis score between the vessels preserved in NSS with and without rhSLPI for 48 hours though there was a trend of reduction in necrosis score in the vessels preserved in rhSLPI group.

Similarly, the score for endothelial degeneration/edema in the vessels preserved for 6 hours showed that the vessels preserved in NSS with rhSLPI had a significantly lower endothelial degeneration/edema score when compared to the vessels preserved in NSS (Fig. 4E). However, the score of complete denudations showed no significant difference in any group (Fig. 4F).

DISCUSSION

Among crucial factors that affect the success of vascular reconstruction, the quality of vessel grafting, primarily influenced by the graft preservative/storage solution, influences the preservation of the endothelial structure and vascular functions (Woodward *et al.*, 2016).

NSS is the most widely used preservative solution in vascular operative procedures (Kazemi *et al.*, 2018; Weiss *et al.*, 2009) as it is convenient and cheap. However, several studies have suggested unsatisfactory outcomes of its use, such as altered integrity of the endothelial layer and abolished vascular dilation (Weiss *et al.*, 2009; Wilbring *et al.*, 2011, Wilbring *et al.*, 2013a,

2013b). Therefore, the preservative efficiency of NSS could be enhanced by adding supplements.

The previous studies have demonstrated that adding various substances such as heparin with papaverine (Santoli *et al.*, 1993) or 5% albumin (Weiss *et al.*, 2009) could enhance the efficiency of NSS for vessel graft preservation. The previous reports have also shown that the cardiovascular-protective effect of rhSLPI could protect cardiomyocytes, cardiac fibroblast, and vascular endothelial cells from ischemia/reperfusion (I/R) injury (Nernpermpisooth *et al.*, 2017; Paiyabhroma *et al.*, 2018; Prompunt *et al.*, 2018a, 2018b). Recently, we have demonstrated that endothelial-derived SLPI could protect cardiomyocytes from I/R injury (Kongpol *et al.*, 2019), which suggests the beneficial effect of rhSLPI for cardiovascular applications. Interestingly, it has been shown that adding rhSLPI in a heart preservative solution could restore myocardial contraction during experimental murine heart transplantation (Schneeberger *et al.*, 2008).

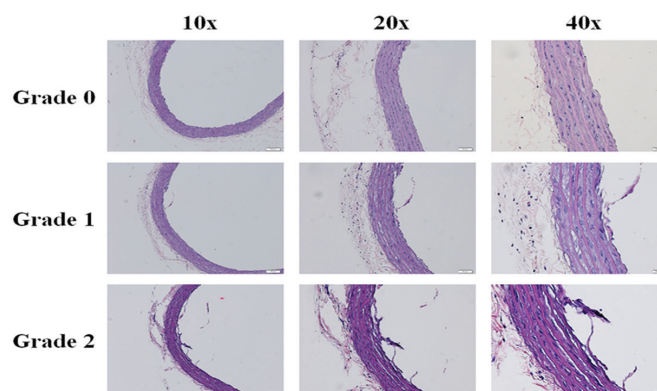
The major findings in the current study showed, for the first time, that rhSLPI could enhance the efficiency of conventional preservative NSS to preserve the isolated rat aortic vessels by reducing injury and inflammation and preserve the vascular structure. Furthermore, the experiments published by Schneeberger *et al.* were designed to test the effect of SLPI, either by gene knockout or treatment with recombinant protein, in transplanted murine hearts. The results showed that 200 μ g of recombinant SLPI given intravenously immediately after transplantation, or diluted in cold-HTK preservation and flushed into the heart, could significantly improve a cardiac score, without the improvement of graft histology, and neither affected organ injury nor the inflammatory response. However, this study showed that only 1 μ g/ml of rhSLPI (200 times less concentrate) dissolved in NSS could preserve vessel allograft by reducing tissue injury and inflammatory response and improve graft histology. Therefore, this study provides evidence that rhSLPI has the potential to be an alternative additive for vessel graft preservation.

The preservation in cold NSS could be affected by both profound low-storage temperature and bloodless-hypoxic conditions. These could induce biochemical and physiological stresses that are detrimental to vessel grafts and, consequently,

Table 1. Nonscoring of vascular histopathology changes in aortic rings preserved in NSS or NSS with rhSLPI for 0, 6, 24, and 48 hours.

		NSS (n)	NSS with rhSLPI (n)	p-value
Endothelial detachment				
Control	No change	5	–	
	Change	1	–	
6 hours	No change	1	5	0.080
	Change	5	1	
24 hours	No change	1	5	0.080
	Change	5	1	
48 hours	No change	3	2	>0.99
	Change	3	4	
Elastic membrane disruption				
Control	No change	5	–	
	Change	1	–	
6 hours	No change	0	4	0.061
	Change	6	2	
24 hours	No change	1	5	0.080
	Change	5	1	
48 hours	No change	0	0	>0.99
	Change	6	6	
Necrosis				
Control	No change	6	–	
	Change	0	–	
6 hours	No change	6	6	>0.99
	Change	0	0	
24 hours	No change	3	6	0.181
	Change	3	0	
48 hours	No change	5	6	>0.99
	Change	1	0	
Endothelial degeneration/edema				
Control	No change	5	–	
	Change	1	–	
6 hours	No change	2	5	0.242
	Change	4	1	
24 hours	No change	5	6	>0.99
	Change	1	0	
48 hours	No change	6	6	>0.99
	Change	0	0	
Complete denudation				
Control	No change	6	–	
	Change	0	–	
6 hours	No change	6	6	>0.99
	Change	0	0	
24 hours	No change	5	6	>0.99
	Change	1	0	
48 hours	No change	5	5	>0.99
	Change	1	1	

cause tissue injury that eventually leads to graft failure. The determination of cellular injury could be confirmed by the detection of released (LDH – a cytosolic enzyme present in almost all cell types in the vasculature) activity. When the plasma membrane is ruptured or damaged, LDH is rapidly released into the surrounding environment (Chan *et al.*, 2013; Kumar *et al.*, 2018). Therefore, the determination of LDH activity is a marker of cellular injury. The vessels preserved in NSS with rhSLPI showed a lower released LDH activity (Fig. 1). This result indicated that

**Figure 3.** Scoring scheme of vascular histopathology. Aortic rings were preserved in 0.9% NSS or 0.9% NSS with rhSLPI preservative solution for 0, 6, 24, and 48 hours. The vessel sections were stained with hematoxylin and eosin (H&E) for histopathology analysis by a pathologist.

rhSLPI could be an alternative additive of choice supplement to NSS by reducing vessel graft injury during storage.

A short episode of ischemia/reperfusion (I/R) injury occurs during the isolation of vessel grafting and the transplantation procedure, respectively (Kazemi *et al.*, 2018; Schneeberger *et al.*, 2008). The I/R injury is an inflammatory process that increases the expression of pro-inflammatory cytokines, particularly the tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6), that cause cellular injury and death and induce leukocyte recruitment and platelet adhesion, which could lead to vascular occlusion and rejection after revascularization. These affect the success of organ transplants (Eltzschig and Eckle, 2011; Epelman *et al.*, 2015; Kazemi *et al.*, 2018). Moreover, ischemic injury could also induce a tissue inflammation by releasing pro-inflammatory cytokines (Yang *et al.*, 2016).

One of the well-known functions of SLPI is anti-inflammation (Doumas *et al.*, 2005), which corresponds to these findings and demonstrated that vessels preserved in NSS with rhSLPI had less TNF- α and IL-6 levels than control (Fig. 2). Similar to the previous study, the pretreatment of rhSLPI in adipocytes indicated the downregulation of LPS-induced IL-6 gene expression and protein secretion in adipocytes (Adapala *et al.*, 2011). Besides, mice hearts preserved in cold preservative with rhSLPI also decreased pro-inflammatory cytokines expression, including TNF- α , Transforming growth factor (TGF)- β , Nuclear Factor Kappa B (NF- κ B), eNOS, Potassium chloride (KCl) (Schneeberger *et al.*, 2008). Therefore, adding rhSLPI to cold preservative could reduce pro-inflammatory cytokine levels, leading to a decreased inflammation of vessel grafts.

The previous studies have shown that NSS is an acceptable preservative although with limitations when compared to other preservative solutions (Kazemi *et al.*, 2018; Zatschler *et al.*, 2009). For example, the determination of preservation-induced changes in smooth muscle cell and *ex vivo* endothelial cell function in isolated porcine carotid arteries showed that NSS is the worst preservative solution, causing failure in the contraction and relaxation of the carotid artery, when compared to other preservatives, such as UW, HTK, Celsior, or a modified HTK solution (Abrahamse *et al.*, 2002). Besides, a comparison between HTK and physiological saline solution (PSS) or the newly developed preservation solutions (solution 8 and solution 9) in isolated rat aorta showed that PSS

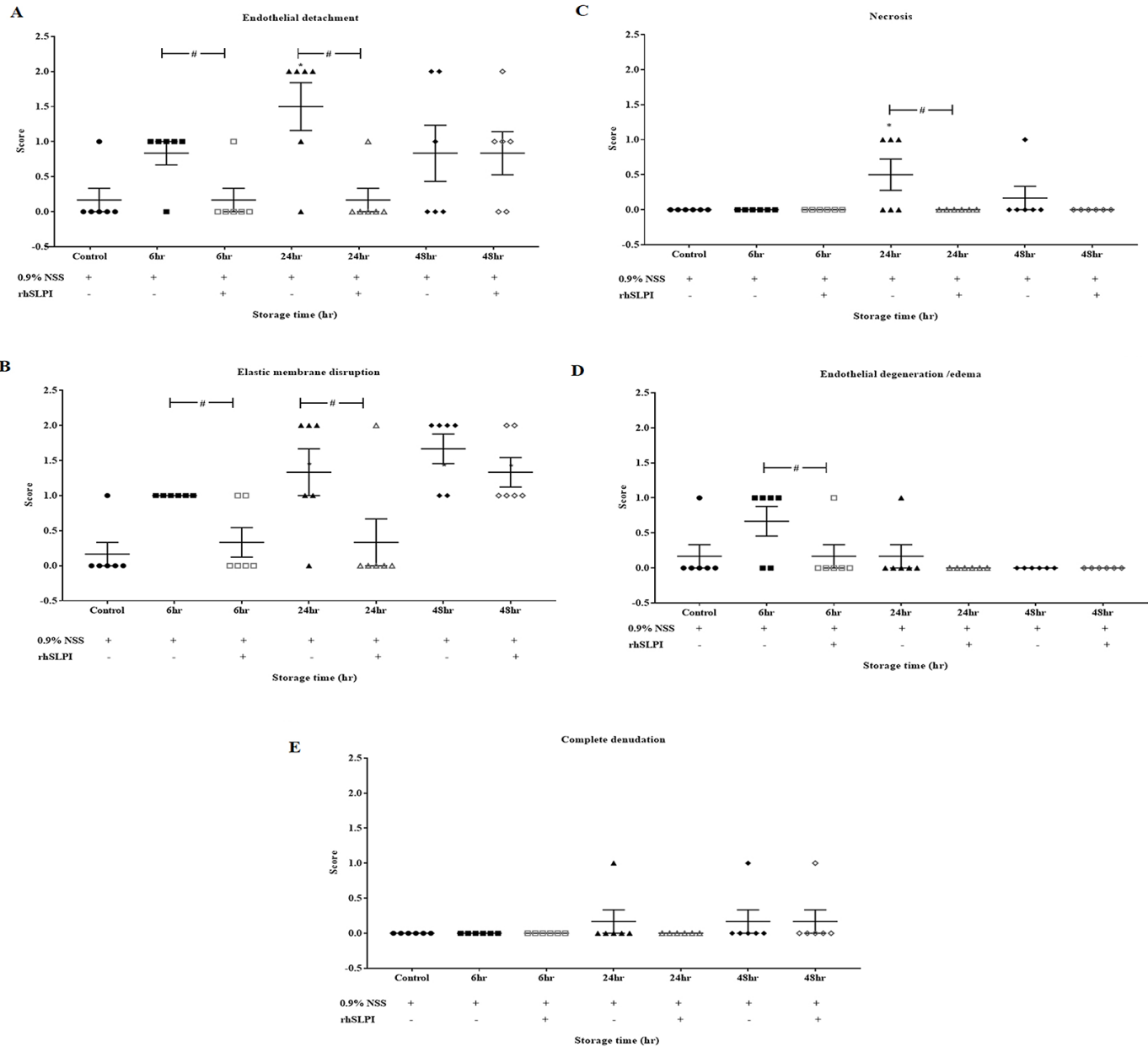


Figure 4. Vascular histopathology score after storage of aortic rings in 0.9% NSS in the presence and absence of rhSLPI for 0, 6, 24, and 48 hours. The vascular histopathology parameters consisted of (A) endothelial detachment, (B) elastic membrane disruption, (C) necrosis, (D) endothelial degeneration/edema, and (E) complete denudation. Data are presented in a scatter plot with the values of means \pm S.E.M. * $p < 0.05$ versus storage at 0 hour (ANOVA), # $p < 0.05$ versus among storage in 0.9% NSS, with or without rhSLPI (ANOVA, $n = 6$).

failed to develop the vascular relaxation in isometric tension and endothelial nitric oxide synthase (eNOS) KCl expression (Zatschler *et al.*, 2009). Furthermore, human saphenous vein preserved in NSS showed a reduction of KCl- and phenylephrine-induced vascular contraction, as well as endothelial-dependent and independent relaxation and cell viability when compared to UW solution, Celsior solution, and autologous whole blood (Wise *et al.*, 2015). However, the aspect of preservation on the vascular structure by measuring vascular histopathology was not considered as there was insufficient information. In the previous study, isolated human femoral and iliac arteries preserved in NSS or UW solutions were assessed for vascular integrity changes using the histopathology technique on the 1st, 5th, 10th, and 21st days (Kazemi *et al.*, 2018). The study

demonstrated that there was no statistical difference in vascular pathological score between NSS and UW groups until 21st day (Kazemi *et al.*, 2018). This was similar to the findings, indicating that there was no significant difference in the complete denudation score for the vessels preserved in NSS in the presence and absence of rhSLPI for 48 hours. However, the preservation for 24 hours resulted in a lower pathological degenerative score, particularly on endothelial detachment, elastic membrane disruption, and necrosis in NSS supplemented with rhSLPI (Table 1).

Interestingly, the histopathological score (Fig. 4A, C, and D) showed discrepancies in histopathological changes between 24 and 48 hours. It has been reviewed that the earliest event of vascular injury is endothelial edema, followed by endothelial

necrosis, resulting in endothelial detachment (Woywodt *et al.*, 2002). Therefore, at 6 hours after storage, we assumed that most endothelial cells were in the edema stage, which reflect why the scoring of endothelial edema at 6 hours was the highest (Fig. 4D). When the injury progressed between 24 and 48 hours, the necrosis of endothelial cells predominated, and therefore, the score of endothelial necrosis was observed as higher, whereas that of edema was lower (Fig. 4C). At 24–48 hours, endothelial detachment occurs (Fig. 4A), where endothelial detachment scores of both 24 and 48 hours predominated, without significant difference between 24 and 48 hours. At 48 hours, endothelial cell detachment occurs, and in particular, necrotic cells also disappeared from vascular tissue; therefore, the score of necrosis at 48 hours was observed less. This result provided novel information concerning the addition of rhSLPI into NSS to protect the vascular structure and could potentially increase the success rate of vascular transplantation.

There are several limitations that could be considered in this study, as follows: first, only the vascular structure was measured by vascular histopathological assessment. However, the effect of preservation with rhSLPI on vascular physiological functions, by using isometric tension, needs to be investigated. Moreover, the expression of adhesion molecules such as cell adhesion molecules, P-selectins, and integrin on activated endothelial cells should be investigated further to evaluate the risk of vessel graft failure under rhSLPI supplement in NSS. This experimental study performed a preclinical research using rats. The sample size ($n=6$) was calculated as appropriate by a statistician with the awareness of unnecessary wastage of resources, which lead to ethical issues. However, the sample size in this study might only be appropriate for a laboratory study and might not provide more clinical significance. Therefore, we suggest using a larger sample size in future published articles, mostly in clinical studies of human samples.

For the first time, this study showed that rhSLPI could enhance the efficiency of NSS as a vascular preservative solution. However, the experimental perspective should focus on the effect of rhSLPI on vessel preservation in an *in vivo* animal model of transplantation. To closely simulate real clinical settings, the clinical outcome of vessel patency post-transplantation of vessel grafts, preserved in a preservative solution supplemented with rhSLPI, should be determined. This could be performed using angiography to determine the vascular parameters such as wall thickness and lumen diameter, as well as atherosclerotic lesions. From a clinical perspective, the outcomes from this study could be implemented for human vessel grafting and other organ transplants, and the long-term effects of transplanted grafts preserved in preservative solution, supplemented with rhSLPI, should be determined. Furthermore, the effect of rhSLPI in a preservative solution for other vital organs, such as the kidneys, brain, and liver, should be considered for further study.

CONCLUSION

The supplementation of vascular preservative solution with recombinant rhSLPI could reduce vascular injury and inflammation while maintaining vascular integrity.

For the first time, the results have shown that adding rhSLPI to the preservative saline solution could prevent vascular injury and possibly extend the duration of graft storage before transplantation.

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AUTHORS' CONTRIBUTIONS

Kantapich Kongpol, Nitirut Nernpermpisooth, and Sarawut Kumphune conceived and designed the experiments. Kantapich Kongpol and Rungrueang Yodsheewan performed the experiments. Kantapich Kongpol, Rungrueang Yodsheewan, and Sarawut Kumphune analyzed the data. Sarawut Kumphune and Nitirut Nernpermpisooth contributed reagents/materials/analysis tools. Kantapich Kongpol, Nitirut Nernpermpisooth, and Sarawut Kumphune wrote and prepared the manuscript. Kantapich Kongpol, Rungrueang Yodsheewan, Nitirut Nernpermpisooth, and Sarawut Kumphune corrected, revived, commented, and responded to reviewer's comments.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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