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Journal of the Chinese Medical Association 79 (2016) 400–408

www.jcma-online.com

Original Article

Low dose of protein A pretreatment can alleviate the inflammatory reaction and the bio-safety was evaluated *in vivo*

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Received July 9, 2015; accepted November 27, 2015

Abstract

Background: Staphylococcal protein A (SPA) is a protein of *Staphylococcus aureus*. Up to now, there have been many studies on the biological activities of SPA. Some reported effects of SPA pretreatment on septic shock in mouse models but there is no study which reports the role of SPA pretreatment on the infected incision.

Methods: According to count results, bacterial suspension was set at a density of ~1.8×10^9 colony forming units/mL. BALB/c mice were anesthetized via intraperitoneal injection with pentobarbital sodium. A longitudinal skin incision was made on the medial side of the right thigh. The length of the incision was 5 mm, and the depth was ~3 mm. The bacterial suspension was gradually dripped and embrocated onto the incision surface to make the wound infection model. Before making the wound infection model for 48 hours and 24 hours, mice were retreated with SPA via intraperitoneal injection. Rats were intraperitoneally injected with SPA 1 mg/kg and the control group was injected with sterile saline to evaluate the biological safety of the best pretreatment dose.

Results: A 1-mL bacterial suspension can be utilized to make the wound infection model of BALB/c mouse lower limb. SPA pretreatment can reduce the inflammatory reactions in wound methicillin-resistant *Staphylococcus aureus* infection mouse model and the best pretreatment dose is 1 mg/kg. Intraperitoneal injection 1 mg/kg SPA does not destroy the functions of the organs. A 1-mg/kg SPA pretreatment can also reduce the inflammatory reactions in wound various bacterial infection mouse models.

Conclusion: SPA pretreatment can effectively decrease the infected severity of a wound infected by various bacteria in a BALB/c mouse model. The best pretreatment dose is 1 mg/kg, and this dose does not damage organ function in rats up to a point.

Keywords: effects; infected incision; low doses of Staphylococcal protein A

1. Introduction

Staphylococcal protein A (SPA) is a protein in *Staphylococcus* aureus.[1–3] It can form precipitation with highly diluted immune sera from an animal inoculated with *S. aureus* or SPA.[4] At present, most species of coagulase-positive *S. aureus* have SPA, whereas few species of coagulase-negative *S. aureus* have SPA.[5,6] Low doses of SPA can cause an allergic reaction and high doses can cause bleeding and arthus reaction.[7] However, SPA can activate the complement. When the body is infected with *S. aureus*, SPA can combine with IgG to activate the complement to localize the infection.[4,8,9] SPA has further important capabilities, including: (1) inhibiting the phagocytosis of macrophages;[10] (2) activating B cells with T cells;[11,12] and (3) inducing B cells to synthesize and secrete polygonal antibody.[11] As an immunomodulator, a low dose SPA pretreatment can adjust the body immune system response. When the body is infected after SPA pretreatment, the body can therefore respond quickly. Along with the study of the effects of SPA on the immune system, some

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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http://dx.doi.org/10.1016/j.jcma.2016.01.010

reports have presented that SPA pretreatment can protect mice from a lethal infection of *S. aureus*.[13] However, no published research currently exists regarding SPA pretreatment on the infected incision. Our study is based on a BALB/c mice infected incision model, exploring SPA pretreatment with different doses on an incision infected by methicillin-resistant *S. aureus* (MRSA), assessing the bio-safety of the best pretreatment dose, and analyzing the effects of SPA pretreatment on the incision infected by different bacteria.

2. Methods

2.1. Bacteria

Methicillin-sensitive *S. aureus* (MSSA) strain ATCC 25923, MRSA strain ATCC 33591, *Pseudomonas aeruginosa* strain ATCC 27853, and *Escherichia coli* strain ATCC 25922 were all from American Type Culture Collection (Manassas, VA, USA).

2.2. Mice and rats

Adult BALB/c mice (20 g) and adult Wistar rats (200 g) were obtained from the Charles River Laboratories (Beijing, China). All procedures were performed in accordance with the guiding principles in the Care and Use of Animals and approved by the Capital Medical University Committee on the Use of Animals in Research and Education. Animals were separately housed in plastic cages in a room maintained at 23.6°C and 35% humidity with 12-hour light/dark cycles (light on at 07:00 AM). Each animal was used only once and fed a standard chow diet with unrestricted water intake. Experiments were conducted in an ABSL-2 laboratory and at the end of the experiments, the animals were anesthetized using pentobarbital sodium and then euthanized.

2.3. Reagents

Recombinant SPA was from the Sino Biological Inc. in Beijing China, product number: 10600-P07E. ELISA kits for the detection of IL-1β, IL-6, IL-10, and TNF-α were from the Dakewe Bioengineering Company in Beijing, China.

Table 1
Day 4 of the observation results of the incision.

	Slightly red and swollen	Obviously red and swollen	Appeared pus
MRSA 1 mL		5	7
MRSA 0.5 mL		8	4
MRSA 0.25 mL	4	7	1
MSSA 1 mL		9	3
MSSA 0.5 mL		10	2
MSSA 0.25 mL	6	6	0
P. aeruginosa 1 mL		7	5
P. aeruginosa 0.5 mL		9	3
P. aeruginosa 0.25 mL	2	8	2
E. coli 1		9	3
E. coli 0.5 mL		10	2
E. coli 0.25 mL	3	8	1

E. coli = *Escherichia coli*; MRSA = methicillin-resistant *Staphylococcus aureus*; MSSA = Methicillin-sensitive *Staphylococcus aureus; P. aeruginosa* = *Pseudomonas aeruginosa*.

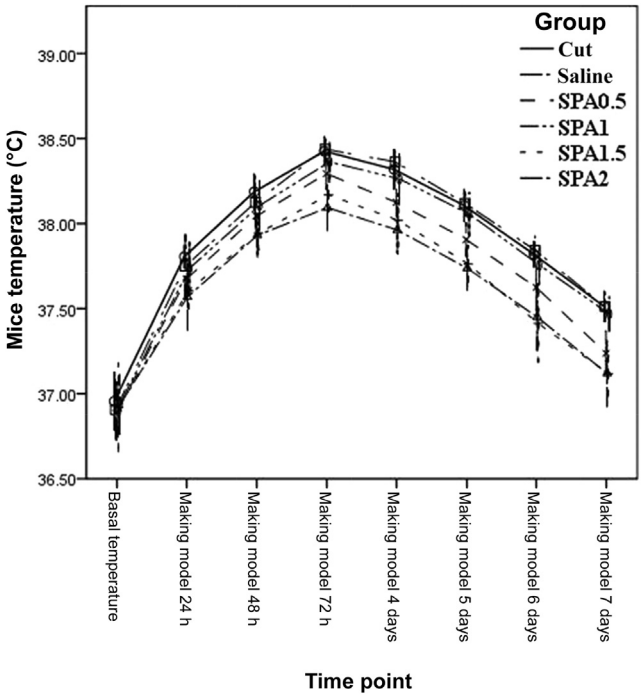

Fig. 1. The temperature variation in different pretreated groups of MRSA infected mice. Cut = incision only; MRSA = methicillin-resistant *Staphylococcus aureus*; saline = sterile saline pretreatment; SPA = staphylococcal protein A; SPA 0.5 = SPA 0.5 mg/kg pretreatment; SPA 1 = SPA 1 mg/kg pretreatment; SPA 1.5 = SPA 1.5 mg/kg pretreatment; SPA 2 = SPA 2 mg/kg pretreatment.

2.4. Preparation of bacteria suspension

Bacteria were cultured in Trypticase soy broth (Beijing, China) which was conducted in an incubator at 37°C in a 95% humidified atmosphere and 5% CO_2. Then, 0.2 mL bacterial culture solution was diluted 1:10 into sterile saline (Biosntech Company. Beijing, China) and measured the optical density (OD) value at 600 nm of the diluted solution every 1 hour. The growth of bacteria was in the exponential phase when the OD value was rapidly increasing.[14,15] Then, the bacteria were segmented (4°C, 6 × 10^3 r/min 15 min), washed, and suspended in sterile saline. A 0.2-mL suspension was diluted 1:10^4 into sterile saline. To 0.2 mL of the diluted suspension 0.4 mL 0.4% trypsin blue solution was added and mixed well to stain for 2 minutes. A 2-μL sample of the stained solution was flowed into the cell counter. According to the count results, bacterial suspension was set at a density of ~1.8 × 10^9 CFU/mL.

2.5. The incision infection model

BALB/c mice were randomly distributed into 13 groups, and each group consisted of 12 male. Mice of the control group received incisions made at the medial side of the right thigh without bacteria suspension dripped onto the surface.

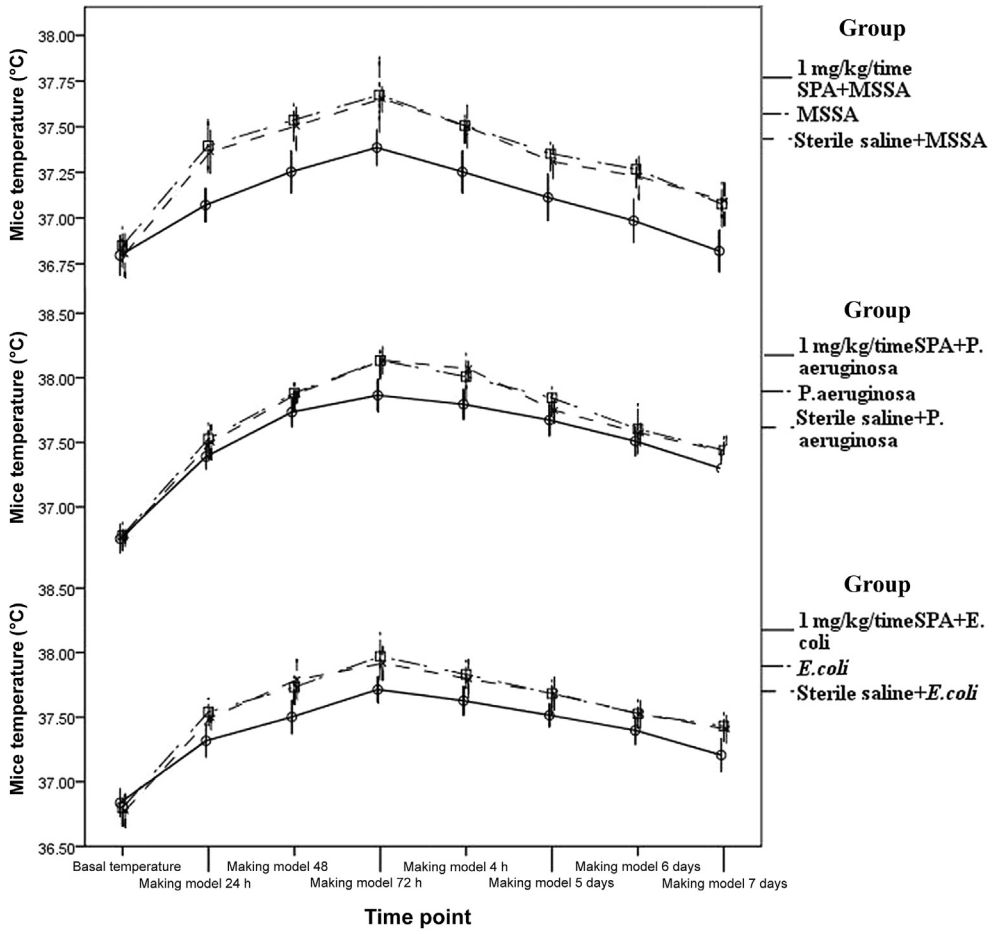

Fig. 2. The temperature variation of mice. *E. coli* = *Escherichia coli*; MSSA = Methicillin-sensitive *Staphylococcus aureus; P. aeruginosa* = *Pseudomonas aeruginosa*; SPA = staphylococcal protein A.

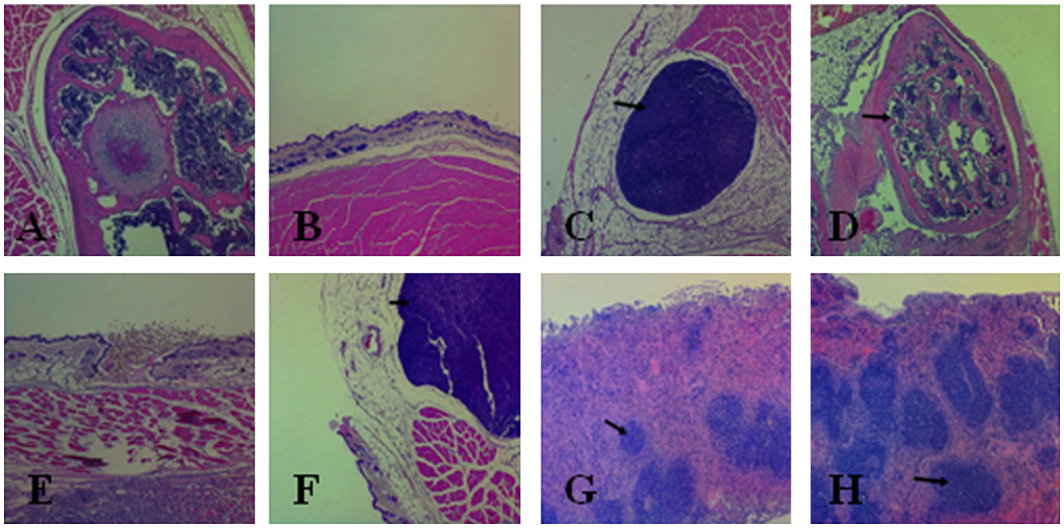

Fig. 3. Groups infected by MRSA. (A) Group A (SPA 0.5 mg/kg pretreatment) subcutaneous tissue was damaged and inflammatory cells infiltrated, × 40; (B) Group B (SPA 1 mg/kg pretreatment) epithelial tissue had healed and inflammatory response was milder, × 40; (C) Group C (SPA 1.5 mg/kg pretreatment) epithelial tissue did not heal and appeared subcutaneous abscess (arrow), × 40; (D) Group D (SPA 2 mg/kg pretreatment) epithelial tissue did not heal and tissue was damaged (arrow), × 40; (E) Group E (sterile saline pretreatment) epithelial tissue did not heal and tissue was damaged, × 40; (F) Group F (cut only) epithelial tissue did not heal and appeared subcutaneous abscess (arrow), × 40; (G) Group B in which splenic lymphoid nodules increased less (arrow in lymph nodules), × 40; and (H) Group E in which splenic lymphoid nodules increased much (arrow in lymph nodules), × 40. MRSA = methicillin-resistant *Staphylococcus aureus*; SPA = staphylococcal protein A.

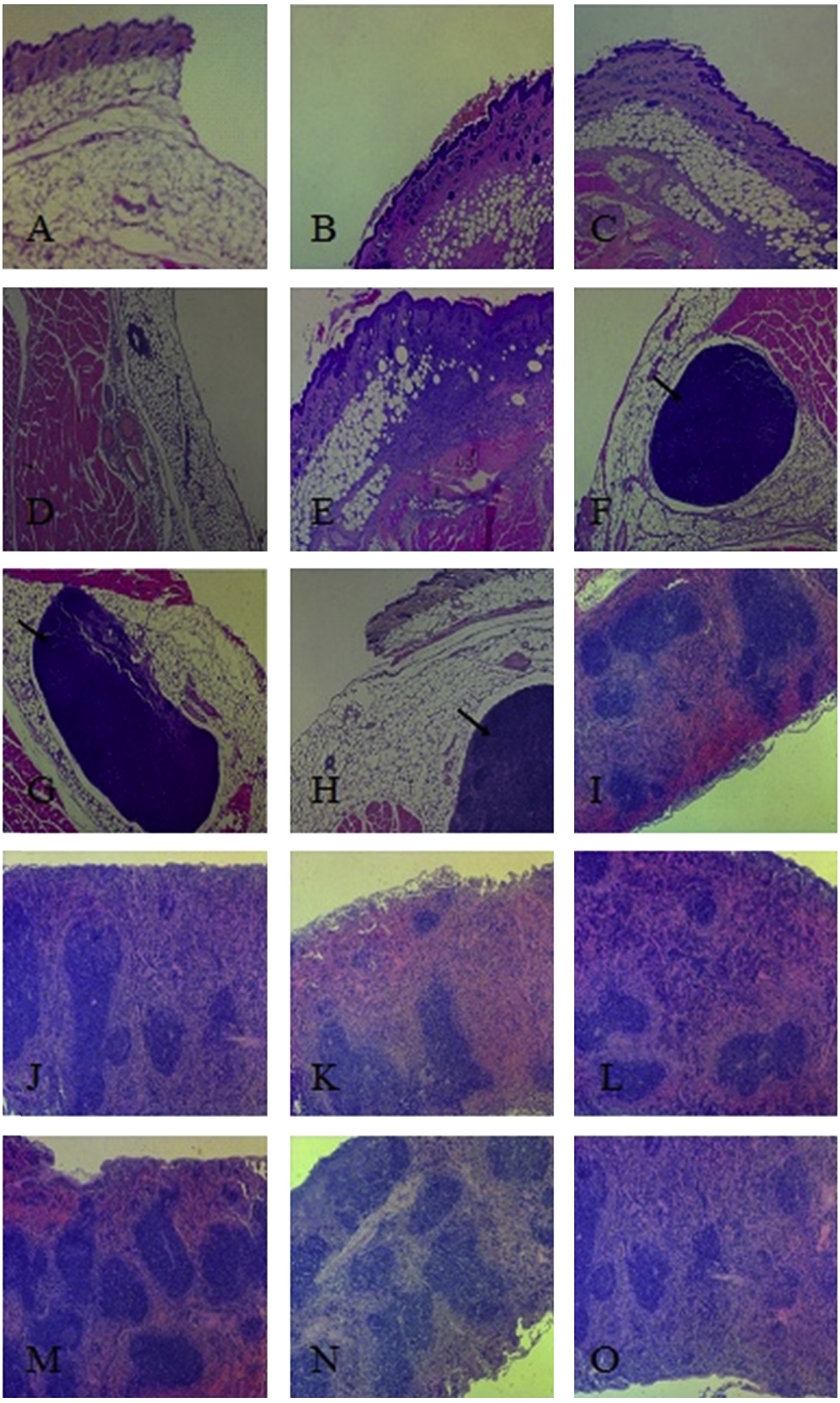

Fig. 4. Groups infected by different bacteria with SPA 1 mg/kg pretreatment. (A) and (I) Group P (MSSA infected), × 40; (B) and (J) Group Q (*P. aeruginosa* infected), × 40; (C) and (K) Group R (*E. coli* infected), × 40; (D) Group R (*E. coli* infected), × 40; (E) and (M) Group T (*P. aeruginosa* infected and sterile saline pretreated), × 40; (F) Group U (*E. coli* infected and sterile saline pretreated), × 40; (G) and (N) Group W (*P. aeruginosa* infected only), × 40; (H) and (O) Group X (*E. coli* infected only), × 40; and (L) Group S (MSSA infected and sterile saline pretreated), × 40. *E. coli* = *Escherichia coli*; MSSA = Methicillin-sensitive *Staphylococcus aureus; P. aeruginosa* = *Pseudomonas aeruginosa.*

Table 2
The white blood cells, granulocyte, lymphocytes, and cytokines in different MRSA infected pretreatment groups.

	Day 3					
	Group A SPA 0.5	Group B SPA 1	Group C SPA 1.5	Group D SPA 2	Group E Saline	Group F Cut
White blood cells	12.5563 ± 0.64804	10.9562 ± 0.74114	11.6562 ± 0.72935	13.4750 ± 0.54345	14.0125 ± 0.96945	14.2813 ± 0.89608
Granulocyte	2.5062 ± 0.57904	2.0563 ± 0.43508	2.1813 ± 0.26133	2.8188 ± 0.53068	3.0375 ± 0.54757	3.3688 ± 0.58163
Lymphocytes	8.7875 ± 0.61847	7.3500 ± 0.53166	7.8250 ± 0.70380	9.7750 ± 0.92268	10.1875 ± 0.92727	10.4688 ± 0.76830
	Days 7					
	Group A SPA 0.5	Group B SPA1	Group C SPA1.5	Group D SPA2	Group E Saline	Group F Cut
White blood cells	8.7000 ± 1.03923	7.5063 ± 0.87975	7.8438 ± 0.73391	9.3250 ± 0.77760	9.8813 ± 1.00546	10.3063 ± 0.66380
Granulocyte	1.7313 ± 0.34779	1.4313 ± 0.33807	1.6063 ± 0.40574	1.7750 ± 0.33166	1.8375 ± 0.43186	2.0500 ± 0.46043
Lymphocytes	6.2500 ± 0.57504	5.4688 ± 0.73368	5.7625 ± 0.88910	6.5500 ± 0.67132	7.0750 ± 0.51833	7.2438 ± 0.45456
IL-1β	221.1250 ± 19.32140	148.3750 ± 27.18793	182.3750 ± 34.98357	258.6875 ± 20.96893	292.2500 ± 32.77702	287.0000 ± 38.61261
IL-6	378.0625 ± 24.39254	287.9375 ± 37.34429	322.0000 ± 27.00617	451.6875 ± 20.19478	500.1250 ± 21.10253	493.6250 ± 25.44766
IL-10	142.0000 ± 18.51126	117.8125 ± 19.66119	129.1875 ± 20.43271	158.3750 ± 21.39120	182.3125 ± 19.71030	179.7500 ± 22.71123
TNF-α	156.1250 ± 18.72209	110.5625 ± 22.29490	121.9375 ± 20.65742	170.6250 ± 18.82507	208.6875 ± 29.16326	206.6875 ± 19.45154

Cut = incision only; saline = sterile saline pretreatment; MRSA = methicillin-resistant *Staphylococcus aureus*; SPA = staphylococcal protein A; SPA 0.5 = SPA 0.5 mg/kg pretreatment; SPA 1 = SPA 1 mg/kg pretreatment; SPA 1.5 = SPA 1.5 mg/kg pretreatment; SPA2 = SPA 2 mg/kg pretreatment.

Mice of the other groups received the bacterial suspension gradually dripped onto the surface of the incision and embrocated with a sterile bacterial inoculation needle after the incision was made.[16] The volume of the bacteria suspension used was 1 mL, 0.5 mL, or 0.25 mL at a concentration with 1.8×10^9 CFU/mL. The length of the incision was 5 mm and the depth was ~3 mm. We did not cut the deep fascia, and the bacterial suspension did not overflow the incision.

2.6. SPA pretreatment

Once again, the mice were randomly distributed into 20 groups and each group was 12 male. Mice of the control group received incision infection without SPA pretreatment. Mice of the other group received SPA intraperitoneally injected before the incision infection was made. The dose of SPA used 0.5 mg/kg/time, 1 mg/kg/time, 1.5 mg/kg/time, or 2 mg/kg/time. SPA was injected at 48 hours and 24 hours before making the incision infection model. A digital thermometer (Shenzhen Life Technologies Corporation, Shenzhen, China) was used to measure rectal temperature and was adjusted to rectal probe to minimize the stress response 2−5 days before the experiment. The mice were gently handled and removed from their cages 10 times daily for 20 minutes every time. The probe was inserted 2 cm into the rectum. Each measurement value recorded was a mean of six, and the temperature was measured at 09:00 AM.

2.7. Assess the biological safety of the best pretreatment dose

Rats were randomly distributed into two groups, with each group consisting of 20.5 male. Rats of the control group received sterile saline intraperitoneally injected, and the other group received 1 mg/kg SPA intraperitoneally injected.

2.8. Statistical analysis

All data are expressed as means ± standard deviation (SD) and results were subjected to statistical analysis using analysis of variance (ANOVA) for repeated measurements or one-way ANOVA. Values of $p < 0.05$ were considered significant.

3. Results

3.1. The incision infection model

A 0.5-mL MSSA, MRSA, *P. aeruginosa*, or *E. coli* suspension could make the incision red and swell. A 0.25-mL suspension could not make the entire incision red. A 1-mL suspension was difficult to control and not overflow the incision (Table 1). These results reproduced our previous findings and suggest that 0.5 mL MSSA, MRSA, *P. aeruginosa*, or *E. coli* bacteria suspension could produce a stable incision infection model.

3.2. SPA pretreatment on incision infection model

SPA pretreatment can effectively reduce the increased amplitude of temperature, white blood cells, blood granulocyte, blood lymphocytes, serum IL-1β, serum IL-6, serum IL-10, and serum TNF-α in mice infected by MSSA, MRSA, *P. aeruginosa*, or *E. coli* (Figs. 1−4 and Tables 2 and 3). A 1-mg/kg/time SPA pretreatment could be more effective in reducing the increased amplitude of these observation indicators than 0.5 mg/kg/time, 1.5 mg/kg/time, and 2 mg/kg/time in mice infected by MSSA, MRSA, *P. aeruginosa*, or *E. coli*.

3.3. Biological safety of the best pretreatment dose

Rat biochemistry included total cholesterol, calcium, amylase, alanine aminotransferase, alkaline phosphatase, and

Table 3
The white blood cells, granulocyte, lymphocytes, and cytokines in different bacteria infected groups with SPA 1 mg/kg pretreated.

	Day 3								
	MSSA + SPA	PA + SPA	EC + SPA	MSSA + saline	PA + saline	EC + saline	MSSA	PA	EC
White blood cells	10.2191 ± 0.21797	11.8004 ± 0.22035	11.2981 ± 0.24156	12.6745 ± 0.24660	13.6032 ± 0.24468	13.0864 ± 0.24475	12.5376 ± 0.21537	13.6157 ± 0.26069	13.1286 ± 0.24875
Granulocyte	1.9922 ± 0.14485	2.2106 ± 0.18973	2.2228 ± 0.17093	2.8122 ± 0.15718	2.9449 ± 0.16502	2.9959 ± 0.20450	2.8421 ± 0.15303	2.9942 ± 0.14555	2.9524 ± 0.17386
Lymphocytes	7.1912 ± 0.26590	8.2479 ± 0.25238	7.8696 ± 0.23463	9.0899 ± 0.19316	10.0414 ± 0.18653	9.6121 ± 0.24427	9.0652 ± 0.27499	10.3832 ± 0.17816	9.6278 ± 0.22727
	Day 7								
	MSSA + SPA	PA + SPA	EC + SPA	MSSA + saline	PA + saline	EC + saline	MSSA	PA	EC
White blood cells	6.6067 ± 0.16970	7.6345 ± 0.25668	7.1835 ± 0.24213	8.8001 ± 0.15808	10.3037 ± 0.25359	9.7867 ± 0.24275	8.8459 ± 0.15846	10.2865 ± 0.25093	9.7165 ± 0.18148
Granulocyte	1.2082 ± 0.15758	1.4838 ± 0.17336	1.2884 ± 0.14809	1.3227 ± 0.16304	1.9957 ± 0.15813	1.6822 ± 0.17669	1.2816 ± 0.17807	1.9101 ± 0.15236	1.7225 ± 0.20574
Lymphocytes	4.9761 ± 0.19309	5.7694 ± 0.19355	5.3015 ± 0.25664	6.6257 ± 0.23478	7.7803 ± 0.21715	7.2210 ± 0.16063	6.7944 ± 0.24684	7.8927 ± 0.21611	7.2972 ± 0.23691
IL-1β	102.7607 ± 5.05716	196.4954 ± 7.20748	159.0481 ± 6.21894	206.0315 ± 4.43192	281.2292 ± 5.72820	252.7364 ± 6.12305	201.9237 ± 7.27873	278.8555 ± 7.23173	253.3289 ± 6.72381
IL-6	152.4182 ± 8.93504	308.0875 ± 6.54658	270.5582 ± 6.23574	349.8513 ± 6.09951	503.8268 ± 7.45727	449.7282 ± 7.33428	348.0479 ± 6.73888	499.4506 ± 5.46794	449.4989 ± 6.30244
IL-10	49.7535 ± 6.13059	100.2269 ± 5.93442	91.4796 ± 6.74735	120.1501 ± 7.10589	171.2849 ± 6.33269	149.7192 ± 6.79272	121.4941 ± 7.46950	170.5110 ± 6.25104	151.8955 ± 6.21908
TNF-α	52.4091 ± 3.05169	109.0857 ± 6.94824	88.0083 ± 5.81353	127.7865 ± 6.27617	198.8187 ± 6.68154	179.1827 ± 6.25702	128.9310 ± 5.97829	202.1593 ± 5.08195	181.6701 ± 7.00167

EC = *Escherichia coli* infected only; EC + SPA = *E. coli* infected and SPA pretreated; *E. coli* + saline = *E. coli* infected and sterile saline pretreated; MSSA = Methicillin-sensitive *Staphylococcus aureus*; MSSA = MSSA infected only; MSSA + saline = MSSA infected and sterile saline pretreated; MSSA + SPA = MSSA infected and SPA pretreated; PA = *Pseudomonas aeruginosa* infected only; *P. aeruginosa* + saline = *P. aeruginosa* infected and sterile saline pretreated; PA + SPA = *P. aeruginosa* infected and SPA pretreated; SPA = staphylococcal protein A.

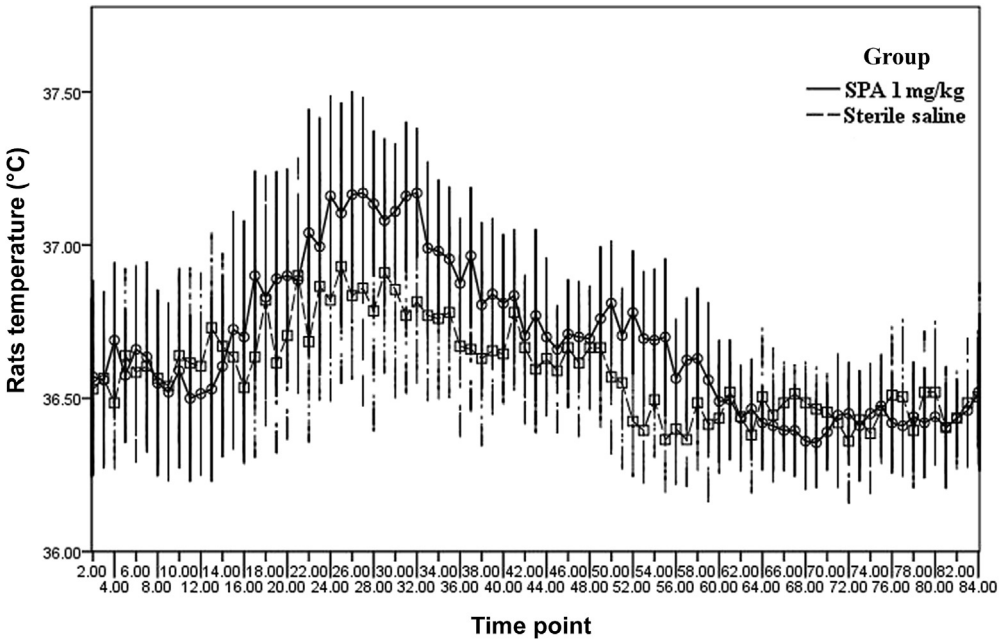

Fig. 5. The temperature variation of rats. SPA = staphylococcal protein A.

albumin, aspartate transaminase, creatine kinase, glutamyl transpeptidase, creatinine, glucose, phosphate and total bilirubin, total protein, and urea. A 1-mg/kg SPA pretreatment did not cause these indicators to be abnormal.

A 1-mg/kg SPA pretreatment could increase the rat temperature, white blood cells, blood granulocyte, blood lymphocytes, serum IL-1β, serum IL-6, serum IL-10, and serum TNF-α, and these indicators returned to normal in 24 hours (Figs. 5 and 6).

Liver tissues of rats pretreated by 1 mg/kg SPA were observed under the microscope and there was no damaged organizational structure.

In all the experiments, the position at which SPA was injected did not appear to have signs of infection at any time.

4. Discussion

SPA is a surface protein on most *S. aureus* strains, and can bind to various host-derived proteins, including the Fc and VH3 domains of immunoglobulins, von Willebrand factor, complement C3, epidermal growth factor receptor, and TNF-α receptor 1. Thus, SPA can potentially modulate the host immune system. Depending on its binding partner and responding cell type in a host, SPA can act as either a proinflammatory or antiinflammatory molecule.

Surgical site infection (SSI) is a common complication after surgery. It immediately influences the curative effect of the operation. Bacteria will form a biofilm on the internal fixation plate, which is utilized during most of the orthopedic surgeries. The biofilm can resist the body immune system and antibiotics.[17,18] Ways to improve cleaning of the bacteria and reduce the biofilm formed continues to be investigated. In recent years, one study reported that SPA can enhance the ability of the body's immune system to resist sepsis and reduce mortality due to infection shock.[6,7] However, the further molecular mechanism involved remains unresolved and continues to generate discussion. In our study we assessed the SPA pretreatment on the incision infection mouse model and selected the best pretreatment dose. On the one hand, we explored the possibility that SPA can alleviate the inflammation of incision infection mouse to possibly reduce SSI; on the other hand, we undertook a further study to find the exact mechanism of SPA on the body immune system to provide a hypothesis.

The medial soft tissue of the thigh was thicker compared with the other site that was considered, and the local anatomical structure was clear. The incision which we chose can make the incision infection model easily established and standard. Bacteria suspension can be flowed via the space between the muscles to diffuse if the fascia was cut. However, we did not cut the fascia to make the bacteria only infect the incision surface, and not diffuse the other site. Under clinical conditions, the source of bacteria of SSI is customarily from the air. Essentially, the bacteria drops onto the incision surface which initiates the SSI process. Therefore, our goal was to simulate that manner of infection.

In our experiment, a low dose of SPA can alleviate inflammation by detecting the body's temperature, white blood cells, granulocyte, lymphocyte, serum cytokines, and wound tissue biopsy. Once bacteria invade, the immune response was rapidly activated, and lymphocytes rapidly proliferate and differentiate. Soon thereafter, the bacteria will be cleared quickly.

We found that 1 mg/kg/time SPA pretreatment has the best protection effect compared with the other doses. This indicates that the spectrum of treatment of SPA is narrow, in part because SPA is toxic to the body. Overall, the SPA treatment mechanism

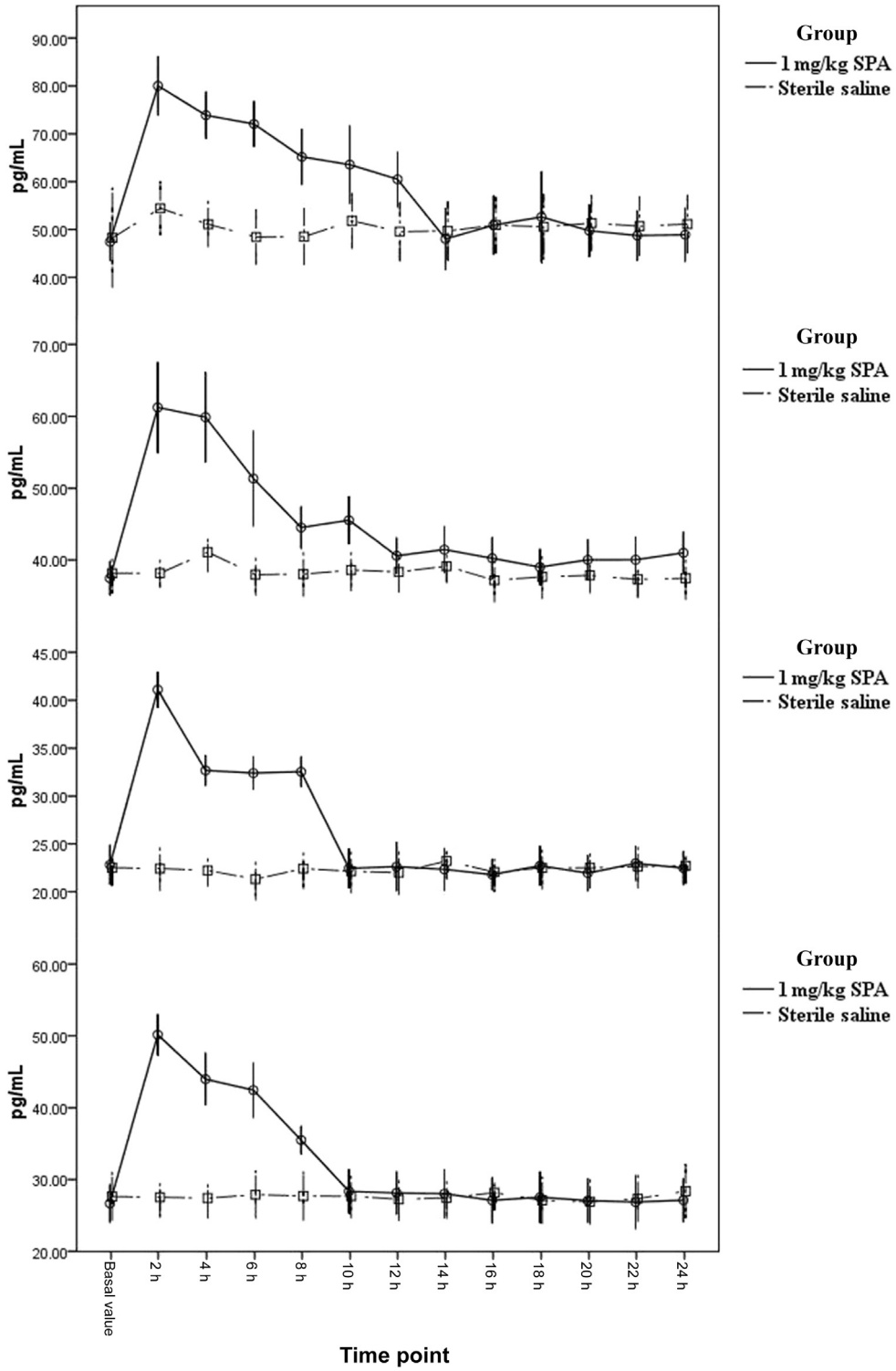

Fig. 6. The variation of lL-1β, lL-6, lL-10, and TNF-α. SPA = staphylococcal protein A.

is complicated and comprehensive. However, we think that SPA can activate some receptors. The activation effects have a dose dependent relationship. The dose below the threshold value may fail to activate the immune system and be clear. Alternatively, the dose that exceeds the threshold value could cause harm. However, this hypothesis needs to be proven by further research.

In conclusion, the findings of this study suggest that SPA pretreatment can effectively reduce the severity of the infected incision of MRSA, MSSA, *P. aeruginosa*, or *E. coli* infection. The best dose of SPA pretreatment is 1 mg/kg, which is a dosage that, up to a point, does not damage the function of the organs in Wistar rats.

Acknowledgments

This study received the support of the Beijing Natural Science Foundation (7152061).

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