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Nano-silver modified porcine small intestinal submucosa for the treatment of infected partial-thickness burn wounds

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ABSTRACT

Background and aim: Silver has been widely used as a topical antimicrobial agent in burn wound care. In a previous study, we demonstrated the introduction of nano-silver particles to porcine small intestinal submucosa (NS-PSIS) led to significant enhancement in antibacterial property in repairing contaminated abdominal defect. In this study, we explored the efficacy of NS-PSIS in the treatment of *Pseudomonas aeruginosa*-infected partial-thickness burn wounds.

Methods: 48 male Sprague-Dawley rats were divided into four groups of equal number. Standardized and reproducible *Pseudomonas aeruginosa*-infected partial-thickness thermal burns wound model were created using these rats. NS-PSIS, PSIS (porcine small intestinal submucosa) or lipido-colloid dressingss (Urgotul™) were tested for 14 days to assess their ability to heal the rats' burn wounds. Control group was without any treatment after the establishment of infected burn-wound. The wound contraction rate, animal body weight change, histological examination, and the quantification of IL-6 and C-reactive protein (CRP) were measured to evaluate the healing effects.

Results: NS-PSIS significantly promoted wound healing and recovered the normal growth of rats. There were significantly lower expression levels of pro-inflammatory cytokine (IL-6) and CRP in NS-PSIS group as compared with the PSIS or Urgotul group in the treatment of infected partial-thickness burn wounds. Histological exams revealed significant less inflammatory cells infiltrating, more re-epithelization and neovascularization in NS-PSIS group. There were also less inflammatory cells infiltrations in the major organs in NS-PSIS group.

Conclusions: Nano-silver modified porcine small intestinal submucosa (NS-PSIS) can be used as a biological derivative dressing for the treatment of infected partial-thickness burn wounds.

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1. Introduction

Scald (65%), contact thermal burns (15%) and flame burns (20%) are frequent causes of trauma, associated with both morbidity and mortality [1,2]. Although skin is the principally affected organ, complications may develop in many other organs and systems. The wound infection is a critical threat in this condition. Invasive infection destroys the microenvironment of the wound and may lead to severe systemic complications such as multiple organ dysfunction syndrome (MODS) [3]. Severe infection and MODS are the two commonest causes of death among patients with burns [4]. Partial-thickness dermal (second-degree) burns are a common, painful injury requiring a great deal of resources in their care. *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the main etiological agents in burn infections [5]. Once the burns wound has been colonized with *P. aeruginosa*, the bacterium rapidly proliferates within the damaged tissue. It often leads to a disseminated infection, which could be life threatening. Antimicrobial medicated dressings (AMD) are often used to reduce bacterial infection of burns [6]. In a previous study, we demonstrated the introduction of nano-silver particles to porcine small intestinal submucosa (NS-PSIS) led to significant enhancement in antibacterial property in repairing contaminated abdominal defect [7]. In the present study, we established a standardized and reproducible *P. aeruginosa* infected partial-thickness thermal burns wound model. Base on this model, we explored the efficacy of NS-PSIS as a biological derivative dressing for the treatment of infected partial-thickness burn wounds.

2. Materials and methods

2.1. Ethics statements

The Animal Experiments Committee at the Second Military Medical University approved all experiments in this study involving animals. All experimental protocols conformed to international regulations and declarations relevant to animal experimentation. All surgical procedures were performed under sodium pentobarbital anesthesia and in a clean surgical room with sterilized instruments. All efforts were made to minimize the suffering of the rats during the experiments.

2.2. Preparation of *P. aeruginosa*

P. aeruginosa (ATCC27853) was kindly provided by the clinical laboratory department of Changzheng hospital. Bacterium were cultured in lysogeny broth (LB) medium overnight, resuspended in 10% glycerin medium, and stored at −80°C. Before animals wound vaccinated, the bacterium were diluted with saline. A spectrophotometer was used to adjust the concentration. The bacterium suspension was subjected to serial dilutions and plated on LB agar plates for the determination of colony-forming unit (CFU).

2.3. Preparation of PSIS and construction NS-PSIS

We prepared PSIS from fresh porcine jejunum. The submucosa layer was isolated by manually scraping off the mucosal and muscular layers. After mechanical cleaning, the intestine was cut into 10cm sections that were processed through a series of chemical cleaning steps, by incubation in ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, hydrochloric acid and sodium chloride. The resulting PSIS was vacuum freeze-dried and sterilized by gamma irradiation. We used self-assembly to fabricate NS-PSIS with nano-silver (15nm in diameter; Uni-impex Korea, Inc. Republic of Korea). In brief, we immersed PSIS into the nano-silver solution (50μg/mL) in a bioreactor with 50rpm/min in shake flask at 37°C for 24h. The resulting NS-PSIS was used for the following experiments [7]. We have already demonstrated the usage of this particular concentration (50μg/mL) in the previous study by assessment of the minimum inhibitory concentration (MIC) of Nanosilver against *P. aeruginosa* (Supplement Fig. 1).

2.4. Cytocompatibility of NS-PSIS: cell viability assay

Cellular viability was determined by the MTS assay, which observes the reduction of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) to formazan in viable cells. Briefly, human dermal fibroblasts (HDFs) were plated onto 96 multiwell plates. After incubation with NS-PSIS for various lengths of time at 37C, formazan absorbance was measured at 490nm. The mean absorbance of the non-exposed cells was the reference value for calculating 100% cellular viability. Cytotoxicity was quantified using a Live/Dead viability/cytotoxicity kit (Invitrogen, CA, USA), according to the manufacturer's protocol. Briefly, cells were treated with NS-PSIS for 24h and the combined Live/Dead assay reagents were added. The cells were incubated with the assay reagents for 30–45min at room temperature. The labeled cells were analyzed by a fluorescence microscope (Olympus, Japan) and flow cytometry (BD biosciences, San Diego, USA) [8].

2.5. In vitro cell migration: scratch assays

HDFs were plated in 24-well plates. The cells were grown to confluence and 3days after confluence were mechanically disrupted with a sterile 200 lL pipette tip with the use of a grid of 3×3mm squares scratched with a pipette tip. The pre-incubation with mitomycin C (10lg/mL) for 2h blocks further proliferation so that only migration is followed. NS-PSIS conditioned media were added (2mL) for 18h. Unconditioned media were added as the control. After capturing images, migrating cells were counted in a field of the three scratch areas [9].

2.6. Animal model of thermal injury and infection

Rats were anesthetized by intraperitoneal injection of 40mg/kg pentothal sodium. The back of the neck hair was cropped. Deep second-degree burns were induced using a Copper hot plate (3×2cm) heated for 5min (100±10°C) and placed for 15s, using an even pressure, on the shaved back of the neck. In order to establish a robust deep second-degree burn model, we had tested a series of contacting time (5s, 10s, 15s, and 20s). The success of creating a reproducible deep second-degree burn model by contacting for 15s was demonstrated by the

pathological results (Supplement Fig. 2). Rat surface areas were 100–120 cm^2, calculated using the San Roman formula. As less than 10% of each surface area was burned, no parenteral fluid was given. When the burned regions were cooled down for about 5 min to normal body temperature, the burned regions were injected with 100 ul (1.3×10^9 CFU) of *P. aeruginose* bacterial suspension using a 1 ml injector. The animals were placed in separate sterilized cages and allowed to recover [10].

2.7. *Animal subjects and study setting*

We used 48 adult male Sprague-Dawley rats weighing 140–200 g, which were randomly divided into four groups, each of 12 rats. The baseline body weights were balanced among groups. Standardized *P. aeruginosa* infected partial-thickness thermal burns wounds were established as described. In the 3 experimental groups (n=12, each), the wounds were covered by the same size of NS-PSIS, PSIS, or lipido-colloid dressingss (Urgotul™), and fixed by sterile sutures, respectively. In the control group (n=12), no treatment was given. After operation, the rats were housed individually, and the environment was kept relatively dry and sterile. All dressings were changed once every other day, and were moistened by sterile saline every day. The wound area, animal body weight changes were measured for 14 days to evaluate the healing effects. Because rats possessed a subcutaneous panniculus carnosus muscle that facilitates skin healing by both wound contraction and collagen formation, wound contraction was the primary healing method of rats as opposed to re-epithelialization in humans. [11,12]. Thus, we used "wound contraction rates" instead of "wound area" for the assessment of wound healing. Wound contraction rate (%)= (Wound area of day 0 − wound area of day x)/wound area of day 0 × 100. The expression levels of serum IL-6 and C-reactive protein (CRP) were detected by ELISA method. Briefly, after collecting blood samples during euthanasia, serum samples were loaded to the microplates and assayed in accordance with the manufacturer's instructions (BD biosciences, San Diego, USA). HE and Masson's staining were used to evaluate wound healing and inflammation changes of burn wounds and major organs. All tissue sections were observed under a light microscope and recorded.

2.8. *Statistical analysis*

Statistical analysis was performed with the aid of SPSS version 18.0. For numerical variables, descriptive statistics are expressed as arithmetic means ± standard deviations (SD). Differences among groups were analyzed via one-way ANOVA with a Tukey post-hoc test. The 95% confidence intervals were calculated and $P < 0.05$ was considered to reflect the presence of statistical significance.

3. Results

In the assessment of the cytocompatibility of NS-PSIS, we found at the concentration of 50 μg/mL, NS-PSIS had negligible cytotoxicity, which demonstrated the biocompatibility of the NS-PSIS dressing (Supplement Fig. 3).

No animal was excluded in the experiment. According to statistical analyses of wound contraction rate (Fig. 1) and body weight (Fig. 2) on 7th day and 14th day, we found that NS-PSIS significantly promoted wound healing and recovered the normal growth of rats. The results of ELISA revealed there were significantly lower expression levels of pro-inflammatory cytokine (IL-6) (Fig. 3) and CRP (Fig. 4) in NS-PSIS group as compared with the PSIS or Urgotul group in the treatment of infected partial-thickness burn wounds.

In histology on 14th day, HE and Masson staining revealed only few re-epithelization, dense inflammatory cell infiltrations, and disordered fibroblasts in the PSIS group. In the Urgotul group, no re-epithelization was observed. In contrast, extensive inflammatory cell infiltrations in subcutaneous granulation tissues were seen. In the NS-PSIS group, histological exams revealed significant less inflammatory cells infiltrating, more re-epithelization and neovascularization. In addition, increased dermal collagen fibers, arranged neat and

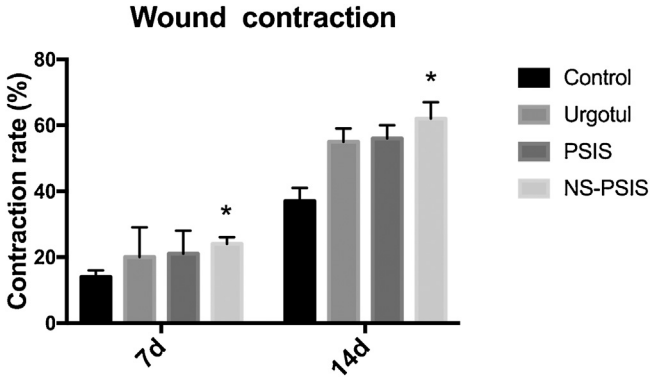

Fig. 1 – Wound contraction rate of the rats on 7th day and 14th day after the creation. *On 7th day, the wound contraction rate was significantly higher in NS-PSIS group ($p < 0.05$), as compared with the control group. NS-PSIS vs. PSIS vs. Urgotul, not statistically significant. **On 14th day, the wound contraction rate was significantly higher in NS-PSIS group ($p < 0.05$), as compared with the control, PSIS, and Urgotul groups.

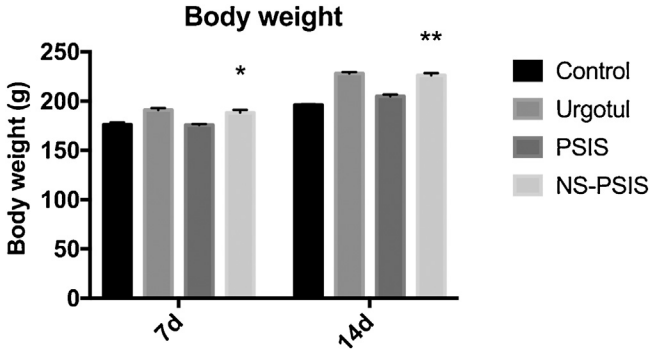

Fig. 2 – Body weight of the rats on 7th day and 14th day after the creation. *On 7th day, the body weight was significantly heavier in NS-PSIS group ($p < 0.05$), as compared with the control, and PSIS groups. NS-PSIS vs. Urgotul, not statistically significant. **On 14th day, the body weight was significantly heavier in NS-PSIS group ($p < 0.05$), as compared with the control, and PSIS groups. NS-PSIS vs. Urgotul, not statistically significant.

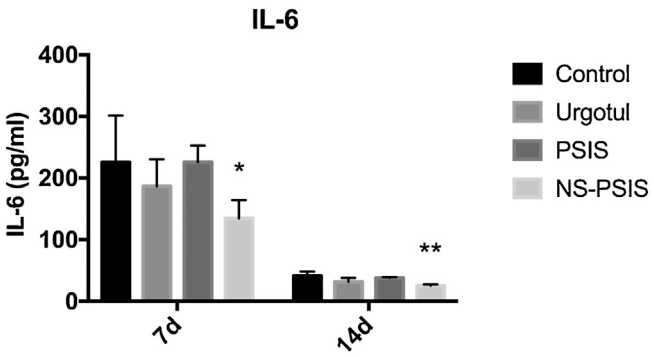

Fig. 3 – The expression level of IL-6 in the serum of the rats on 7th day and 14th day after the creation. *On 7th-day, the expression level of IL-6 was significantly lower in NS-PSIS group ($p < 0.05$), as compared with the control, PSIS, and Urgotul groups. **On 14th day, the expression level of IL-6 was significantly lower in NS-PSIS group ($p < 0.05$), as compared with the control, PSIS, and Urgotul groups.

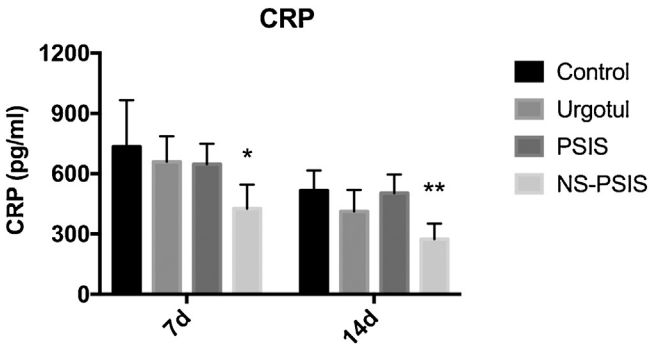

Fig. 4 – The expression level of CRP in the serum of the rats on 7th day and 14th day after the creation. *On 7th day, the expression level of CRP was significantly lower in NS-PSIS group ($p < 0.05$), as compared with the control, PSIS, and Urgotul groups. **On 14th day, the expression level of CRP was significantly lower in NS-PSIS group ($p < 0.05$), as compared with the control, PSIS, and Urgotul groups.

compact, were also seen in the NS-PSIS group (Fig. 5). The results were further supported by the in vitro cell migration assay, which showed, when treated with NS-PSIS, the migration of fibroblasts increased by 35% (Supplement Fig. 4).

For major organs (lung, liver, kidney), there were also less inflammatory cells infiltrations in NS-PSIS group, as compared with PSIS or Urgotul™ groups (Fig. 6).

4. Discussion

In this study, we explored the efficacy of NS-PSIS in the treatment of *P. aeruginosa*-infected partial-thickness burn wounds. As hypothesized, our findings show NS-PSIS significantly promoted wound healing and can be used as a biological derivative dressing for infected partial-thickness burn wounds.

Because of the biologic properties of PSIS, its abilities to support tissue regeneration, repopulation with fibroblasts, revascularization, new collagen deposition, and eventual absorption and replacement with native tissue, it has been widely used in the treatment of deep burn wounds [13]. However, PSIS dose not have an effective antimicrobial property, which precludes its usage in the infected burn wounds. Therefore, we incorporated nano-silver into PSIS, which enables it to be effective against bacteria, yeast, fungi and viruses [14]. Silver exerts its biocidal effects by interfering with microbial respiratory cytochromes, electron transport,

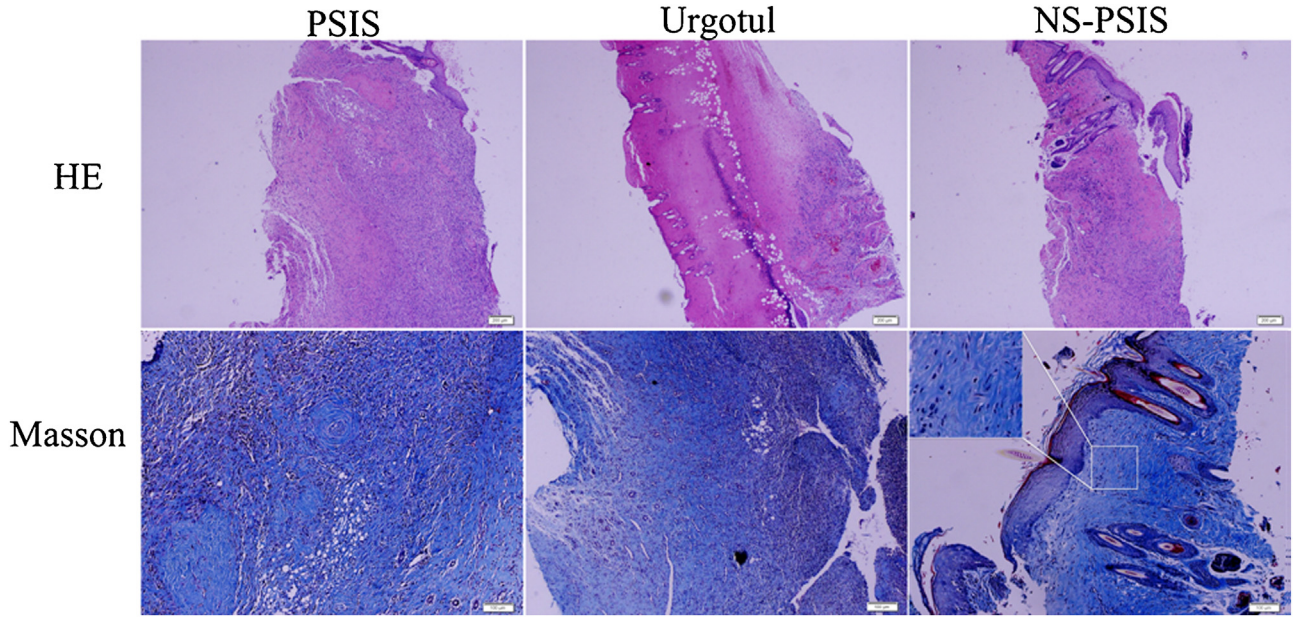

Fig. 5 – HE and Masson staining of the burn wounds on 14th day after the creation. HE staining, scale bar: 200 μm; Masson staining, scale bar: 100 μm.

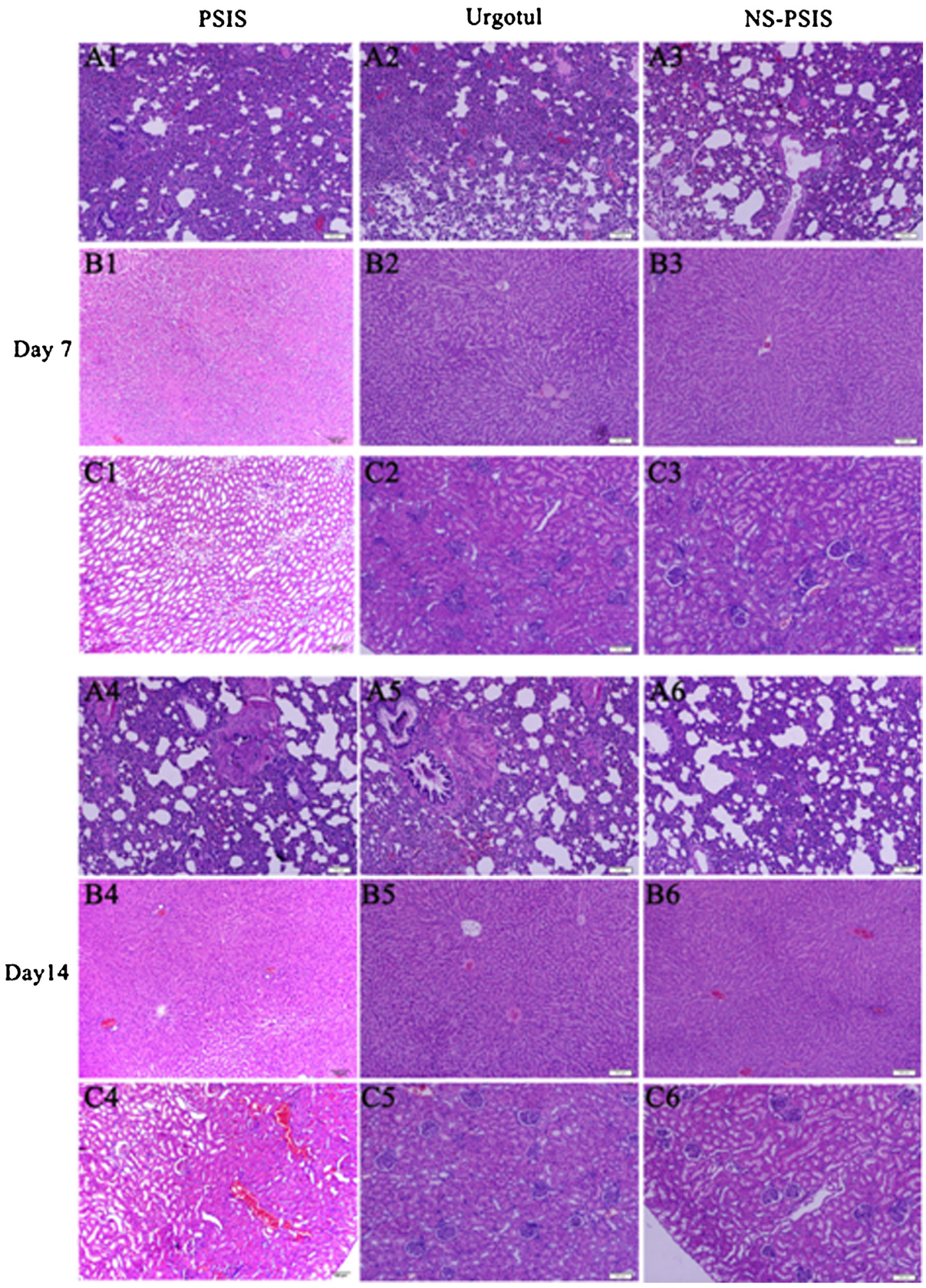

Fig. 6 – HE staining of the major organs (lung, liver and kidney) on 7th day and 14th day after the creation. Scale bar: 100 μm.

and DNA replication [15]. It also appears to have anti-inflammatory properties [16] and can increase re-epithelialization rates by over 40% [17]. Sustained and steady silver release is crucial for a wound dressing with a prolonged antibacterial role. Our previous study showed the antibacterial effect of NS-PSIS could last for 14 days [7]. The use of nano-silver also raised the concern of the cytotoxicity of such dressings. Recent studies have found that the cytotoxicity of nano-silver is dose dependent and low doses could be nontoxic [18,19]. In the present study, our cytotoxicity assay showed, at the concentration of 50 μg/mL, NS-PSIS had negligible cytotoxicity, which demonstrated the biocompatibility of the NS-PSIS dressing.

There are several possible reasons to explain the accelerated wound healing by NS-PSIS. The epithelialization process depends on self-renewal, proliferation, and migration of keratinocytes residing at the basal cell layer [20]. In this study, NS-PSIS showed to induce a higher rate of re-epithelialization that allowed for accelerating wound healing. Furthermore, the formation of a new epithelial layer over the wound treated with NS-PSIS was clearly seen. Collagen is a main component of skin that plays an important role in tissue healing by providing tissue strength and an extracellular matrix framework for cell adhesion and migration [21]. Our results showed that NS-PSIS induced the production of collagen fibers, which were arranged neat and compact, with a similar pattern to normal skin. In addition, in vitro cell migration assay showed increased migration of fibroblasts when treated with NS-PSIS, which supported NS-PSIS helping in wound healing process. At molecular level, this study showed that NS-PSIS modulated the pro-inflammatory cytokines and protein involved in wound healing. Deep burn wounds were associated with severe inflammation, which could lead to worsening of the tissue damage caused by the initial thermal injury [22]. Furthermore, excessive expression of pro-inflammatory cytokines and protein within the burn wounds impairs the wound healing process [23].

Taken together, the results of this study indicated that the NS-PSIS was found to be effective for the healing of burn wounds in rats, compared with PSIS and the medical gauze control (Urgotul™). The accelerated wound healing can be attributed to the effects of NS-PSIS in suppressing inflammation and stimulating re-epithelialization during the wound healing process. It can be a promising biological derivative dressing for the treatment of infected partial-thickness burn wounds.

Conflict of interest statement

The authors have no conflict of interest to disclose for this study.

Author contribution

Haiyang Zhou and Zhiqian Hu designed study. Yanyan Zhang, Jian Xu, and Yunsheng Chai performed experiments and collected data. Jian Zhang analyzed data. Yanyan Zhang and Jian Xu wrote the draft. Haiyang Zhou and Zhiqian Hu revised it critically. All the authors approved the version to be published.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.burns.2018.12.002.

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