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Original Article

Maggots promote the healing of infected cutaneous wounds by activating the TGF-β/SMAD signaling pathway

Lei Huang[1], Jin Chu[2], Song Liu[2], Gang Li[2], Yong Jiang[1], Decheng Lv[1]

[1]Orthopedics Department, First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Xigang District, Dalian 116011, Liaoning, China; [2]Dalian Medical University, 9 West Lüshun South Road, Lüshunkou District, Dalian 116027, Liaoning, China

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Abstract: Maggots can be used as a representative biological therapy. They possess many varied pharmacological properties that make them useful for the wound healing process. However, the mechanism underlying how maggots contribute to the healing of infected cutaneous wounds remains unknown. In this study, infected cutaneous wound models were employed to evaluate how maggots contribute to wound healing. We conducted wound area measurements and wound infection measurements, the granulation tissue was graded, and we used HE staining to assess the effects maggots have on rat wounds. Then, the effect of maggots on the TGF-β/SMAD signaling pathway was determined. Moreover, the effects of maggots on neovascularization and neuro-regeneration were also observed. The maggots improved the condition of the wound tissue and decreased its bacterial content, and they regulated the TGF-β/SMAD signaling pathway to affect the wound healing process. The promotion of neovascularization and neuro-regeneration in new granulation tissue was also a treatment effect of the maggots. In summary, this study found that maggots significantly promote the healing of infected cutaneous wounds by activating the TGF-β/SMAD signaling pathway.

Keywords: Maggots, TGF-β/SMAD, infected cutaneous wound, wound healing

Introduction

Acute skin and soft-tissue defects caused by severe trauma are common in clinical practice [1]. Since some wounds come in contact with unclean objects in the injury process, and other wounds are directly exposed to the air after the injury, bacterial infections are highly probable [2, 3]. Notably, severe bacterial contamination always delays wound healing, aggravates the extent of wound tissue necrosis, and even causes serious systemic infections that can be life-threatening. Hence, common treatments such as debridement and the use of antibiotics are adopted to decrease the risk of wound infections [4-6]. Unfortunately, antibiotic resistance and poor healing happen sometimes and make the treatment of wound infection more challenging [7]. Therefore, the existing treatments are no longer meeting the need for wound healing.

Maggot therapy, a representative biological therapy, can effectively avoid the adverse reactions of resistance that are seen in conventional therapies [8]. The history of maggot therapy has a long history and its application can be traced back to the 16th century [9]. Maggot therapy is widely used in diabetic foot ulcers [10], acute and chronic wound infections [11], pressure ulcers [12], and other refractory wounds [13, 14].

We know that wound healing is a complex process in which a variety of transcription factors and related molecules participate [15, 16]. These factors are not independent, but interrelated. Transforming growth factor-β (TGF-β) is known to stimulate collagen production in dermal fibroblasts by fibroblast-to-myofibroblast transition [17, 18]. Whether the mechanism underlying maggot therapy is related to the TGF-β signal pathway is still not clear. This study

aims to explore the mechanism responsible for maggot therapy in wound healing.

Material and methods

Drugs, reagents and main instruments

Living maggot bag: 30 maggots were sealed in a 2 cm × 2 cm nylon bag, purchased from the Biowim (China) Technology Development Co., Ltd. Mupirocin ointment was obtained from the Sino-US Tianjin Shike Pharmaceutical Co., Ltd. Medical Vaseline gauze was obtained from the Xinxiang Huaxi Weishi Material Co., Ltd. Analytical grade chloral hydrate was purchased from the Tianjin Komi Chemical Reagent Co., Ltd.; the batch number was 20120110. *Staphylococcus aureus*: The standard strain was provided by the Department of Microbiology, Dalian Medical University, and the bacterial solution was diluted to 10^9/mL spare. Columbia sheep blood agar plates were obtained from the Shanghai Solarbio Biotechnology Co., Ltd. The main instruments, such as the syringes, tweezers, scissors, dissection plates, and the homogenizer and paraffin slicers were provided by the Central Laboratory of the First Affiliated Hospital of Dalian Medical University.

Animal studies

The animals were provided by the Center of Dalian Medical University (SCXK 2013-0003). All the animal experiments were conducted in accordance with the established guidelines regulating the care and use of experimental animals in universities. Six-week-old male Sprague-Dawley rats (200-220 g) were purchased from the Laboratory Animal Center of Dalian Medical University and selected to be used in this study. The experiment was conducted after 1 week of adaptive feeding at room temperature; the rats were housed in separate cages and had free access to drinking water and were exposed to natural light. All the animal experiments complied with the principle of replacement, refinement, or reduction (the 3Rs). The 60 rats were randomly divided into three groups (n=20 each): a negative control group (the Vaseline gauze covering group), a positive control group (the Mupirocin treatment group), and an experimental group (the maggot treatment group). The materials and related indicators were tested on the 4th, 8th, and 12th days after the wound treatments. Referring to the method of Fu et al., the infected wound model was established on the back of each rat by integrating the wound [19]. The rats were anesthetized using an intraperitoneal injection with 10% chloral hydrate at a dose of 0.3 g/kg. In each group, the procedure was performed on the right side of the spine, in an area of about 2 cm × 2 cm. During the operation, the area was routinely disinfected. The whole skin layer, the subcutaneous superficial fascia, and the muscle layer were incised layer by layer. After the hemostasis treatment, we added 2 mL of bacterial solution to each wound. We made sure the bacterial solution was evenly spread all over the wound. When the bacterial solution was completely infiltrated into the wound, we covered the medical Vaseline gauze, and we added a sterile dressing to fix it properly in place. The dressing and Vaseline gauze were removed after 48 h, and the wound surface was covered with a purulent secretion. The granulation tissue was grayish-white, and the local skin edges were red and swollen. All 60 rats were successfully modeled. And no rat exhibited any signs of peritonitis, pain, or discomfort following the administration of the anesthetic.

For the Vaseline gauze group, we used Vaseline gauze to cover the wound surface, and then we wrapped it with a sterile dressing. In the treatment group, the wounds were covered with nylon bags containing living maggots, and then we covered the wound with a sterile dressing. The mupirocin ointment was applied to the mupirocin ointment group; it was externally used and then the wound was covered with a sterile dressing. The three groups of rats had their dressings changed once every 2 days, and the above treatments were repeated each time we changed the dressing.

An intraperitoneal injection of chloral hydrate was also used for anesthesia prior to the wound tissue collection (10% chloral hydrate at a dose of 0.3 g/kg). After 12 days of treatment, all the rats were euthanized using cervical dislocation and with an intraperitoneal injection of chloral hydrate. Respiratory arrest and the absence of blinking confirmed the deaths. The wound tissue was collected and stored at -80°C for use in the subsequent experiments.

Wound area measurement

The transparent membrane method was used to record the areas of all the wounds on the

4th, 8th, and 12th days after the operations. We applied a 5 cm × 5 cm transparent biofilm center to each wound surface and traced it along the edge of the wound with a marker. After scanning the images, we imported them into Photoshop CS 6 (Adobe, USA). Then the selection function was used to calculate the groups' wound areas at the different time points. Granulation scoring of the wounds was performed [20]. At every bandage change, the granulation tissue was graded. (1 = below skin edge; 2 = level with the skin edge; 3 = above the skin edge but not overlapping; 4 = overlapping the skin edge). The assessment of the granulation tissue was performed by two surgeons (Lei Huang and Jin Chu) throughout the study.

Wound infection measurement

A small amount of living tissue from each group's wound model centers was taken before the treatments and on the 4th, 8th, and 12th days after the treatments. 99 times the weight of the sterile saline was added. We homogenized the saline contained within the tissue in a homogenizer, and then we diluted the homogenate (1:10). 100 μL of the solution was inoculated onto a 10 cm sheep blood agarose plate and was incubated for 24 h in a 37°C incubator. Subsequently, the number of bacterial colonies on the plate was observed and recorded.

Western blot analysis

The tissue was lysed with a RIPA buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins. Then, the proteins were transferred onto PVDF membranes (Millipore Co., USA). Next, the membranes were blocked with a blocking solution, which contained 5% skimmed milk and 0.07% (v/v) Tween 20. The incubation of the membranes was treated with primary antibodies against β-actin (Abcam; 1:1000), TGF-β1 (Abcam; 1:1000), SMAD4 (Abcam; 1:1000), SMAD2/3 (Abcam; 1:1000), Ras (Abcam; 1:500) and c-myc (Abcam; 1:1000) overnight at 4°C. The secondary antibodies were horseradish peroxidase-conjugated anti-mouse (Abcam) or anti-rabbit (Abcam) antibodies. LAS-3000 (Fujifilm), a luminescent imaging system, was used to quantify the protein expressions.

Hematoxylin and eosin (HE) staining

The tissue was embedded in paraffin and cut into 4-mm sections. The slides were deparaffinized in three xylene gradients and rehydrated using a gradient ethanol series. The sections were treated with hematoxylin for 5 minutes, and then we used eosin for 1 minute (HE staining kit, Shanghai Solarbio Biotechnology Co., Ltd., China). The slides were dehydrated through a series of fractionated alcohols and clarified in xylene. The HE stained slides were measured using optical microscopy in a random field.

Immunohistochemistry (IHC)

Following the dewaxing and hydration, the 4-mm paraffin-embedded sections were treated overnight at 4°C with primary antibodies against TGF-β1 (Abcam 1:500), SMAD4 (Abcam; 1:500), SMAD2/3 (Abcam; 1:500), vascular endothelial growth factor A (VEGFA) (Abcam; 1:1000), (Abcam; 1:500), and protein gene product 9.5 (PGP9.5) (Abcam; 1:500). Moreover, the sections were treated with secondary antibodies at room temperature for 30 min, and then a diaminobenzidine tetrahydrochloride (DAB) kit (Zhong Shan Jin Qiao, Beijing, China) was used to incubate the sections. Then, a digital camera (Motic BA400, Xiamen, China) was used to capture the image. More details are given in the previous study [21]. Finally, integrated optical density (IOD) was used to compare the differences in the protein expression among the different treatments.

Statistical methods

All the statistical analyses were conducted using the Prism program (Version 6.0.1, San Diego, USA), and the data are expressed as the mean ± SD. The comparisons between groups were performed using t-tests. The comparisons between groups were performed using completely randomized analyses of variance. All the statistical tests were two-sided. A difference was considered statistically significant when $P<0.05$.

Results

Maggots promote the healing of infected cutaneous wounds

To evaluate the effects of the maggots on the infected cutaneous wounds, we observed the general condition of the wounds in the rats on the 4th, 8th, and 12th days after the treatment. As shown in **Figure 1**, by observing the wounds on the 4th, 8th and 12th days, we found that the rats in the Vaseline group had more puru-

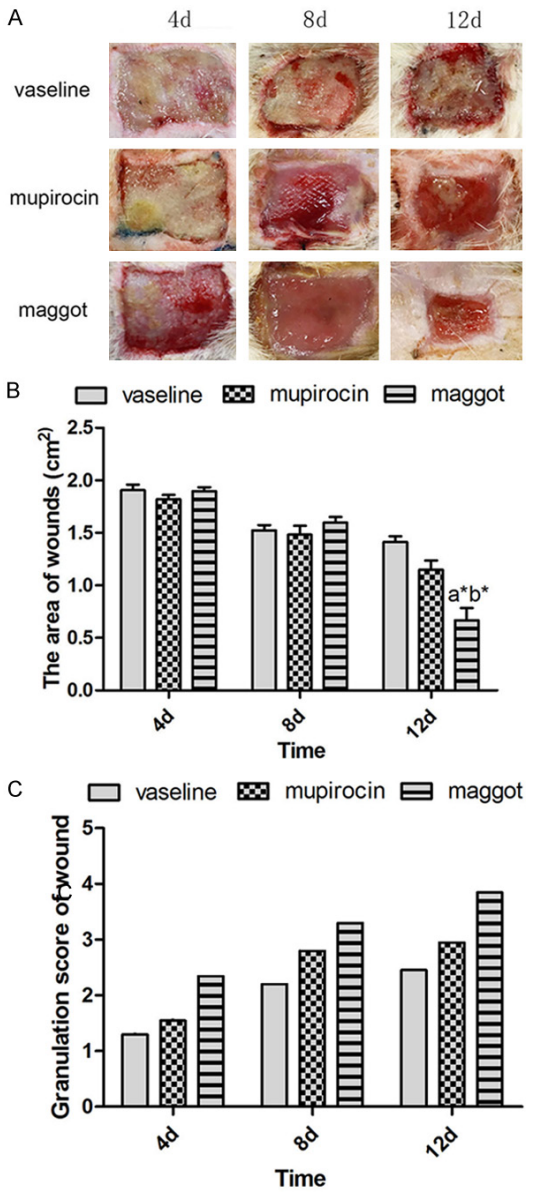

Figure 1. Changes in the infected wounds after the different treatments. A: General changes in the infected wounds in the different groups. B: The wound areas at different times in the Vaseline, maggot, and mupirocin groups. The mean ± SD of the areas are shown. (a: maggot group versus the Vaseline group; b: maggot group versus the mupirocin group; *: $P<0.05$). C: granulation wound scores in the different groups (the bar chart represents a comparison of the means).

lent material, more severe exudation, slower wound reduction, and poor healing. The mupirocin treatment group and the maggot treatment group exhibited a better effect than the Vaseline group. More importantly, on the 12th day, compared with the mupirocin treatment

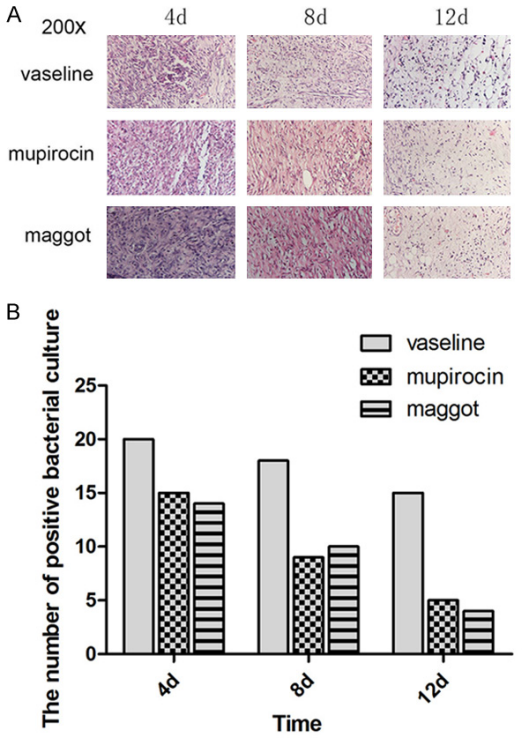

Figure 2. HE staining and cultures of the wound bacteria. A: The HE staining sections were prepared from the tissue samples for microscopic observation. B: The granulation was collected for culturing the bacteria on blood agarose plates (the bar chart represented a comparison of the means).

group, the rats in the maggot treatment group had significantly smaller wounds and showed good recovery. In addition, the average granulation score of the wounds in the maggot group was higher than it was in the other two groups. Fast-growing granulation tissue was found easily in the wounds treated with maggots. According to the scores, the granulation growth promoting abilities of Vaseline and mupirocin were poor.

Meanwhile, HE staining was performed to assess the wound repair and regeneration. On the 4th day, large amounts of inflamed cells in all three groups were observed. On the 8th and 12th days, in the maggot and mupirocin groups, we observed clear granulation tissue and fibrous connective tissue hyperplasia, as well as a reduced presence of inflammatory cells compared with the Vaseline group. Additionally, compared to the mupirocin group, the maggot group exhibited a better effect on wound healing (**Figure 2A**).

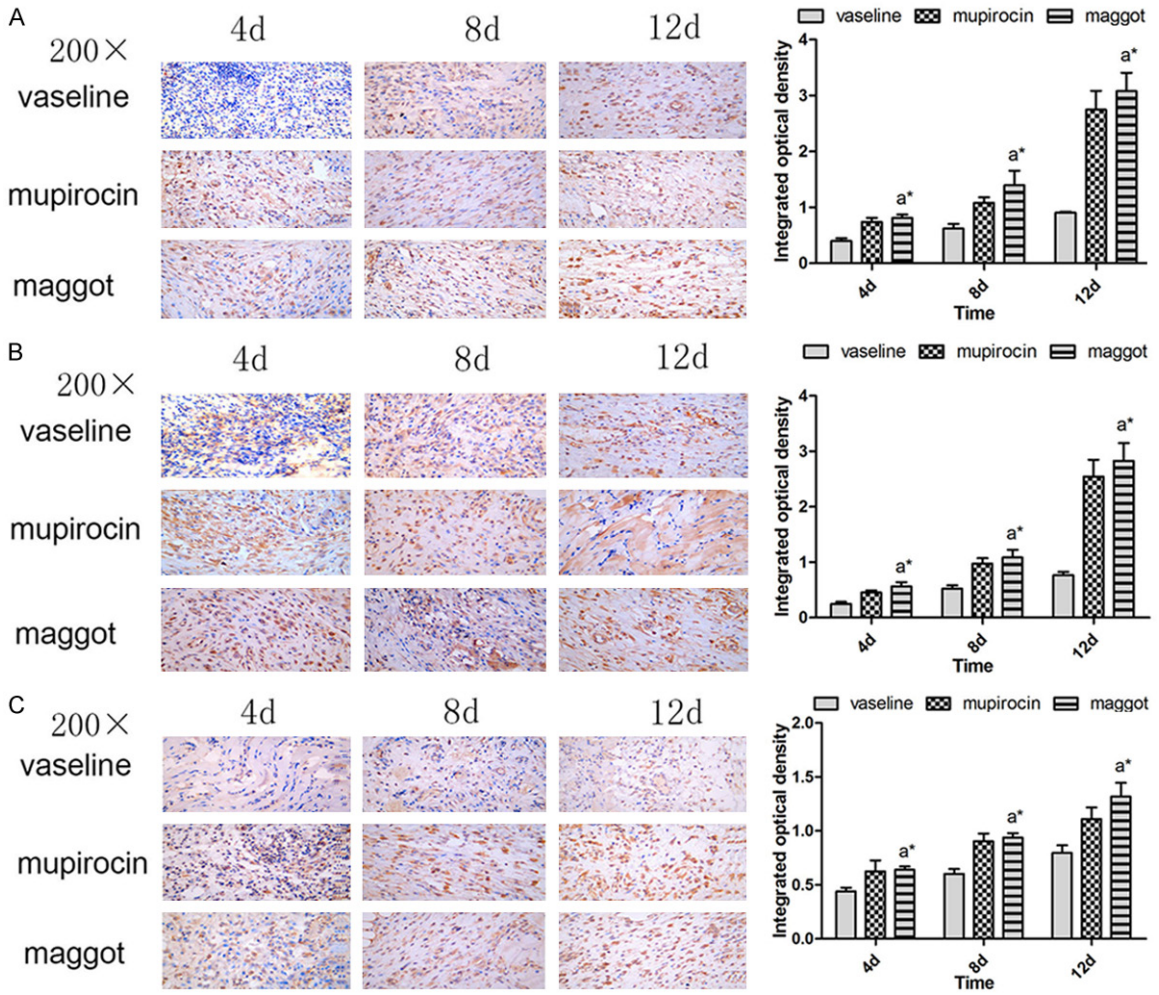

Figure 3. The immunohistochemical results of TGF-β (A), SMAD 2/3 (B) and SMAD4 (C). The means ± SD of IOD are shown. (A: The maggot group versus the Vaseline group; B: The maggot group versus the mupirocin group; *: P<0.05).

To examine the effects of the maggots on the bacterial content, living tissue was taken and incubated at 37°C for 24 h. As shown in **Figure 2B**, on the 4th day, the wounds pertaining to the three groups consisted of a lot of bacteria; on the 8th and 12th days, the number of positive bacterial cultures in the Vaseline group was higher than it was in the mupirocin and maggot groups. Also, comparing the mupirocin group and the maggot group, the maggot therapy also showed a good anti-bactericidal capacity.

Maggots increase the TGF-β, SMAD2/3, and SMAD4 expressions in the wound healing process

To illuminate the molecular mechanism of the effect of maggots on wound healing, we performed immunohistochemistry to investigate the expression of the TGF-β/SMAD signaling pathway during the wound healing process in the rat models. Infectious full-thickness wounds were created on the rats' backs to determine the TGF-β, SMAD2/3, and SMAD4 levels. Among all the comparisons, the IODs of TGF-β, SMAD2/3, and SMAD4 in the maggot and mupirocin groups were higher than they were in the Vaseline group (**Figure 3**).

Maggots activate the TGF-β/SMAD signaling pathway in the process of wound healing

We measured the expressions of the five main TGF-β/SMAD signaling pathway proteins in the process of wound healing using western blot. As shown in **Figure 4**, on the 4th day, the TGF-β1, SMAD2/3, SMAD4, Ras, and c-myc expres-

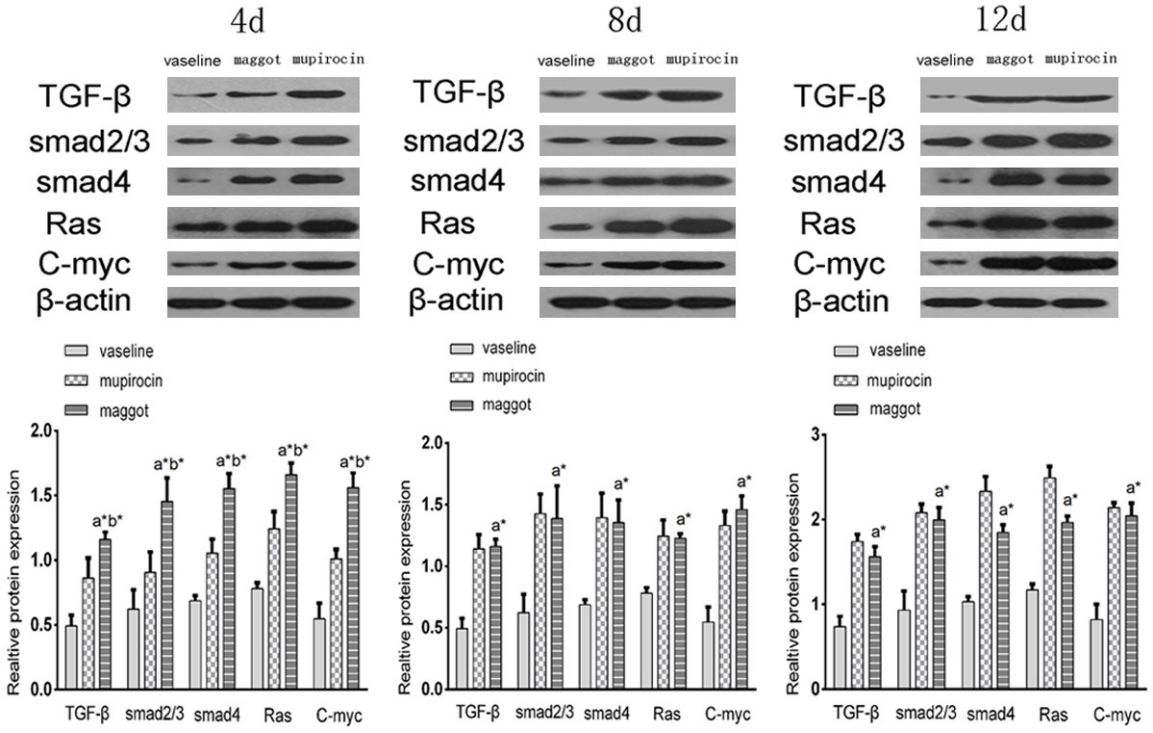

Figure 4. The Western blot results of the five main proteins of the TGF-β/SMAD signaling pathway on the 4th, 8th, and 12th days after the different treatments. The means ± SD of the relative protein expressions are shown. (a: the maggot group versus the Vaseline group; *: P<0.05).

sions increased in the maggot group and reached their highest expressions on the 8th and 12th days. Moreover, the SMAD 4 and Ras expressions in the maggot group were higher than they were in the mupirocin group on the 12th day. The results show that during the course of the treatment, the TGF-β/SMAD signaling pathway played important roles in both the maggot and mupirocin treatments.

Maggots increase the expressions of VEGFA and (PGP9.5) in the wound

Because the expression of TGF-β is related to angiogenesis, we conducted immunohistochemistry and Western blotting to investigate the VEGFA expressions. There was no significant difference in the VEGFA expressions among the three groups on the 4th day; on the 8th and 12th days, in the groups treated with maggot or mupirocin, the expressions increased compared with the Vaseline group. The VEGFA expression in the maggot group reached its maximum level compared to the other two groups (**Figure 5A**). To further understand whether the maggots were involved in the neuro-regeneration, we measured the PGP9.5 expressions in the rats' wounds. As shown in **Figure 5B**, on the 4th, 8th and 12th days after the treatment, the PGP9.5 expressions in the maggot group wounds were more significant than they were in the other groups. It is worth noting that the expressions on the 12th day were most significant in the maggot group.

Discussion

Wound healing has long been a hot topic in scientific research and clinical practice. Infected wounds fall into the common and refractory types. For such wound cases, the continuous use of antibiotics may easily lead to antibiotic resistance. According to published studies, maggot treatment, a safe, effective, and green method, has the potential to solve this difficult problem involving infected wounds [8]. Studying the mechanism of maggot treatment for infected wounds is very important for the promotion and application of this therapy.

The TGF-β/SMAD signaling pathway mechanism is well-known among researchers who study wound healing. In a previous study on acute wounds, Li et al. found that the maggot extract enhances the TGF-β/SMAD3 activities [22]. Our study intended to investigate the rela-

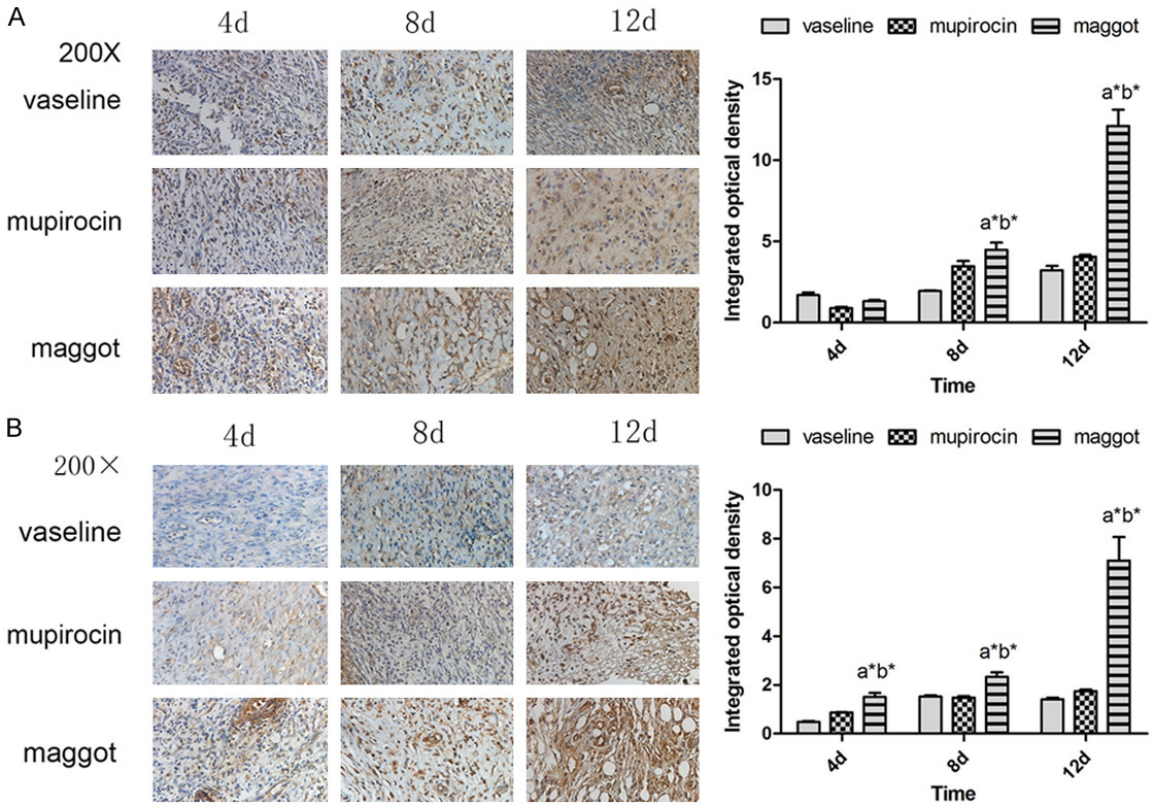

Figure 5. The immunohistochemical results of VEGFA (A) and PGP9.5 (B). The means ± SD of the IOD are shown. (a: maggot group versus the Vaseline group; b: maggot group versus the mupirocin group; *: $P<0.05$).

tionship between maggot therapy and the TGF-β/SMAD signaling pathway in infected cutaneous wound healing. The TGF-β/SMAD signaling pathway is known to be an important pathway that regulates cell proliferation [23, 24]. Many studies have shown that TGF-β1, a pro-fibrotic cytokine, is important in the inflammation [25], fibrosis [26], and wound processes [27].

It is generally believed that TGF-β promotes cell proliferation, differentiation, and migration depending on the expression and activation of the SMAD3 protein [28]. Moreover, TGF-β activates SMAD3 and subsequently forms a complex based on R-SMAD/co-SMAD by attracting a series of SMAD mediators (SMAD4) [29]. These complexes enter vascular endothelial cells, skin cells, and fibroblasts in wound tissue [30]. The activation of related genes promotes wound healing, including the promotion of extracellular matrix secretions, the proliferation of vascular endothelial cells, the formation of fibroblast permeates, and the migration of skin cells [31-33]. In our study, we observed the up-regulation of TGF-β, SMAD2/3, and SMAD4 in the maggot group. Compared with the Vaseline group, the TGF-β/SMAD signaling pathway kept a high and stable expression in the maggot group during the treatment process.

Meanwhile, previous studies confirmed that medical maggots have multiple biological activities, including antibacterial and antibiofilm functions [8]. In this study, the bacterial content on the 4th day after treatment, per gram of granulation tissue in the treatment group of mupirocin ointment, was evidently less than it was in the Vaseline and maggot groups. However, on the 12th day, the bacterial content of the granulation tissue in the maggot group was markedly less than it was in the mupirocin and Vaseline groups. We compared the use of mupirocin ointment, a representative antibacterial preparation, with the use of maggots to treat infected cutaneous wounds. The results indicated that the antibacterial activity of maggots is as effective as mupirocin. More importantly, one advantage of the maggot therapy is

that it decreases the risk of antibiotic resistance compared with traditional antibacterial preparations. Previous studies have demonstrated that antibacterial proteins from maggots (the extracts isolated from the maggots) showed an inhibitory function against both standard strains and clinically isolated antibiotic-resistant strains of *S. aureus* [34].

Moreover, the results from the in vivo wound healing also confirmed that maggot therapy accelerates the wound healing process. We demonstrated that the wound area of the mupirocin group seemed to be smaller than the wound areas of the Vaseline and maggot groups on the 4th day. This was most likely due to the maggots' debridement effects. The wound area became larger or did not change in the early stage of treatment. Subsequently, with the rapid growth of the granulation tissue and the acceleration of epithelialization, the wound area shrunk fast. On the 12th day, the wound area of the maggot group showed a brilliant recovery compared with the other two groups. In terms of assessing the granulation tissue, the ability of maggots to promote granulation growth is surprising. In the maggot group, the granulation covered the wounds quickly and provided the necessary conditions for epithelialization. Meanwhile, the HE staining results showed that, compared with the Vaseline group, the number of inflamed cells decreased rapidly in both the maggot and mupirocin groups. This indirectly suggests that the maggots can inhibit the inflammatory response as well as accelerate the transformation of the wound healing stage.

Neovascularization and neuro-regeneration, two main cascade reactions of the TGF-β/SMAD signaling pathway, play important roles in the development and progression of wound healing [35, 36]. For neovascularization, VEGFA is a key molecule that modulates many signaling pathways and promotes neovascularization [37]. It is more closely related to the TGF-β/SMAD signaling pathway. PGP9.5 is an ubiquitin hydrolase, which is a neuron-specific antigen [38]. It is widely expressed in all stages of neuronal differentiation, especially in the nerve fibers of skin wounds. Previous research has demonstrated that PGP9.5 is a sensitive indicator for observing neuro-regeneration [39]. In the current study, we found that the maggot group exhibited high expressions of VEGFA and PGP9.5 compared with the Vaseline and mupirocin groups. This result suggests that maggots can promote neovascularization and neuro-regeneration by activating the TGF-β/SMAD signaling pathway.

As described above, maggots have three ways of dealing with infected wounds. Bacteria control, debridement, and the promotion of wound tissue growth played different roles in the different stages of wound healing. In this study, the results of the protein level study showed that maggot therapy can accelerate wound healing by up-regulating the TGF-β/SMAD signaling pathway, which is likely to be involved in the mechanisms of neovascularization and neuro-regeneration.

However, there are still some limitations to this study. Maggot therapy has distinctive features, and the secretions and excreta are very complex. Here, it is impossible to determine the specific components that really play the role of signaling pathway regulation and wound healing promotion. Moreover, it is still possible that maggot therapy regulates other signaling pathways to resist infection, remove necrotic tissue, and repair wounds. Therefore, further studies are urgently needed, especially at the gene and transcription levels, etc. In addition, it is very important to determine the specific effective components for promoting the application and development of maggot therapy. There's reason to believe maggot therapy will an important alternative to the current wound treatment methods in the future.

Conclusion

This study demonstrated that maggots can treat infected cutaneous wounds. In rats treated with maggots, bacteria control, the debridement of necrotic tissue, and wound tissue growth were all completed. Additionally, maggots increased the TGF-β/SMAD signaling pathway levels in the wounds and improved the wounds' conditions. Furthermore, maggots promoted neovascularization and neuro-regeneration by increasing VEGFA and PGP9.5 expressions. In this way, maggot therapy might be a more effective clinical strategy to heal infected cutaneous wounds.

Acknowledgements

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The study was approved by the Ethics Committee of Dalian Medical University. An experimental animal use license (NO: SCXK 2013-0003) from the Laboratory Animal Center of Dalian Medical University was obtained.

Disclosure of conflict of interest

None.

Abbreviations

PGP9.5, protein gene product 9.5; TGF-β, transforming growth factor-β; HE, hematoxylin and eosin; IHC, immunohistochemistry; VEGFA, vascular endothelial growth factor A; IOD, integrated optical density.

Address correspondence to: Yong Jiang and Decheng Lv, Orthopedics Department, First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Xigang District, Dalian 116011, China. E-mail: jiangsurgeon@126.com (YJ); dechenglv@126.com (DCL)

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