TOXICITY TESTING AND WOUND HEALING EFFICACY OF FIBROIN GEL IN ANIMAL MODEL

Nikhom Naksupan[1], Nuttawut Saelim[1,*], Pornarin Taepavarapruk[2], and Niwat aepavarapruk[2]

[1]Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand
[2]Department of Physiology, Faculty of Medical Sciences, Naresuan University, Phitsanulok 65000, Thailand
*e-mail: nut456zz@yahoo.com

Abstract

The objective is to study skin wound healing effect and toxicity of silk fibroin gel *in -vivo* animal model. Thirty-eight male Sprague-Dawley rats with 200 to 250g in weight were used in this study. In wounding procedure, briefly, four full thickness wounds (0.6 cm Ø) were generated using a biopsy punch on the back of each animal. Each of four wound treatments, fibroin 1% (w/v) gel in PBS (10 mM), fibroin 2.5% (w/v) gel in PBS, PBS (control), or CMC 1% (w/v) gel in PBS (control for occlusion effect) was then applied onto designated wound at 20 µl/wound (single dose). Afterwards, animals were euthanized and wounds were harvested on day 1, 3, 5, 7, and 14 post-wounding (N=8, each). Anatomical and histological evaluation and expressions of specific proteins, Ki67, CK14, CK1/10, and collagen-I of each wound were studied to evaluate the wound healing effect and toxicity of the treatment groups. The results demonstrated that wounds treated with a single dose of 20 µl fibroin gel 2.5% (w/v) showed better wound healing than wounds treated with fibroin gel 1% and control groups (10mM PBS and CMC gel 1% (w/v)). The maximal efficacy in %wound coverage of silk fibroin gel could be observed within 3 days after wound treatments, wounds treated with fibroin gel 2.5% (w/v) significantly showed the highest of %wound coverage at 70% with 3/8 complete healing compared to 47% in fibroin gel 1% (w/v) with 1/8 complete healing, 49.5% in CMC gel 1% (w/v), and 42.7% in PBS (ANOVA, $P<0.01$). Interestingly, we also discovered that silk fibroin significantly promoted higher expression of Ki67 protein expression (a cell proliferation marker) during skin wound healing progression, and lowered degree of granulation area during wound remodeling. Therefore, we concluded that fibroin gel treatment accelerated skin wound healing in animal (rat) model by accelerating epidermal (keratinocytes) migration, increasing wound coverage, and promoting wound remodeling. Furthermore, there was no anatomical change, no signs of cell death and tissue toxicity, or wound healing inhibition significantly observed among the treatment and control groups, indicating that the treatment of silk fibroin on wound was safe. Results from this study indicate that silk fibroin gel has a high potential for wound dressing application in clinical trial.

Keywords: wound healing, fibroin, animal model, silk

Introduction

Silk fiber secreted from silkworm (*Bombyx mori*) almost consists of two major proteins, sericin and fibroin. Fibroin is a fibrous protein composed of parallel beta-sheet structure, with high amount of amino acid glycine (43%), alanine (30%), and serine (12%). Such unique structure and composition makes fibroin a thermodynamically stable and high tensile strength protein. Silk fibroin, so far, has been utilized in various applications, apart from clothing, such as biodegradable scaffolds, membrane materials and biomedical materials (i.e. tissue engineering and drug delivery system).

It's been documented that silk fibroin offers special properties in animal and clinical uses, namely non-allergy, tissue compatibility, bio-degradability, and safety profile (Vepari and Kaplan 2007; Lawrence and others 2009; Chung and Chang 2010). Recently, some studies suggested that fibroin may contain ability to promote wound healing by increasing rate and amount of proliferating fibroblasts and keratinocytes, inhibiting free radicals, and protecting cells from apoptosis (Minoura and others 1995; Tsubouchi and others 2003; Yamada and others 2004). These evidences, however, were mainly from *in-vitro* models. Together with our previous findings, fibroin from difference sources generated different physical, chemical, and biological activities.

Therefore, we have developed our own process to extract and purify fibroin from Thai yellow silk cocoon (Thailand petty patent no.6272, May 31, 2011). In brief, dried de-pigmented and de-gummed silk fiber was cautiously dissolved in 9.0M lithium bromide at 5%w/v and then incubated at 60°C for 4 hours. The fibroin solution was filtrated and dialyzed with dialysis tubing (10kDa MWCO) in sterile de-ionized water for 4 to 5 days. Finally, dialyzed fibroin was dried by freeze-dried method. Our silk fibroin has high quality, molecular weight homogeneity, water solubility, heat and wide pH range tolerability. This in-house fibroin showed ability to increase cell proliferation on both cultured primary fibroblasts and keratinocytes, and promoted wound healing in *ex-vivo* porcine skin wound healing model (unpublished data).

The objective of this study is to demonstrate the efficacy of purified silk fibroin gel preparation on skin wound healing effect and its toxicity in animal model. The success of this study will pave the way for silk fibroin to clinical trial as a wound healing promoting drug.

Materials and Methods

Animal preparation and wounding procedure

In this experiment, 38 male Sprague-Dawley rats with 200 to 250g in weight were used as a model for skin wound healing study. Animals were randomly assigned by numbers preset for operating dates, wound treatment patterns, and wound harvesting dates. Animals were kept in individual cage in temperature-controlled room (25±2°C), 12 hour dark-light cycles. Food and water were provided *ad lib* (the protocol was approved by Naresuan University Animal Ethics Committee). In wounding procedure, animals were anesthetized by 40 mg/kg Nembutal (ip) and given antibiotic 50 mg/kg ampicillin (ip), hair at the back of the animals were removed by electric clipper and surgical blade, proposed wound positions were marked using a guiding grid, four full thickness wounds (two wounds/side, Fig. 1A, 1B) were made using a biopsy punch (0.6cm. Ø), 1 cm. apart from each other and 0.5 cm. away from animal vertebral line (Fig. 1A).

Fibroin gels and CMC gel preparation

Fibroin gel was simply prepared by dissolving dried fibroin powder in 10mM phosphate buffer saline solution (PBS) at 1% and 2.5%w/v. Carboxy-methyl cellulose (CMC) also was prepared in the same way at final concentration of 1% (w/v) in PBS. All gels and solutions were sterilized by autoclaving.

Wound treatment

Soon after wound generation procedure was finished, four wound treatments, (1) fibroin 1% (w/v) gel in PBS, (2) fibroin 2.5% (w/v) gel in PBS, (3) 10mM PBS (control), and (4) CMC (carboxy-methyl cellulose) 1% (w/v) gel in PBS (control for occlusion effect) were applied onto designated wounds at 20 µl/wound. All wounds in each animal were secured with elastic Tegaderm^{TM} film (3M^{TM}) 6x7 cm. to protect the treatments from leakage and wound contamination. Neck ring was then put on to avoid wound scratching and tearing by the animal.

Efficacy and toxicity evaluation

Wounds were harvested at specified date post wounding, on day 1, 3, 5, 7, and 14 (D1, D3, D5, D7; N = 8, only D14; N = 6). Animals were euthanized, and each wound was inspected, photographed, and measured before wounds harvesting and storage for histological and immunological studies. Abnormal signs and symptoms on skin such as allergy, inflammation was evaluated by the experts throughout the study. To evaluate the wound healing effect of treatment groups, both histological evaluation and expressions of specific proteins, Ki67, CK14, CK1/10, and collagen type I of each wound were extensively studied.

Statistical analysis

The percentage of wound re-epithelialization (%RE) or wound coverage of each wound was carefully assessed by measuring the length of the wound covered by newly formed epidermis using the AxioVision 5.0 and Motic program (Zeiss, Germany), and calculated, pooled and expressed as mean %RE±SD using the following equation.

$$\%\text{Wound re-epithelialization (\%RE)} = [(M_R+M_L/W_O] \times 100$$

Where W_O is original wound diameter (0.6 cm.), M_R and M_L are the length of epidermal migration from the right and left margins of the wound. Significant difference among experiment groups was tested at 0.05 and 0.01 significant levels by ANOVA.

Results and discussion

The toxicity and healing effect of silk fibroin in gel formulation in rat model were evaluated by both skin tissue and molecular levels. General physical observation, all animals were healthy, gaining weight, no stress signs. All wounds showed no signs of infection, allergy, severe inflammation, or tissue toxicity event from any received treatment preparations (Fig. 1C). All wounds were completely healed after 14 days post wound operation (Fig. 1C). The expressions of basic protein markers in skin wound healing processes including Ki67 (indicating cell proliferation), CK1/10 (indicating differentiated keratinocytes), CK14 (indicating un-differentiated keratinocytes), and collagen type I (matrix protein) were used to help wound evaluation at molecular level during the healing process. Immuno-staining study of those protein markers (data not showed) clearly demonstrated that all wounds in all groups were healed normally in the same patterns of expression of protein markers. These therefore clearly demonstrated that fibroin preparation for skin application is low in toxicity causing no harm to the animal health and wound healing process retardation.

The percentage of epidermal layer migration (%RE) or wound coverage is used for fibroin gels efficacy evaluation. Histologic study of all wounds revealed no significant difference among treatment groups after 1 day post wounding in term of % wound coverage (%re-epithelialization, %RE). All groups were around 11 to 13% in wound coverage. This is because keratinocytes in the epidermis and fibroblasts in the dermis normally need some time to respond to the injury (wound).

Three days post-wounding (D3), wounds treated with fibroin gel 2.5% significantly showed the highest of %RE at 70% compared to CMC 1% (49.5%), fibroin gel 1% (47%), and PBS (42.7%) by ANOVA ($P<0.01$). Complete skin wound healing (100 %RE) was clearly observed 3/8 wounds (37.5%) in fibroin gel 2.5% treated-group and 1/8 wounds (12.5%) in fibroin gel 1% treatment-group (Fig. 2). However, no complete skin wound healing was observed in control groups (wounds treated with CMC 1% or PBS). This finding indicates that the third day post-wounding is optimal time for wound healing efficacy study in rat model since the healing process is fully active in this period.

After 5, 7, and 14 days post-wounding, there were no statistical difference of %RE in any groups because wounds started to heal completely (Fig. 2). Interestingly, on day 14, fibroin treated-wounds (both 1% and 2.5%) revealed lower degrees of granulation area significantly when compared to the control groups (Fig. 3). We also found that silk fibroin preparations significantly increased degrees of expression of Ki67, especially on day 3 and day 5 compared to control groups ($P<0.05$) (data not showed). These findings suggest that fibroin promotes wound re-epithelialization by increasing keratinocytes proliferation (as %RE and expression of Ki67 increased) and enhancing wound remodeling by fibroblasts (as granulation area reduced). Therefore, our study strongly supported that silk fibroin gel is effective as promoting skin wound coverage and skin tissue remodeling.

In conclusion, this study clearly reveals the efficacy of silk fibroin gel formulation on wound healing in animal model by accelerating skin wound healing rate and wound coverage, and possibly promoting skin tissue remodeling. Fibroin gel also demonstrates no cell or tissue toxicity, or inhibition of wound healing process. The results are further confirm the healing effect of silk protein fibroin previously reported in primary cell lines and in *ex-vivo* porcine skin wound healing models. Finally, our results reveal that fibroin gel has a high potential for clinical trial as a wound promoting drug.

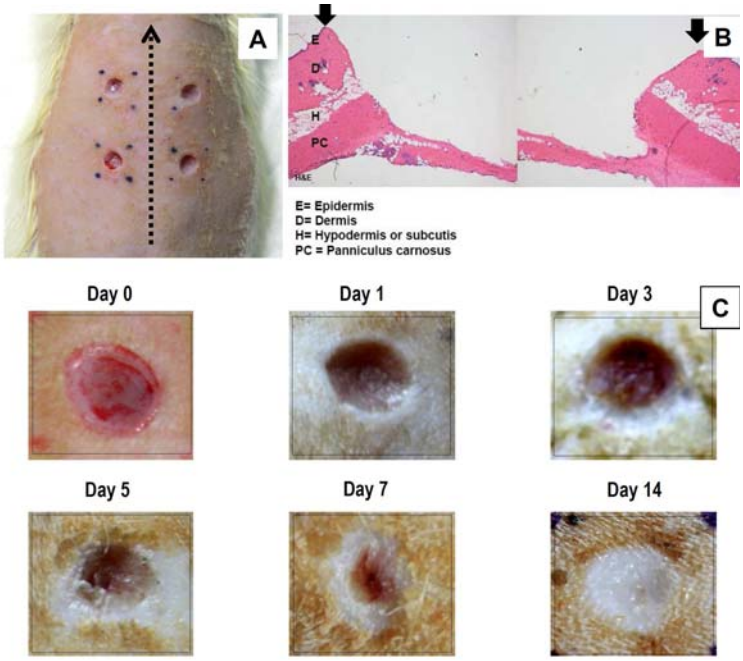

Figure 1 Skin wound healing experiment in Sprague-Dawley rat model. (A) wound positions along animal vertebral line. (B) full thickness wounded skin (D0). (C) wound healing progression from D0 to D14.

A

Day	PBS	CMC	FN1	FN2.5
1	12.3±1.3	12.6±1.1	11.3±1.2	13.5±1.1
3	42.7±4.4	49.5±5.2	47±7.9	70±10.2
5	93.8±4.2	91.9±3.9	96.6±2.4	89.3±5.2
7	95.4±4.5	85.7±7.2	100±0	85.2±7.4
14	100±0	100±0	100±0	100±0

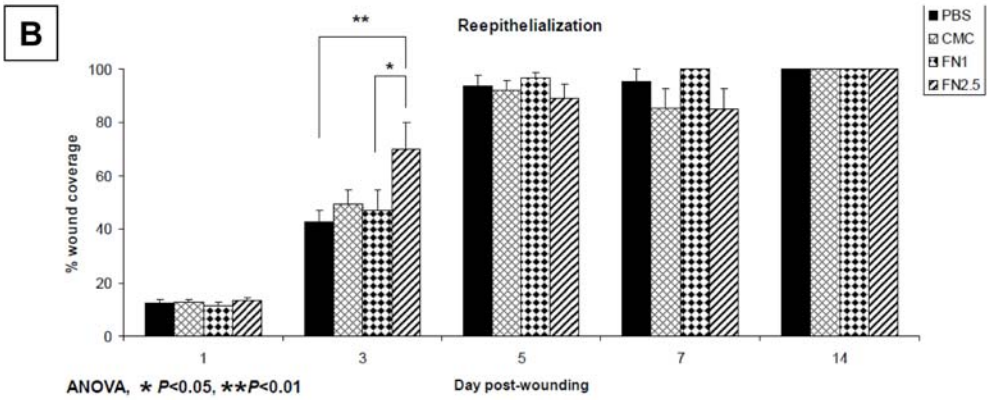

Figure 2 Fibroin gels promote wound coverage (%RE). (A) %RE of four treatment groups on different post-wounding dates. (B) ANOVA analysis histograms of %RE (n=8, only D14 n=6). All data were presented as mean ±SD.

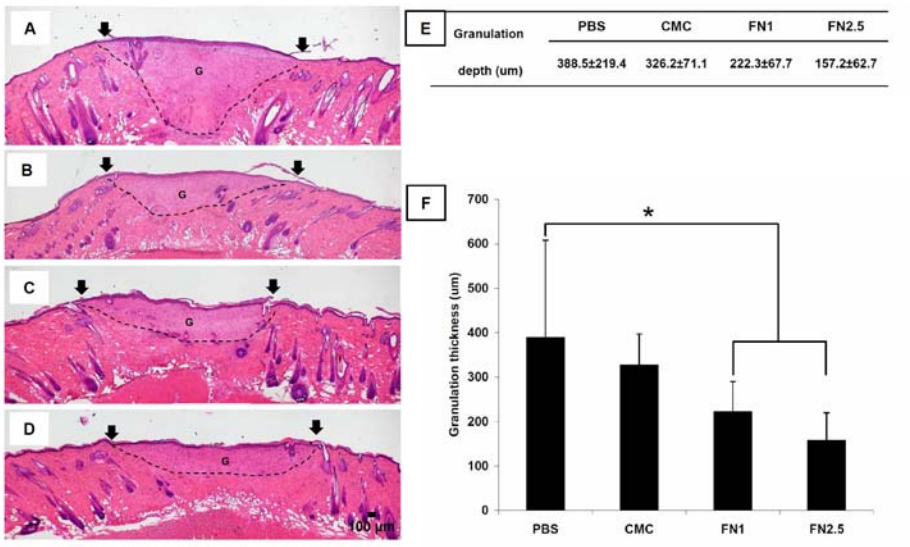

Figure 3 Fibroin gels enhance skin wound remodeling. (A) histologic study showing wound granulation depth on day 14 in wounds treated with (A) PBS, (B) 1% CMC, (C) 1% FN, and (D) 2.5% FN. (E) %granulation depth of four treatment groups on D14. (F) ANOVA analysis histograms of %granulation depth (n=6, **P* <0.05). All data were presented as mean ±SD

Acknowledgements

Financial support from the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education is gratefully acknowledged. Acknowledgment is also to the Faculty of Pharmaceutical sciences and Center of Excellence in Medical Biotechnology (CEMB), Naresuan University.

References

1. Chung TW, Chang YL. 2010. Silk fibroin/chitosan-hyaluronic acid versus silk fibroin scaffolds for tissue engineering: promoting cell proliferations in vitro. Journal of Materials Science: Materials in Medicine 21(4): 1343-51.
2. Lawrence BD, Marchant JK, Pindrus MA, Omenetto FG, Kaplan DL. 2009. Silk film biomaterials for cornea tissue engineering. Biomaterials 30(7): 1299-308.
3. Minoura N, Aiba SI, Higuchi M, Gotoh Y, Tsukada M, Imai Y. 1995. Attachment and Growth of Fibroblast Cells on Silk Fibroin. Biochemical and Biophysical Research Communications 208(2): 511-6.
4. Tsubouchi K, Nakao H, Igarashi Y, Takasu Y, Yamada H. 2003. *Bombyx mori* Fibroin Enhanced the Proliferation of Cultured Human Skin Fibroblasts. Journal of Insect Biotechnology and Sericology 72(1): 65-9.
5. Vepari C, Kaplan DL. 2007. Silk as a biomaterial. Progress in Polymer Science 32(8-9): 991-1007.
6. Yamada H, Igarashi Y, Takasu Y, Saito H, Tsubouchi K. 2004. Identification of fibroin-derived peptides enhancing the proliferation of cultured human skin fibroblasts. Biomaterials 25(3): 467-72.