J Mater Sci: Mater Med (2011) 22:2497–2504
DOI 10.1007/s10856-011-4420-6

Efficacy of chitin-PAA-GTMAC gel in promoting wound healing: animal study

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Received: 18 April 2011 / Accepted: 8 August 2011 / Published online: 19 August 2011

Abstract Acrylic grafted chitin (chitin-PAA) was modified with glycidyltrimethylammonium chloride (GTMAC) with the aim of promoting wound healing. The chitin-PAA-GTMAC gels with different GTMAC contents were compared with the original chitin-PAA gel and Intrasite gel for their efficacy in deep wound healing of Wistar rats. Four full-thickness wounds were made on the dorsal skin of rats and then each was treated with 4 materials; chitin-PAA, chitin-PAA-GTMAC(1:4), chitin-PAA-GTMAC(1:10) and Intrasite gel. During 18 days of treatment, the wounds were visually observed and calculated for wound size using image analysis program. Skin wound tissues of sacrificed rats were processed for routine histological observation and immunohistochemistry of proliferating cell nuclear antigen (PCNA). The wounds covered with the chitin derivatives either with or without GTMAC showed a significant reduction in wound size in day 9 in comparison with day 12 for those covered with Intrasite gel. The faster rate and the better pattern of epidermal development observed in histological study as well as the higher dermal cell proliferation (PCNA expression) also demonstrated the better efficiency in wound healing of the chitin derivatives than Intrasite. The earliest epidermal development of the wounds treated with chitin-PAA-GTMAC (1:4) among the tested materials suggested the most promising of this material for the treatment of full-thickness open wound.

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1 Introduction

Chitin, one of the most abundant renewable resources, is a very attractive substance for medical uses due to its biodegradability, biocompatibility and bioactivity [1, 2]. It is easily and economically prepared from the shells of crab, shrimp and squid pens which are waste from seafood industry. In order to turn this industrial waste into a valued wound dressing, we have produced chitin-PAA-GTMAC that imparted functional groups encouraging a moist wound healing environment and discouraging wound infection [3]. β-Chitin was modified with acrylic acid and glycidyltrimethylammonium chloride (GTMAC) to increase, respectively water sorption and antibacterial properties. The in vitro investigation of biological properties of chitin-PAA-GTMAC gel demonstrated the potential of the material in promoting wound healing. In this study, the wound healing efficiency of chitin-PAA-GTMAC was further evaluated in vivo.

There have been a number of studies on wound healing of chitin and its derivatives using a rat model [4–7]. Dibutyryl chitin was tested in full thickness wounds in rats and no adverse effect on the healing process was observed histologically [5]. Chitosan-crosslinked collagen sponge containing recombinant human acidic fibroblast growth factor (CCCS/FGF) showed accelerating wound healing in diabetic rat model as compared to collagen sponge alone and FGF alone [6]. The earliest wound closure and tissue collagen generation as well as the highest tissue growth factor-β1 and dermal cell proliferation found in CCCS/FGF treated

wound indicated the most efficient healing of CCCS/FGF among various treatments. Dermal cell proliferation was determined by the method of immunohistochemical staining of proliferating cell nuclear antigen (PCNA). PCNA is an essential factor for DNA replication [8, 9] and repair [10]. Therefore, it is abundantly expressed in proliferating cells and can be used as a marker protein of proliferating cells [11]. In our recent studies, β-chitin grafted poly (acrylic acid) (chitin-PAA) has shown a biocompatible property in vitro [12] and in the rat model [13]. The chitin-PAA also demonstrated a comparable healing efficacy to alginate and lipido-colloid dressings in the partial-thickness skin graft model [14]. With the aim of further improving the wound healing efficiency of the chitin-PAA dressing, chitin-PAA-GTMAC that incorporated GTMAC into the gel was developed. The presence of GTMAC has shown to be effective for the suppression of bacteria and promoting wound healing by stimulating cytokine secretion and facilitating cell proliferation [3].

The present study was therefore undertaken in order to evaluate the effect of incorporation of GTMAC into the chitin-PAA gel on the wound healing efficiency in experimental full-thickness wounds in Wistar rats. The gel without GTMAC (chitin-PAA) including a clinically available dressing (Intrasite) were used for a comparative study. Intrasite gel is a clear amorphous hydrogel containing a modified carboxymethylcellulose polymer, sodium, propylene glycol and water [15]. Clinical study on pressure ulcers has shown that debridement was achieved more quickly in patients treated with Intrasite [16]. It has been recommended not only for the treatment of leg ulcers but also surgical wounds, extravasation injuries, and pressure sores. In this study, the wound healing efficiency was assessed and compared among different types of dressing in three aspects; the appearance and the size of wound area, the routine histological study of the wound tissue by hematoxylin and eosin staining (H&E), and the immunohistochemistry of PCNA.

2 Materials and methods

2.1 Preparation of chitin-PAA-GTMAC gels

The chitin-PAA-GTMAC gels have been prepared as previously reported [3]. In brief, chitin was chemically modified with acrylic acid at the weight ratio chitin: acrylic acid of 0.25. The resulting product (chitin-PAA) was further reacted with glycidyltrimethylammonium chloride (GTMAC) using the feed molar ratios of glucosamine to GTMAC at 1:4 and 1:10. The chitin derivatives with and without GTMAC; chitin-PAA-GTMAC (1:4) and (1:10), and chitin-PAA were then prepared in the form of gel at 5% (w/v) in deionized water and sterilized by steam at 121°C for 15 min.

2.2 Wound preparation and treatment

Thirty two female Wistar rats (250–300 g), 12 weeks old were used in this study. All experiments were conducted under protocols approved by the animal research committee of the Armed Forces Research Institute of Medical Sciences (Royal Thai Army Component) and monitored by the facility veterinarian to ensure compliance with guidelines for the care and use of laboratory animals of National Research Council (1996). The animals were anesthetized with ketamine (75–100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. The dorsal hair was then shaved using a clipper and the skin was sterilized. Four full-thickness dorsal skin excisions with the size of 1×1 cm^2 and depth through the entire dermis and into subcutaneous tissue were performed, 2 wounds for each side of the dorsal mid-line. In order to create a wound with consistent and uniform size in all animals, a pattern of 4 wound areas was traced on dorsum of each rat at the same position as referenced from its dorsal midline. The excised wounds were then covered with the gels of chitin-PAA, chitin-PAA-GTMAC (1:4), chitin-PAA-GTMAC (1:10) and Intrasite. Then, a sterilized sheet (10×12 cm^2, Tegaderm) was employed to cover the wounds, following by Neotape which was fixed on the dorsum of a rat with silk, U.S.P.2/0 at the corners. The treated animals were housed in individual cages with no regular dressing changes to avoid any damage to regenerated epithelial tissue. Four Wistar rats were sacrificed on the postoperative days 1, 3, 5, 7, 9, 12, 15 and 18 that covered through the period of proliferation in wound healing. The healing of excised wounds was assessed by wound size measurement, histological examination and immunohistochemistry of PCNA.

2.3 Evaluation of wound healing

2.3.1 Wound observation and measurement

The wounds were visually inspected and photographed by a digital camera soon after wounding and at 1, 3, 5, 7, 9, 12, 15 and 18 days of treatment. The wound circumference was outlined using the Adobe Photoshop software, and the wound area was measured in pixels using a computer program of image analyzer (Image-Pro Plus, version 5.1, Media Cybernetics). The area of the wound at each postoperative day was calculated as percentage of the initial wound area using the following equation:

$$\text{Wound area } (\%) = A_t/A_0 \times 100,$$

where A_t and A_0 represent the wound area in pixels at certain postoperative day and at initial, respectively. The wound areas in each dressing group were then compared for reduction in wound size using one-way ANOVA analysis and Scheffe test with $P < 0.05$ considered significant.

2.3.2 Histological examination

The wound area of 2.5 × 2.5 cm^2, deep into the dermis was removed, fixed in 4% neutral buffered formaldehyde, embedded in paraffin and sectioned to a thickness of 5 μm by a rotary microtome (Leica, RM2135). The sections were stained with hematoxylin and eosin (H&E) and examined light microscopically to observe inflammation and the extent of epidermal growth. Inflammation was detected by the presence of polymorphonuclear leukocytes (PMN) mostly neutrophils. To determine the extent of re-epithelialization, newly formed epidermis that migrated over the wounds from the wound edges as well as the presence of skin appendages such as hair follicles and sebaceous glands were followed. The number of keratinocyte layers as well as the pattern of dermal papilla was also compared among wounds dressed with different materials.

2.3.3 Immunohistochemistry of PCNA

The number of proliferating cells was determined by immunohistochemical staining for PCNA (Santa Cruz Biotechnology, Inc.). Briefly, tissue sections were deparaffinized and blocked endogenous peroxidase activity by incubating in 3% H_2O_2. After washing twice in PBS, the sections were incubated with primary antibody (PCNA) at a dilution of 1:100 at room temperature, and then washed three times in PBS. The sections were subsequently incubated with biotinylated secondary antibody at room temperature. After washing three times in PBS, avidin and biotinylated horseradish peroxidase solution was applied. The sections were then washed three times in PBS, stained with few drops of peroxidase substrate and counterstained in haemotoxylin solution. After being stained, PCNA that normally locates in the nucleus of proliferating cell appears distinct golden-brown. These PCNA-positive cells were counted under a light microscope using the magnification of ×100. For the analysis of PCNA distribution, three sections with five representative areas each were examined for each wound. The representative areas were randomly chosen at the same areas for each group, i.e., 1 area at the left wound edge, 1 area at the right wound edge and 3 areas at the center of the wound. The data obtained were evaluated for statistical significant using one-way ANOVA analysis and Bonferroni test with $P < 0.05$ considered significant.

3 Results and discussion

3.1 Wound observation and measurement

The visual observation of wounds covered with different gels at days 1, 3, 5, 7, 9, 12, 15 and 18 after treatment indicated comparable performance of all gels. They all showed ability in absorption of wound exudates and easily to be removed from wound by normal saline without disruption of the wound tissue. All wounds showed no surface desiccation indicating a moist wound healing environment which facilitated the migration of epithelial cells. No evidence of infection that might contribute to healing failure, was observed throughout the study. During few days after operation, all wounds became hyperemia indicating an acute inflammatory reaction. This inflammation was a normal response of healing process after wounding. All wounds were found almost completely healed by day 15.

Wound defect area for each time interval was calculated and expressed as wound area (%) compared to its initial wound size in Fig. 1. The wounds covered with chitin-PAA and Intrasite gels were found increasing in wound size from its original until day 3 after the operation whereas the wounds covered with chitin-PAA-GTMAC (1:4) and (1:10) prolonged their wound swelling till day 5. Moreover, the chitin-PAA-GTMAC (1:4) and (1:10) dressed wounds appeared greater swelling compared with the mild swelling of chitin-PAA and Intrasite. The previous study of biological properties of chitin-PAA-GTMAC showed increased cytotoxicity with increasing GTMAC content [3]. Thus, the long period of wound swelling of chitin-PAA-GTMAC (1:4) and (1:10) was likely induced by inflammation response to GTMAC. After day 5 of treatment, the wounds in all groups showed a progressive decrease in size with time indicating that all wounds were in the proliferative phase of wound healing process.

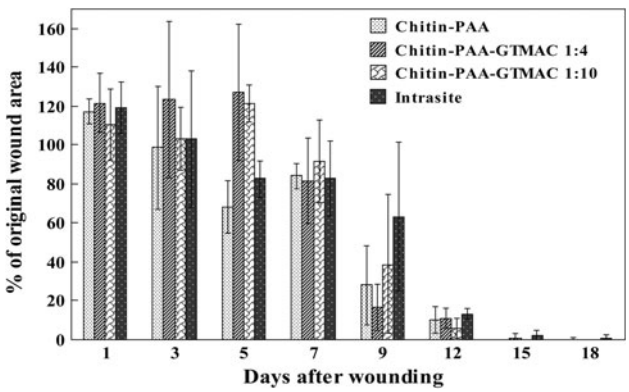

Fig. 1 Comparison of the area change of the wounds covered with chitin derivatives and Intrasite

A significant difference in wound reduction was observed on day 9 for the wounds covered with chitin derivatives; chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10) whereas those covered with Intrasite gels were found statistically significant in wound reduction on day 12. The wounds treated with chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10) showed a progressive decrease in size at the greater extent than those of Intrasite and obtained, respectively the greater extent of healing at 72, 83 and 61% in comparison with 37% for Intrasite on day 9. These results implied the rapid healing of chitin derivatives compared to Intrasite gel. There was, however, no statistical difference in the wound reduction among the chitin derivatives contained different amount of GTMAC. On day 18, all wounds treated with chitin-PAA-GTMAC (1:4) and (1:10) achieved 100% wound closure whereas the chitin-PAA and Intrasite treated wounds were found almost completely healed with the average remaining wound area of 0.25 and 0.69%, respectively.

3.2 Histological examination

Histological observations showed faster epithelization of the wounds dressed with chitin derivatives; chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10) than those dressed with Intrasite gel. The epithelization was early observed on day 3 at the edges of the wounds dressed with chitin derivatives. All Intrasite dressed wounds, on the contrary, showed rarely number of developing epidermis on day 3. There were also remaining residues as well as inflammatory cells, mostly neutrophils presented in all wounds on day 3.

On day 9, the wound dressed with chitin-PAA-GTMAC (1:4) was completely covered with newly organized epithelium, comprising of keratin (Kr), dermal papillae (Dp) and hair follicles (Hf), as shown in Fig. 2. Its residue (Rd) and inflammatory cells that observed in large amount on day 3 were mostly disappeared. Those keratin and dermal papillae, but hair follicles were observed in wounds dressed with chitin-PAA and chitin-PAA-GTMAC (1:10). The small amount of residual dressing still remained with no inflammatory cell observed in the chitin-PAA-GTMAC 1:10 dressed wounds while there was no residue left in the wound covered with chitin-PAA. The Intrasite dressed wound showed the poorest developing epidermis (De) among the tested materials. Although its wound edges revealed the developing epidermis with slight keratinization and some dermal papillae, the center of the wound was still uncovered. Moreover, a large number of inflammatory cells and material residue were observed on the wound surface and the underneath subcutaneous layer. These findings were in agreement with the wound reduction results that observed the significant reduction in the chitin

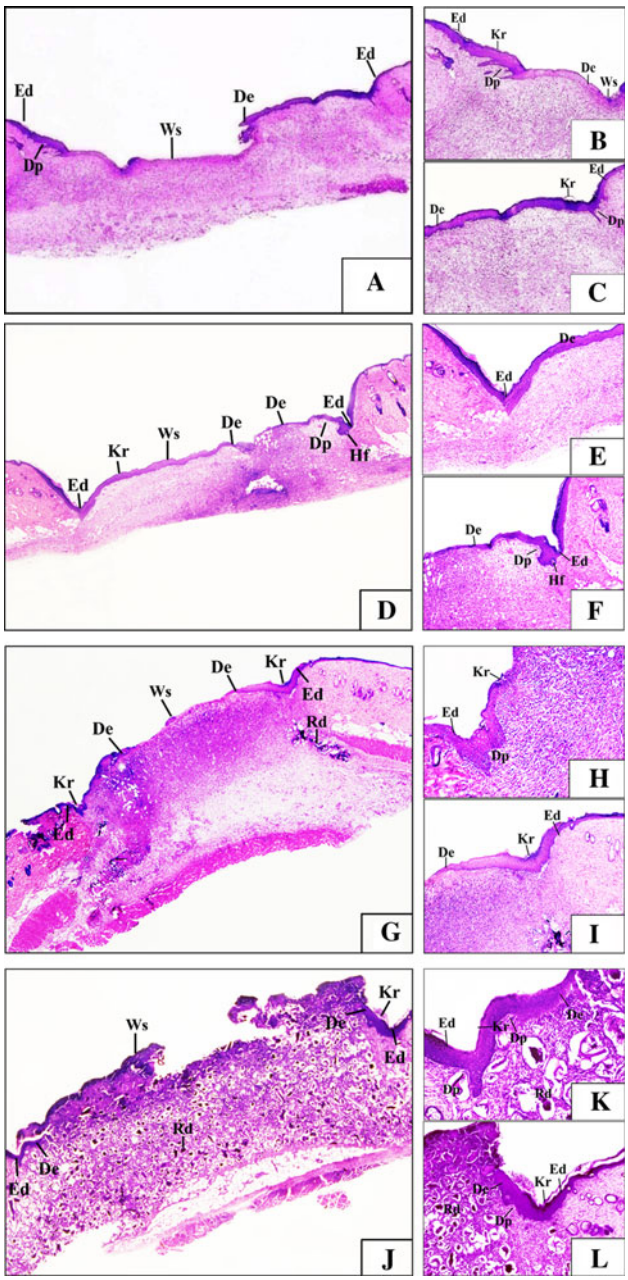

Fig. 2 Histology of skin wounds on day 9 dressed with Chitin-PAA (**a**, **b**, **c**), Chitin-PAA-GTMAC (1:4) (**d**, **e**, **f**), Chitin-PAA-GTMAC (1:10) (**g**, **h**, **i**) and Intrasite (**j**, **k**, **l**). The overall surface of the wound ×1.25 (**a**, **d**, **g**, **j**); the left wound edge ×4 (**b**, **e**) ×10 (**h**, **k**); the right wound edge ×4 (**c**, **f**, **i**, **l**). Wound edge (*Ed*); wound surface (*Ws*); dermal papillae (*Dp*); developing epidermis (*De*); keratin (*Kr*); hair follicle (*Hf*); residue (*Rd*). (H&E stain)

derivatives dressed wounds on day 9. It indicated that the wounds dressed with chitin derivatives reached inflammatory phase and proliferative phase faster than those dressed with Intrasite.

Hair follicles were observed later on days 15 and 18 in the wounds dressed with chitin-PAA-GTMAC (1:10) and chitin-PAA, respectively. Furthermore, sebaceous glands

(Sb) were found developed on day 15 in those wounds dressed with chitin-PAA-GTMAC (1:4) and (1:10). On day 18, the surfaces of all wounds dressed with different materials were completely covered with epidermis, as shown in Fig. 3. The chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10) dressed wounds appeared well-developed epidermis containing multilayered keratinocytes with keratin, dermal papillae and hair follicles resembling the intact normal skin. Their tissues beneath the wound surfaces were found compact and strong with no dressing residue observed in chitin-PAA dressed wounds, but tiny amount of residue in the chitin-PAA-GTMAC (1:4) and (1:10) dressed wounds. The wounds dressed with Intrasite showed inferior epithelization to those dressed with chitin derivatives. Although, its wound surface was covered with new epidermis (Ne) comprising keratin and dermal papillae, the formation of hair follicle and sebaceous gland were not observed. The large amount of material residue still remained in the wound, resulting in loosely arranged tissue beneath the wound surface.

It has been reported that a moist environment promoted epithelization and wound healing [17, 18]. All dressings used in this study were capable of providing a moist wound environment, thus they all appeared to be helpful in accelerating wound healing. However, the wounds dressed with chitin derivatives; chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10) appeared more advanced tissue formation and epithelization than those dressed with Intrasite. The chitin derivatives dressed wounds showed high quality of the newly formed epidermis with well organized tissue underneath whereas Intrasite dressed wounds showed rather loosened tissue structure in wound cavity. This was not surprising due to the previous report of high activity as a wound healing accelerator of chitin [19]. The chitin that modified with acrylic acid (chitin-PAA) and both acrylic acid and GTMAC (chitin-PAA-GTMAC), still possessed the beneficial properties of chitin with enhancing wound healing ability by increasing absorption of wounds exudates and antibacterial ability [3, 12]. GTMAC although has been known of its effectiveness against a broad range of micro-organisms [20–22], it slightly irritated cells as observed previously from the greater wound swelling of chitin-PAA-GTMAC (1:4) and (1:10) than the chitin-PAA gel during inflammatory phase. Moreover, the residue of chitin-PAA-GTMAC dressings still appeared till the end of experiment, not entirely absorbed as that of chitin-PAA. These results indicated that chitin-PAA-GTMAC was less biocompatible and biodegradable than chitin-PAA. For Intrasite dressed wound, the residue was found remained in the largest amount on day 18, and that indicated the poorest biocompatible and biodegradable of Intrasite among the tested materials. The remained residue of Intrasite likely interfered and delayed proliferative phase and the remodeling or maturation phase of wound healing process. As a result, the wound dressed with Intrasite showed rather poor pattern of epidermal development and required a longer time to complete skin derivative formation.

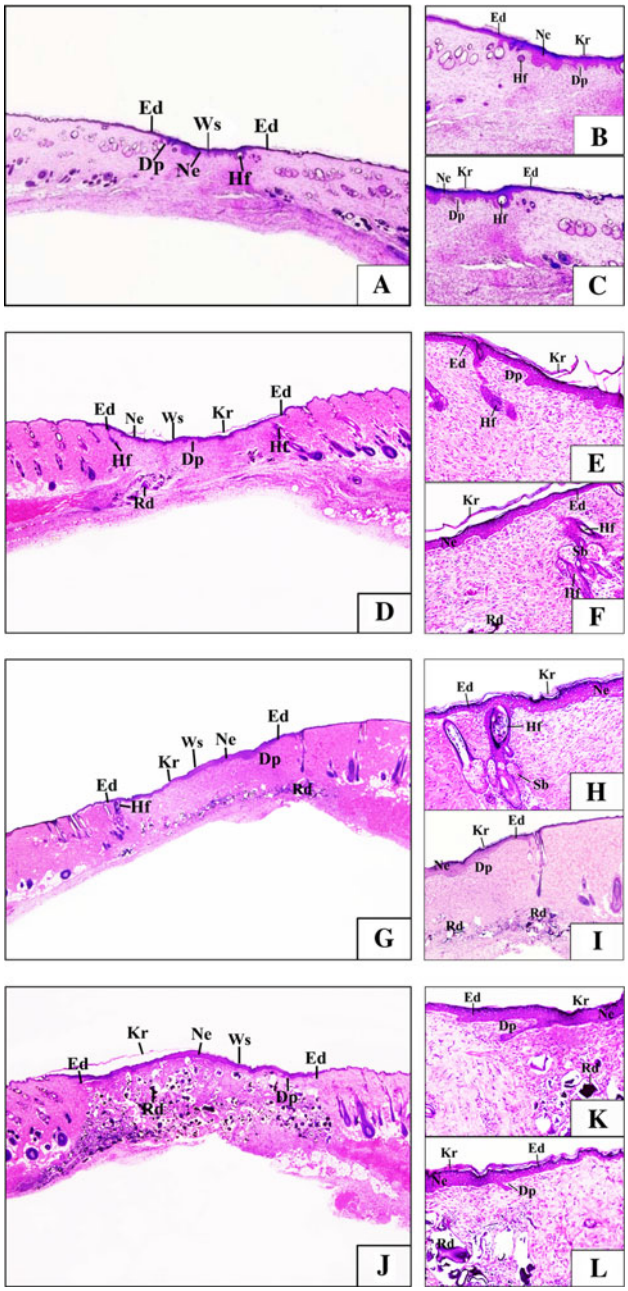

Fig. 3 Histology of skin wounds on day 18 dressed with Chitin-PAA (**a**, **b**, **c**), Chitin-PAA-GTMAC (1:4) (**d**, **e**, **f**), Chitin-PAA-GTMAC (1:10) (**g**, **h**, **i**) and Intrasite (**j**, **k**, **l**). The overall surface of the wound ×1.25 (**a**, **d**, **g**, **j**); the left wound edge ×4 (**b**) ×10 (**e**, **h**, **k**); the right wound edge ×4 (**c**, **i**) ×10 (**f**, **l**). Wound edge (*Ed*); wound surface (*Ws*); dermal papillae (*Dp*); keratin (*Kr*); hair follicles (*Hf*); residue (*Rd*); new epidermis (*Ne*); sebaceous gland (*Sb*). (H&E stain)

From the histological finding, the GTMAC incorporated dressing was evidenced to improve wound healing.

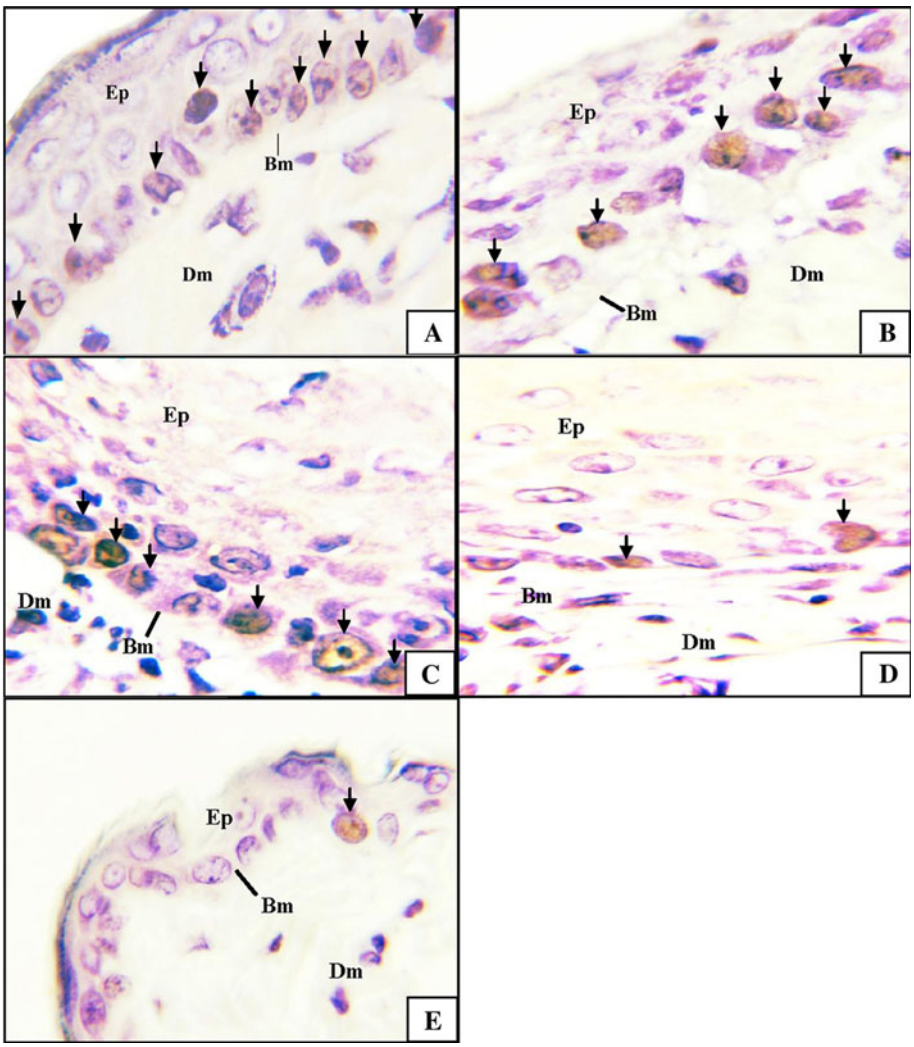

Fig. 4 Wound sections on day 9 dressed with Chitin-PAA (**a**), Chitin-PAA-GTMAC (1:4) (**b**), Chitin-PAA-GTMAC (1:10) (**c**), Intrasite (**d**) and normal skin section (**e**) stained by immunohistochemistry of PCNA. Epidermis (*Ep*); basement membrane (*Bm*); dermis (*Dm*); PCNA-positive cell (*arrow head*)

The newly formed epidermis of wounds dressed with chitin-PAA-GTMAC both (1:4) and (1:10) showed a well organized structure with the formation of hair follicles and sebaceous glands since day 15 which progressed faster than the chitin-PAA dressed wounds. It implied the possibility of GTMAC in stimulating the wounds to re-epithelize. The stage of epithelization that appeared earliest for the chitin-PAA-GTMAC (1:4) dressed wounds suggested the most promising of this material for the application in full thickness skin wound.

3.3 Immunohistochemistry of PCNA

The efficiency of chitin-PAA-GTMAC dressing on the epithelization was confirmed by utilizing the indirect immunoperoxidase staining technique for detection of the PCNA-positive cells. It had been reported that the PCNA expression was closely related to the cell migration, and it could be used as an indication of the cell proliferation in the wound healing process [11, 23, 24]. The PCNA-positive cells could also be observed in normal skin that undergoing self-renewal and proliferation of epidermal cells. In this study, the average percentages of the PCNA-positives cells were found in wounds in the greater amount than in normal skin for the whole period of study as seen in Fig. 4. It indicated the proliferation of epidermal cells in the wound tissue was more extensive compared with those in the normal skin.

The average percentages of PCNA-positive cells of the wounds dressed with chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10), and Intrasite are compared in Fig. 5. The number of PCNA-positive cells of Intrasite dressed wounds was significantly lowest among the test groups during the whole period of study. There were no differences among the average percentages of PCNA-positive cells of the wounds dressed with chitin-PAA, chitin-PAA-GTMAC (1:4) and (1:10) except on days 7, 9 and 12. On days 7 and 9, the average percentages of the PCNA-positive cells of chitin-PAA-GTMAC (1:4) dressed wounds were found significantly higher than those of chitin-PAA-GTMAC

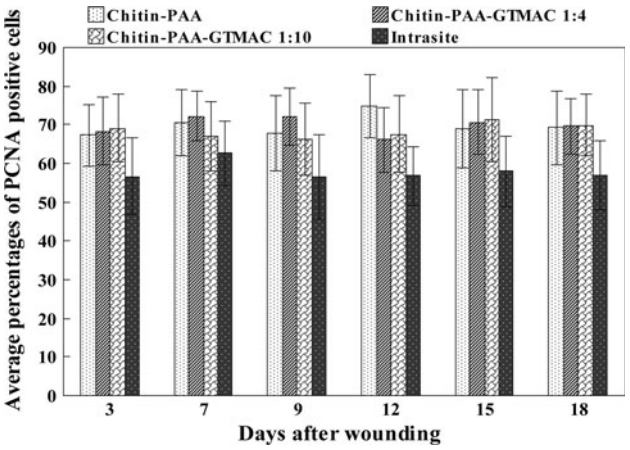

Fig. 5 Comparison of the average percentages of PCNA-positive cells of the wounds dressed with Chitin-PAA, Chitin-PAA-GTMAC(1:4), Chitin-PAA-GTMAC(1:10) and Intrasite

(1:10). On day 12, the highest number of PCNA-positive cells was observed in the chitin-PAA dressed wound.

The PCNA findings in this study suggested the better wound healing efficiency of chitin derivatives than Intrasite. The effect of GTMAC on promoting wound healing was unclear since the number of PCNA-positive cells among different chitin derivatives was likely comparable. However, the statistical analysis of PCNA-positive cells on days 7 and 9 suggested the greater effect on stimulating the proliferation of epidermal cells of chitin-PAA-GTMAC (1:4) than chitin-PAA-GTMAC (1:10). These results were correspondent to the histological findings that observed the most advanced epidermal development of chitin-PAA-GTMAC (1:4) among the test groups on day 9. Thus, it confirmed the enhanced wound healing rate and the earliest epidermal development of chitin-PAA-GTMAC (1:4).

4 Conclusions

The effect of incorporating GTMAC to chitin-PAA gel on wound healing efficiency was investigated and compared with the commercial gel, Intrasite. The wound healing ability was assessed by the extent of wound size reduction, histological observation and immunohistochemical technique of PCNA. All results demonstrated the higher efficiency in wound healing of chitin derivatives either with or without GTMAC than Intrasite. The evidences in promoting the re-epithelization and accelerating the proliferative phase of GTMAC incorporated gel were revealed under histological and immunohistochemical observations. The wounds dressed with chitin-PAA-GTMAC (1:4) displayed the most advanced epidermal development among the tested materials. The finding of PCNA-positive cells in the wounds confirmed the epithelization that was early performed in the wounds dressed with chitin-PAA-GTMAC (1:4) and suggested its high potential in promoting wound healing.

Acknowledgments This research work was financially supported by National Metal and Materials Technology Center, Thailand.

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