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Expression of Human Epidermal Growth Factor in *Escherichia coli* by Intein Approach

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Abstract

Different approaches are used to express recombinant proteins without the requirement of post-translational modification in *Escherichia coli* (*E. coli*). Though *E. coli* may have a drawback in producing endotoxin, its short division time and high expression of the final product are significant in making use as a host to produce various recombinant proteins. Inteins have been discovered in multiples microorganisms in facilitating the expression of homologous proteins and it has been used as one of the crucial tools for the production of recombinant proteins due to its unique feature in auto-excising its fusion partner, which also known as exteins. In this communication, we employed a well-established gp41-1 mini-intein to facilitate the expression of epidermal growth factor (EGF). The study revealed that though the epidermal growth factor cannot be excised from the gp41-1 mini intein during the expression, it showed the capability of gp41-1 mini intein in processing intracellular expression of soluble EGF fusion protein. Different conditions for inducing the cleavage of exteins from inteins has been studied by many research groups, and reducing condition by using the DTT works well on the C-terminal cleavage of EGF from the gp41-1 mini intein. The final purified, different concentration of EGF was mixed with homemade aqueous cream and showed to be highly active in accelerating the healing rate of patients suffering from bedsores, diabetic foot ulcers and skin rupture.

Keywords: Epidermal Growth Factor (EGF) • Inteins • Healing

Introduction

Epidermal growth factor (EGF) is a 53 amino acid oligopeptide with three disulfide bonds, which has been discovered 60 years ago. EGF can bind on the EGF receptor; thereby activate the downstream signalling transduction cascade in triggering the proliferation of epidermal cells. It has been shown that EGF works well not only in the improvement of wound healing, but also participates in various physiological pathways like tissue and bone regeneration [1-4].

Epidermal growth factor has a high affinity towards epidermal growth factor receptor (EGFR). The EGFR is located at the cell surface, which are inactive in monomeric form. Upon binding to EGF, the monomeric form undergoes transition into homodimers which activates the tyrosine kinase domain and downstream pathways. This signalling of EGFR is essential in skin re-construction. The activation of EGFR promoted the following cellular responses: migration, proliferation, cytoprotection and epithelial-mesenchymal transition [5].

Since EGF has multiples valuable biological functions, it has been used in the treatment of hard-to-heal wounds, including diabetic foot ulcers and bedsores [6-8]. EGF can also stimulate the production of collagen and elastin, which further extend its application on cosmeceutical use [9,10]. Though there are various applications of EGF, the commercial utilization of EGF is very low due to the high cost in extraction of adequate amount in natural hosts with only a low abundance of EGF.

Many groups have tried to produce EGF using the recombinant technology via different approaches, including synthesis, intracellular expression and secretion, in order to expand its application on medical or cosmeceutical industry [11-13]. However, only fusion protein may result because of the failure to remove signal peptides or affinity tags.

Our group has been concentrated on employing different inteins to produce various recombinant proteins, including but not limited to interleukin 3, interleukin 4 receptor, interleukin 6, stem cell factor (manuscript in preparation) and basic fibroblast growth factor as well as focusing on *in vitro* translation (IVT) system for expressing recombinant proteins [14]. Our group has been searching for different ways, especially inteins and IVT system, to produce soluble and human bio-identical EGF from bacterial or mammalian host. Some of the group also tried to employ intein approach in facilitating the expression of EGF, however, only insoluble EGF fusion proteins was obtained and protein refolding is needed during the purification of EGF [15]. The protein conformation may be incorrect during the refolding process and eventually greatly affect the bioactivity of the EGF [16]. In this study, we aimed to produce soluble EGF via intein mediated system by controlling the C-terminal cleavage of chosen intein or ideally by auto-cleavage without the need of protein refolding process. A GST tag was fused on the N-terminus of chosen intein, gp41-1, to facilitate the downstream purification and also, increase the solubility of EGF fusion protein. A strong T7 promoter was employed to initiate the transcription and improving the yield of soluble EGF fusion protein. The result was satisfactory under IPTG induction at low temperature. No significant change was found in upscaling the production of fusion from 1 L shake flask to 45 L fermentation. EGF was shown to be able to separate from the fusion protein under incubation with low concentration of DTT in natural pH by performing the on-column cleavage. The mitogenicity result also showed that the purified EGF was showed to be highly bioactive.

To explore the effectiveness of EGF, various concentration of EGF with the addition of bFGF was added into homemade aqueous cream in treating patients with different levels of skin ruptures. EGF was proven to be effective in treating patients with bedsores, skin injuries and diabetics in a short period. Our study may further enable and promote the use of EGF in both medical and cosmeceutical sector.

Materials and Methods

Bacterial strains and chemicals

E. coli strain, DH5α and T7 express, and restriction enzymes were

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purchased from New England Biolabs (Ipswich, MA). The synthetic DNA fragments and antibody against EGF were purchased from Thermo Fisher Scientific (Ipswich, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Construction of EGF expression vector

The expression construct, pET42a (+)-GST-gp41-1-EGF, was constructed in the following way. A synthetic DNA fragment, encoding the sequence of NotI-stop codon-EGF-gp41-1-thrombin site-SpeI from 5' to 3' end, was synthesized by Thermo Fisher Scientific. The synthetic DNA fragment was amplified by PCR extension using the forward primer 5'- AAAAAGCGGCCGCTTAGCGCAGTTC-3' and backward primer 5'-AAAAAACTAGTCTGGTGCCACGCGGTAGTT-3'. The amplified PCR product was purified by Axygen AxyPrep PCR Clean-Up Kit and digested with NotI and SpeI. The digested fragment was purified from 1% agarose and ligated into pET42a (+) digested with the same restriction enzymes. The plasmid sequence of pET42a (+)-GST-gp41-1-EGF was confirmed by Sanger sequencing.

Expression of EGF fusion protein in shake flask

The plasmid, pET42a (+)-GST-gp41-1-EGF, was transformed into the T7 Express. A single colony of transformant was grown at 37°C, (with rotations at 250 rpm) in 1 L LB medium supplemented with 40 µg ml^{-1} of kanamycin. When the A_{600} value reached 0.5, the growth temperature was reduced to 16°C and a final concentration of 0.1 mM IPTG was added. The culture was allowed to grow overnight. 1 ml cell pellet was collected and resuspended in 200 µl of resuspension buffer (50 mM Tris-Cl, 200 mM EDTA, pH 8.0) supplemented with 1x PMSF, aprotinin, benzamidine and leupeptin, followed by incubation on ice for 5 min. The mixture was then treated with 120 µl of lysozyme solution (1 mg mL^{-1}) at 37°C for 20 min. 80 µl of lysis buffer (10 mM EDTA, 10% Triton X-100, and 50 mM Tris-Cl, pH 8.0) was then added. The tube with solutions was inverted gently, followed by centrifugation at 14,800 rpm for 5 min. The cell lysate samples were analyzed by Western blotting.

Up-scaling expression of EGF fusion protein in fermentor

The T7 Express, pET42a (+)-GST-gp41-1-EGF transformant was grown at 37°C, (with rotations at 250 rpm) in 1 L LB medium supplemented with 40 µg ml^{-1} of kanamycin. When the A_{600} value reached 1, the entire volume of culture was subculture into a 50 L fermentor containing 44 L LB medium. The culture was allowed to grow at 37°C, (with rotations at 100 rpm, 1.5 vvm) until A_{600} value reached 0.5, the growth temperature was reduced to 16°C and a final concentration of 0.1 mM IPTG was added. The culture was allowed to grow overnight with 1 M H_2SO_4 and 1 M NaOH in maintaining the pH at 7.0. Cell pellet was harvested by continuous centrifugation and washed with buffer A (1x PBS with 1x PMSF, aprotinin, benzamidine and leupeptin) two times prior to long term storage

Protein purification and inducted cleavage of EGF

The harvested cell pellet was resuspended in 400 ml buffer A. Resuspension was lysed by sonication (10s sonication with 30s intervals for 30x), and then centrifuged at 10,000 rpm for 30 min. Supernatants were clarified and loaded onto the glutathione agarose 4B resin column, followed by washing with buffer A for 10 bed volume. The induction buffer, buffer C (50 mM Tris–Cl, 1 mM EDTA, 300 mM NaCl, 2 mM DTT, pH 8) was added onto the column and allowed incubation at room temperature for 24 hours. Elution was done by adding 3 bed volumes of buffer C. Eluates were saved for Western blot analysis and dialyzed with 0.1x PBS before lyophilization for other purposes.

Biological assays of EGF

The mitogenic effects of reconstituted EGF on the proliferation of NIH/3T3 fibroblast cells were analyzed by the MTT assay [14-16].

Source of bFGF

Transfection, purification and bioassay of human basic FGF were done following Kwong et. al protocol as described previously [14-16].

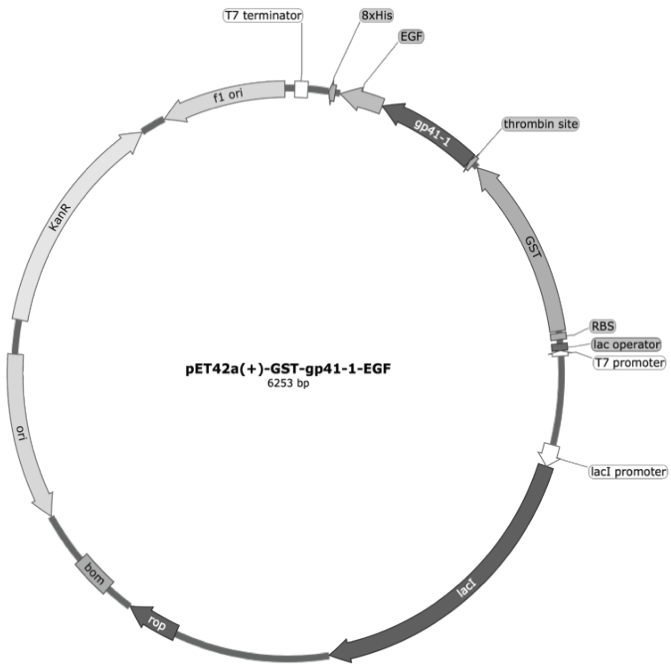
Preparation of rejuvenating cream and directions

Different concentration of lyophilized EGF and bFGF [14], (1) 0.005% EGF, (2) 0.02% EGF and 0.00015% bFGF, (3) 0.04% EGF and 0.0003% bFGF, were mixed with homemade aqueous cream (aqua, phenoxyethanol, cetostearyl alcohol, ceteareth-20, liquid paraffin and white soft paraffin) for 10 mins. The mixed cream was topically applied to various wounds, including bedsore, wound and diabetic foot ulcers of different stages. All treatments were carried at patients' home. Wounds included diabetic foot ulcers, bedsores, laceration, etc. The wounded area was cleaned completely following the Hong Kong Hospital Authority procedures. In brief, 1) clean the wound area thoroughly, 2) apply a small amount of cream to the disinfected area 3) apply sterile adhesive dressing. 4) monitor the healing process closely. Repeat step 1) to 4) until the wounds were fully healed. The aforesaid procedures are repeated twice daily.

Results and Discussion

To maximize both expression and solubility of the fusion protein, pET42a (+) vector with the T7 promoter and N-terminus GST tag was chosen to be the plasmid backbone for expression of the EGF fusion protein. The insert, encoding the gp41-1 and EGF, was synthesized and further amplified by PCR extension. A stop codon was inserted right after the coding sequence of EGF to prevent the translation of downstream C-terminus 8x His embedded in the vector backbone. The GST tag was designed to fuse on the N-terminus of gp41-1 for the purification of the whole fusion protein after expression, while EGF was fused on the C-terminus of a well-studied gp41-1 mini intein, in which the C-terminus cleavage can be done by addition low concentration of DTT. A relatively low induction temperature and low concentration of IPTG was selected to enhance the solubility of EGF fusion protein further Results showed that construct pET42a (+)-GST-gp41-1-EGF (Figure 1) expressed a high level of soluble fusion protein under low temperature and low concentration of IPTG in both shake flask and fermentative scale, with no significant difference in expression level was found (Figure 2).

For purification of EGF fusion protein, the harvested cell pellet was first lyzed and the cell lysate samples were subsequently loaded on the glutathione agarose 4B. EGF fusion proteins with GST tag could be specifically captured while non-specific bound protein was washed off by continuous washing of buffer A. Results showed that under low pH conditions, the EGF C-extein, could be effectively cleaved off from the gp41-1 mini intein after prolonged



Note: This figure shows plasmid constructs vector (6.2 kb) expressing GST-gp41-1-EGF insert cassettes. Symbols for the genetic components shown are: *ori* = origin of replication in *E. coli*; *KanR* = kanamycin resistance gene; *GST* = glutathione S-transferase tag; *gp41-1* = gp41-1 intein; *EGF* = EGF gene. Arrows indicate directions of gene expression.

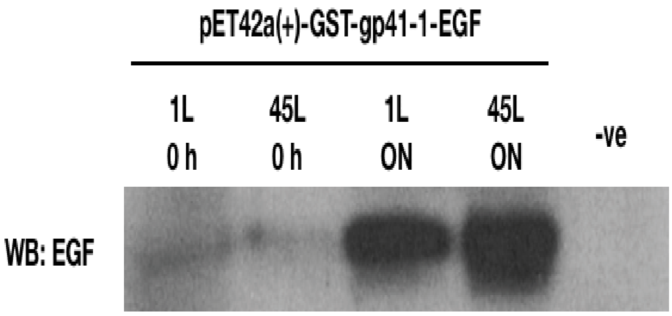
Figure 1. Schematic representation of DNA constructs expressing GST-gp41-1-EGF.

incubation (Figure 3). The obtained EGF was dialyzed by low salt buffer with neutral pH and lyophilized to prolong its storage time.

Since EGF is able to trigger the cell proliferation by binding on cell EGF receptor, same approach in examination of the mitogenicity of EGF on NIH/3T3 cells like bFGF was carried out. From the MTT assay result, the reconstituted EGF was observed to be biologically active in triggering the cell proliferation of NIH/3T3 cells (Figure 4). Apart from *in vitro* biological assay, clinical case studies were also carried out. We prepared homemade aqueous cream with the addition of various concentrations of EGF and bFGF expressed previously by our group in treating patients suffered from various skin ruptures as described in Materials and methods. Results showed that our expressed EGF could effectively enhance the healing process of patients with bedsores, epidermal wound as well as diabetic foot ulcers (Figures 5-9). All the participants were recruited by DreamTec Limited and all figures were approved for publication used. The case studies are shown in the following:

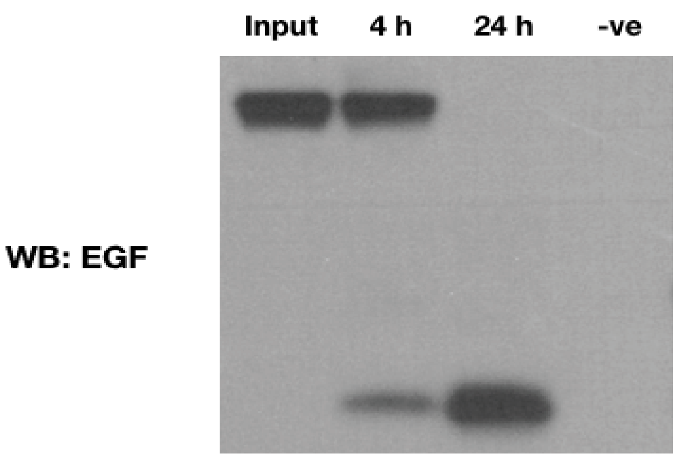
Treatment for pressure wound

Depending on the wound size, a wound typically takes a few weeks to heal. For a wound take longer time than normal one to heal, it is defined as hard-to-heal wound. Poor circulation, infection, aging, mobility dysfunctions, oedema, and repetitive trauma to wound area are the typical reasons for causing hard-to-heal wound. A 83 years old woman suffering from bedsores (Figure 5A) was treated with 0.005% EGF aqueous cream (1). A promising result is shown when treatment started at the early stage of pressure wound development. Indeed, the wound condition might take a turn for the worse but



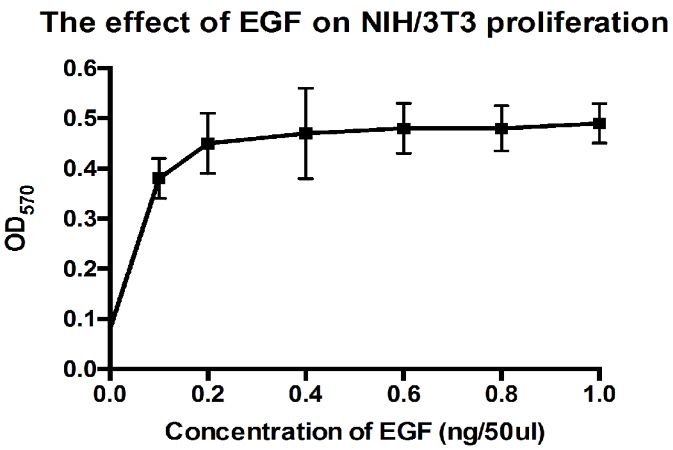
Note: pET42a(+)-GST-gp41-1-EGF transformant post IPTG induction samples collected from different time intervals were analyzed. Lanes 1L 0 h, 45L 0 h, 1L ON, 45L ON : samples collected from cultures induced for 0 h and overnight from 1L and 45L culture respectively, each lane loaded with 10 µl of cell lysate; Lanes -ve: 10 µl of cell lysate from pET42a(+) vector cultures induced for overnight. The Western blot analysis was triplicated.

Figure 2. Western blot analysis of soluble EGF fusion protein.



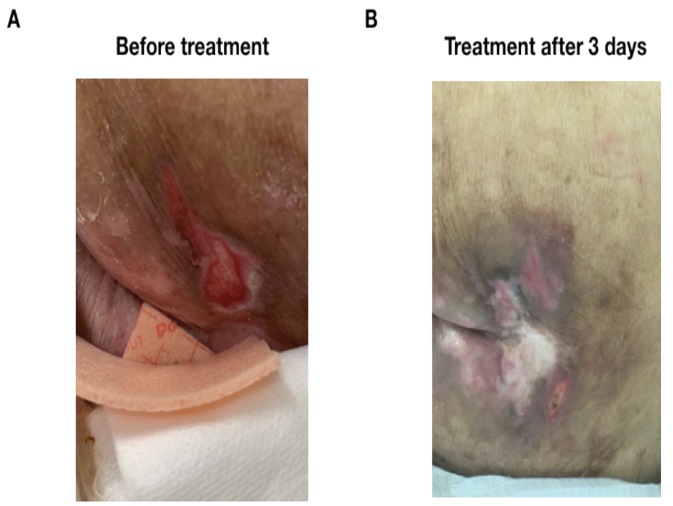
Note: The efficacy of on-column cleavage of EGF from the fusion protein were analyzed. Lanes Input: 10 µl of cell lysate samples collected from the 45L cultures; Lanes 4 h, 24 h: eluate collected after incubation of buffer C after 4 h and 24 h respectively; Lanes -ve: 10 µl of cell lysate from pET42a(+) vector cultures induced for overnight. The Western blot analysis was triplicated.

Figure 3. Western blot analysis of on-column cleavage of EGF.



Note: Mitogenic effects exhibited by different concentrations of reconstituted EGF sample (—■—) is shown. The activity assay was triplicated and standard error bars are shown.

Figure 4. Mitogenicity of EGF.



Note: Mitogenic effects exhibited by different concentrations of reconstituted EGF sample (—■—) is shown. The activity assay was triplicated and standard error bars are shown.

Figure 5. Treatment of bedsore by aqueous cream. (A) A 83 years old woman suffered from bedsores (B) The wound was completely healed after 3 days of treatment by aqueous cream.

fortunately received the treatment at early stage, the bedsore was completely healed in around 3 days (Figure 5B).

Treatment for laceration

Scalp laceration (SL) is one of the most common head injuries. Polytraumatized patients often missed and overlooked by clinicians; moreover, there are less sensitive sensory nerves at scalp skin, which would contribute to worsening the symptoms. The epidermal of a two years old kid's head was accidentally injured. The kid was immediately treated with 0.02% EGF and 0.00015% bFGF aqueous cream (2) (Figure 6A). Bleeding was stopped right after treating aqueous cream (2) for 5 mins and clots were formed after an hour (Figure 6B). The formation of scabs happened after 12 hours (Figure 6C) and after two weeks of treatment, the wounds were completely healed without scarring (Figure 6D).

Treatment for diabetic foot ulcers

One of the common complications of diabetes patients is diabetic foot ulcer (DFU). Vascular and neuropathic complications are the main cause of DFU. Patients with DFU usually lead to longer hospitalization period. It affected not only patients' life quality but also a functional decline in terms of social, physical and psychological.

Figure 6. Treatment of skin rupture by aqueous cream. (A) A 2 years old kid's head was injured and immediately treated with aqueous cream (2). (B) Clots formed in an hour after treatment. (C) Scabs formed in 12 hours after injured. (D) Wounds healed completely without the formation of scars.

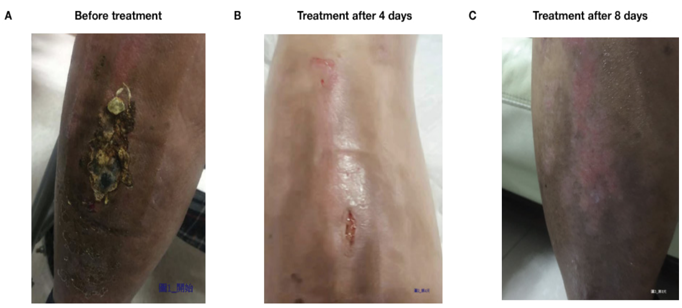


Figure 7. Treatment of diabetic foot ulcer by aqueous cream. (A) A 76 years old man suffered from diabetic foot ulcer for 3 months. (B) Treatment by aqueous cream after 4 days (3). (C) After 8 days treatment, the wound was completely closed.

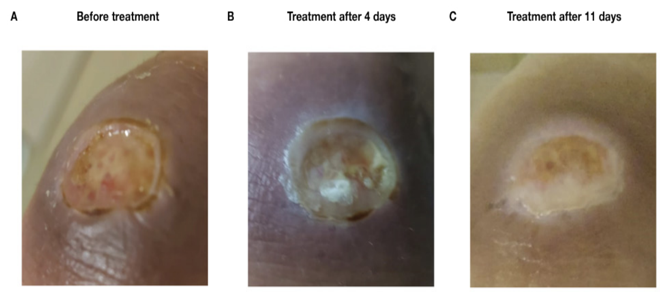


Figure 8. Treatment of diabetic foot ulcer by aqueous cream. (A) A male patient suffered from diabetic ulcerated wound on his lower right leg. (B) Treatment by aqueous cream after 4 days (1). (C) After 11 days treatment, the wound was completely healed.

Under the treatment of growth factor cream, the recovery rates of DFU were massively enhanced within different patients. A 76 years old man suffered from diabetic foot ulcer for three months (Figure 7A). The patient was then treated with 0.04% EGF and 0.0003% bFGF aqueous cream (3). After four days of treatment, the size of wound significantly reduced (Figure 7A). The treatment was continued for four more days and the wound was completely closed (Figure 7B).

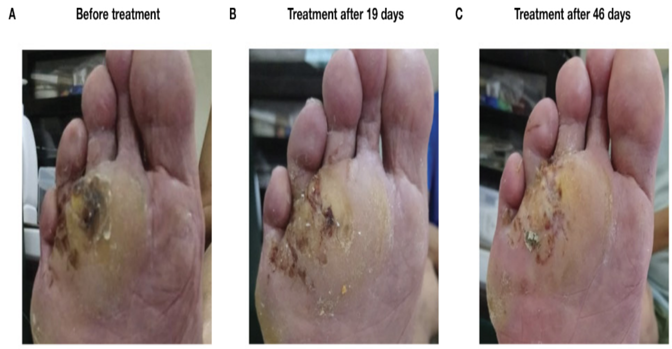


Figure 9. Treatment of diabetic foot ulcer by aqueous cream. (A) A male patient suffered from diabetic ulcerated wound on his left forefoot. (B) Treatment by aqueous cream after 19 days (1). (C) After 46 days treatment, the wound was 75% healed.

A male patient with diabetic ulcerated wound on left knee was received 0.005% EGF aqueous cream (1) as a treatment (Figure 8A). After treatment for around 11 days, a complete healing of wound was reached (Figures 8B and 8C). While a male patient with diabetic ulcerated wound on left forefoot was also received 0.005% EGF aqueous cream (1) as a treatment (Figure 9A). Patient received treatment for 19 days, a noticeable improvement of wound is found on fore foot. It took around 46 days for wound to have 75% healed (Figures 9B and 9C). The result signified the healing rate of DFU utilizing the combination of bFGF and EGF would dramatically enhance.

Conclusion

From the results of the above case studies, the EGF purified from the fusion protein was proven to be highly active. Our findings prove that the final reconstituted EGF obtained by intein approach is bioactive in treating patients with different skin injuries, though the healing process may highly depends on the physiological conditions of the tested subject. More participants may be recruited for investigating the efficiency of EGF in treating different skin injuries in the near future to minimize the variables between different subjects.

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