

Original Research Article

Enhancement of Wound Healing by Topical Application of Epidermal Growth Factor in Animal Model**Abstract**

Wound healing is a complex process of biological events involving re-epithelialization and granulation that are mainly mediated by several endogenously released growth factors such as epidermal growth factor. This work was undertaken to study the effects of various doses of locally applied recombinant epidermal growth factor (rhEGF) on wound healing in rats. In the test groups, rats were given daily with a solution containing 2, 5, 10, 50, 100 µg of EGF spray and 40 µg of EGF ointment. We presented evidence that a significant decreased healing time in wound was observed in all rhEGF groups when compared with the control, and reach to its maximal efficacy at 10 µg/ml of rhEGF spray. The rate of wound closure was over 50 per cent at initial 3 days of treatment. Treatment with rhEGF significantly decreased the length of time to over 50 per cent healing by approximately 4-5 days, and that to 70 per cent and 90 per cent healing by approximately 3-4 days and 3 days, respectively. A stimulatory, dose-dependent effect of EGF on wound healing was observed with increased rhEGF concentration. In toxicological test group, a dose of single 200 µg or 300 µg within 24 hours of subcutaneous and intramuscular rhEGF injection was given respectively, there were no significant adverse side effects. We recommended a proposal of clinical drug doses at 2 µg, 5 µg and 10 µg/ml of rhEGF spray, and 10 µg and even higher 40 µg rhEGF/g of ointment. The results indicated that prepared rhEGF by our group is safe, and is emerging in clinical effective use in assisting wound healing time.

Keywords: Wound healing recombinant epidermal growth factor Animal wound model

Introduction

Epidermal growth factor (EGF) is a broad variety of cellular regulator. EGF was firstly isolated by Cohen S(1) from a mouse submaxillary gland, and further purified mouse EGF. Its amino acid sequences was found to the relationship to urogastrone (2,3). EGF is a single polypeptide chain of 53 amino acid residues and contain these intramolecular disulfide bonds that are required for biological activity. EGF is a heat stable protein and were destroyed during the isolation procedure by boiling. EGF could be still shown its biological activity at storage in room temperature for 2 years(4)(Chen LQ's data in Chinese, 2008). It has been reported that EGF promotes the proliferation and differentiation of cultured cells originated from ectoderm and mesodermal layers, such as epidermal cells, fibroblasts, myofibroblasts, keratocytes, corneal epithelial cells and angiogenesis, and a key importance on maintenance and impact organ development and a cascade of cellular events. In recent, It has been suggested that EGF could be beneficial in burn(5-7), wound healing(8), diabetic foot ulcers(9-11) and digestive ulcer(12), and provide an attractive

perspective. In addition, cosmetic containing EGF was found to be effective to improve the plasticity, to remove wrinkle, to show whitening and anti-aging, and control of erythem amount and sebum amount on the human skin care. In local district, Zhu G in this field has successfully prepared a series of 53 bottles of Shampo liquid (New Wash) into market, and responder rate with satisfied or perfect satisfied over 95 per cent. Actually, EGF is the secreted protein by epidermal cells in epidermis. Based on these data, the present work was undertaken to study the effects of various doses of locally applied EGF on rat wounds.

Materials and Methods

1.1. Wound model

10 female Kunming(KM) strain rats (age 8 weeks, body weight 30-35g) were purchased from Hunan SJA Laboratory Animal Co., Ltd, Changsha, Hunan. The dorsal skin of the rats was sterilized with iodophor and dorsal hair was shaved with clippers. An incision, 2cm length x 1.5cm width in diameter was made in the dorsal midline at the caudal portion of the back using medical scissor and forceps. After full-thickness wound created on the back of the female back, wound sites were photographed daily. Wound area was measured with a ruler on day 0, 1, 2, 3, 4, 7, 8, 10, 13, 15 after initial treatment. The surface area of the wound was calculated using the formula for calculation of the regular geometric figure that best approximated to the wound shape ($area = a \times b$), where a and b represent the perpendicular length and width dimension, respectively. During the experiments, the animals received a normal diet and water *ad libitum*, and were housed individually in cages in animal quarter. All animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, Nanjing, China (OB1409).

1.2. Preparation of recombinant epidermal growth factor(rhEGF)

rhEGF used in the study was obtained as a donation from Prof. Zhi QW(13) in Institute of pharmacology and toxicology, academy of military medical sciences, Beijing. Using *Bam HI* and *EcoR I* restriction enzymes, by genetic engineering(13-17) in 2002-2004 period of previous prepared methods, hEGFcDNA was cloned into pGEX-4T-1 plasmid system, pGEX-4T-1-hEGF prokaryotic expression vector bearing a tac promoter was constructed and production of human epidermal growth factor by *Escherichia Coli* BL21 cells containing the pGEX-4T-1-hEGF recombinant plasmid. The expression product was partially maintained in form of inclusion body. After the supernatant of cell lysate subjected to Glutathione Sepharose 4B affinity chromatography, the purified rhEGF was obtained (figure 1), and its biological activity was detected by MTT methods. In the next step, the drug formulation was prepared by manufacturer Dr. Zhu G in this study at dose of 2, 5, 10ug of rhEGF spray, and 10 and 40ug rhEGF/g of ointment. The rhEGF dose (10ug/g) was selected according to the therapeutic guideline of Brown's in USA(5) and Wong's in HongKong(11) and in Korea Kwon's(18) previous reports. Accordingly, as Gregory RA(2) described urogastrone earlierly, Dr.

Zhu have also prepared rhEGF injection. Laboratory data: Anti-HIV 0.077. No bacteria growth was shown as to rhEGF spray and liquid Shampo in bacteriological examination.

All animals with a similar-sized wounds were randomly assigned to 7 groups of 10 mice. Group 1 was treated with saline solution twice daily as the control. Group 2-4 was treated with 2、 5、 10ug of rhEGF spray twice daily, respectively. High doses of testing group 5 and 6 received 50 and 100ug/ml of rhEGF spray, 5-10 times its clinical drug dosage, twice daily for 15 days, and then once a day up to one month. Group 7, the drug rhEGF ointment (40ug rhEGF/g) was also administered topically to the animal every 7-12 hour for 15 days and then once daily for next 15 days beginning on the day of the incision. Macroscopic assessment was carried out by an independent observer and recorded. Animal body weights and adverse reaction were recorded after the end of experiments.

1.3. Data collection and statistical healing rate analysis

Initial study data were gathered in table 2 and 3. Percentage wound closure was calculated using the formula:

Wound healing rate = [1- Area of present wound / Area of initial wound] x 100% . Wound healing time and crude healing rate of wound were used to evaluate the efficacy of the treatment. The overall period of experimental procedure including genetic engineering in crude product of rhEGF was beginning from November, 2018 to June 25, 2019, and pharmacological preparation of rhEGF spray and its animal wound test was from August 29, 2019 to January 13, 2020.

Results

2.1. The biological activity of rhEGF

For investigation, to examine the biological activity of 3T3 culture cells to a various dose of rhEGF using MTT methods, it has been uncovered that purified rhEGF spray was a potent stimulation of cell growth and proliferation when added to the cultured 3T3 cells at 6.4ng/ml of rhEGF concentration (table 1A). In homogenous preparation of the same batch drugs, when storage as a frozen solution (-20°C), rhEGF could be shown its potent proliferative activity even at 3.13ng/ml of EGF concentration (table 1B 3). As can be shown from table 1A and B, a dose-dependent cellular stimulation was observed with increased rhEGF concentration. The results indicated that purified rhEGF was proved validly in animal experimental wound model and clinical use. In this study, when we carried out the experiments by room temperature, rhEGF has still been found to maintain obviously its biological activity when stored at 4°C for over 5 months. Therefore, if condition permit, rhEGF drug was preferably selected to stored at 4°C refrigerator.

2.2. rhEGF enhance wound healing

Data of the effect of rhEGF on wound healing parameters were shown in table 2 and 3 and figure 2. After daily application of various doses of rhEGF spray, a significant decreased healing time was observed when compared to the

control. The rate of wound healing in all rhEGF groups was over 50 per cent at initial 3 days of treatment. Treatment with rhEGF significantly decreased the length of time to reach 50 per cent healing by approximately 5 days, and that to 70 per cent and 90 per cent healing by approximately 3-4 days and at least 3 days, respectively. In comparative analysis curve between these rhEGF groups (figure 3), wound healing at 2, 5, 10 µg of rhEGF concentration was generally better effective than that in control, and reach to its maximum efficacy at 10 µg/ml of rhEGF spray (the healing rate: 57.5% at initial 3 days, 69.7% at days 5), while no difference was found as to the effects of rhEGF spray at 10-13 days. A higher dose of 100 µg/ml of rhEGF bid for 5 days appear to produce faster initial healing of the wound compared with control. During the duration of wound closure, a stimulatory effect of local rhEGF daily on granulation tissue formation was observed, accompanied with occasional microvillus hair. In contrast, two wounds were treated with only saline solution as control. There were 3 times of repeated bleeding in local surface of a wound, whereas superficial seeping blood was found in the next day of incision in another mouse wound. This bloody wound was treated with iodophor and hemostasis with cotton ball pressure. The results suggested that rhEGF spray may play an antibiotic role and its possible hemostatic effect.

Surprising finding, the wound site was treated topically with 1% silver-sulfadiazine cream containing EGF (40 µg/ml). It has been noted a significant higher efficacy of wound closure. Wound closure in wound was obviously accelerated at initial 3 days, and at 5 days the most parts of wound were scabbed, whereas scab abscission occurred at 7 days. Therefore, we concluded that the EGF has the ability to become an efficient therapeutic drug for superficial or deep partial-thickness wound in skin.

In throughout experiments of rhEGF spray at doses between 2- 100 µg/ml, no significant adverse side effects has been observed in association with the use of EGF in this study regarding body weight and other (hair) growth-inhibiting effects even the incidence of infection in rats wounds. The complete wound healing including hair-growth was observed at 25-28 days in all experimental rats.

To further test whether rhEGF can be used as EGF saline injection, we carried out the additional experiments of subcutaneous and intramuscular injection of rhEGF solution in 2 rats, respectively. Initial dose of rhEGF was 20 µg daily for 5 days, and escalated to 30 µg daily for 5 days, then 50 µg daily for 5 days, and the final volume 100 µg daily for 5 days. Total dosage of rhEGF was 1 mg within 20 days. In toxicological test, a dosage of single 200 µg or 300 µg/24 hours of subcutaneous and intramuscular rhEGF injection was given respectively in another 4 rats, which 20-150 times its clinical drug at doses between 2-10 µg. As we expected, there was no significant adverse side effects, except that a suspicious subcutaneous nodule was noted in one rat receiving continuous application of rhEGF subcutaneously. Animal behavior of rats after a single injection at dose of 200 µg or at 300 µg/24 hours of rhEGF solution was blunt in movement. The rats recovered their activity within 24 hours, whereas an injection of rhEGF at doses of 400 µg a day, 40-200 times higher

than its use in clinical drug doses, is lethal within 12-24 hours. The results indicated that prepared rhEGF is safe and available in clinical wound healing.

Discussion

In this study, we have demonstrated the potential efficacy of rhEGF as an adjunct to conventional wound repair, which have previously been confirmed by a number of *in vitro* and animal experiments (18,19), even though strain-specific differences in wound healing rates may influence the true effect of EGF in the mouse models. The results are encouraging.

From the dose effects curve, it has been shown that wound healing rates were generally better effects at 2-10ug of rhEGF, and reach to a peak value of 10ug/ml of rhEGF concentration. A comparison of dose-response rates also appears to the length of the best healing time at 10ug/ml of rhEGF. Certainly, daily application of 2 and 5ug of EGF were also available. The results were consistent with others Brown's (5) and in HongKong Wong's (11) observation. Brown and colleagues (5) carried out a large trial efficacy on 12 chronic wounds, they treated their patients initially with silvadene alone, which was ineffective in spite of its antibacterial action, for a period of 3 weeks to 6 months. This was followed by treatment with topical silver sulfadiazine cream containing EGF (10ug/g), the addition of EGF producing a highly significant response. EGF significantly decreased the average length of time to 50 per cent healing by approximately one day and that to 75 per cent and 100 per cent healing by approximately 1.5 days ($p < 0.02$).

The same group (20) in Finland also proved the stimulation of wound healing by EGF in dose-dependent effects. In the test group, wounds were injected daily with a solution containing 0.2, 1 or 5 ug of EGF in 0.1% albumin. EGF significantly increases the accumulation of DNA and RNA, and accumulation of granulation tissue cells, collagen and glycosaminoglycans in experimental rat wounds. Dr. Zhu once experienced a 2.5x 2.5cm brush burn, the wound healed without scar at 5ug of rhEGF spray for 1 week. Indeed recent, my colleague Dr. Tang HL caught a knife wound (2 x 2.5cm) by a table knife accident. The entire epidermis was striped off his forefinger. Blood flowed profusely from his right index finger. The wound healed without scar by the combination of 5ug of rhEGF spray every other day and Yunan Baiyao spread within 20 days. Moreover, in Zhu's personal communication, a bone fracture attack by accident, caused severe swollen on the right leg of an animal chicken. The obvious disappearance of serious swollen was found at 3 days after local use of 5ug/ml of rhEGF spray, and 1 week later, the chicken could go on foot with a broken leg. The wound was gradually healing through the combination of local rhEGF spray and topical EGF-silvadene ointment (10ug/g) for 6 days. The results suggested that rhEGF may play its antibiotic role and its some benefits in a bone fracture

Silver sulfadiazine (Ag-SD) is a useful antibacterial agent for wound treatment. Silver sulfadiazine indicated as an adjunct for the prevention and treatment of wound sepsis in patients with second- and third-degree burns. It is bactericidal

for many gram-negative and gram-positive bacteria. Silver sulfadiazine acts only on the cell membrane and cell wall to produce its bactericidal effect. Silver ions bind to nuclear DNA, nucleophilic amino acids, as well as sulphydryl, amino, phosphate, and carboxyl groups in proteins, causing protein denaturation and enzyme inhibition. Silver binds to surface membranes and proteins, causing proton leaks in the membrane, leading to cell death. However, recent findings(21) indicate that Ag-SD delays the wound-healing process, while the addition of rhEGF could reverse the impairment. Therefore, A drug delivery system containing both EGF and Ag-SD, such as 1% silver sulfadiazine containing EGF(10ug/g) in this study, may be clinically relevant. In the study, we have shown EGF-Silvadene in the dual collaboration of both rhEGF on wound granulation tissue formation and antibacterial of silvadene. EGF-Silvadene ointment significantly decreased the length of healing time within initial 3 days, and that to over 90 per cent scabbed healing by 5 days. But at 1 week scab abscission occurred. Therefore, it is need to cover the wound with sterile gauze after topical rhEGF-silvadene cream, then fixed it with medical tape in order to its better drug efficacy.

Many topics on the mechanism of hEGF action(5,12,22,23). Exogenous EGF could selectively bind to its receptor(EGFR) on cell membrane of keratocytes and fibroblasts in skin, and EGF induce initiation of DNA synthesis, activation of RNA and protein synthesis, and activation of the synthesis of extracellular macromolecules. It was reported that interaction between rhEGF and its receptor has to be maintained for 10-12 hours in order to achieve an effective cellular response in terms of better-organized granulation tissue, a greater DNA and protein content and a higher rate of cell replication(22). Using acetic acid-induced gastric ulcer in rats, Liu and Xu(12) have reported that the numbers of EGF and its receptors obviously increased around ulcer margin at 2 days and reach its increased expression to a peak value at 4 days, and consequentially the peak was decline to a normal pattern. Gu et al (Gu YM's data in chinese) also observed that in health skin EGF and EGFR were weak positive, with its distribution of epithelial basal cells, and afterward the expression in wound became positive at 4 days, and at 10 days the EGF and EGFR were strong positive, with mainly distribution of epithelial basal cells around wound edge, dermal endothelial cells and fibroblasts. In addition to EGF-EGFR complexes, other increased PDGF and hydroxyproline contents were also locally acting factors.

To evaluate the further stimulation of rhEGF in malignant ulcer, *In Vitro* transfection of NIH3T3 with a functional EGF receptor resulted in no significant alteration(0.4% colonies in soft agar) in growth properties(24,25). The same observation was shown that the 32D/EGFR cells which exhibited high levels of functional EGFR remained nontumorigenic during a 2-months period(26). However, EGF addition led to the formation of densely growth tranfected foci($\sim 2 \times 10^2$ ffu/pM of DNA) in liquid culture and colonies(19.7%) in semisolid medium(25). Moreover, isolation of two distinct epithelial cell

line K248C and K248P from a single feline mammary carcinoma with different tumorigenic potential in athymic mice, the K248C cells with amplification of the EGFR gene and elevated levels of RNA and protein were highly tumorigenic. The K248P were poorly tumorigenic(27). An oncogenic receptor EGFRvIII (28-33) which was first identified in primary human glioblastoma was revealed to be capable of abrogating IL-3-dependent pathway with tumorigenic activity(26). Thus, these results were consistent with the autocrine loop model postulated by Professor DF Stern and RA Weinberg (34) at Massachusetts Institute of Technology in which constitutive production of a mitogenic growth stimulatory signals by the EGFR in response to its normal ligand(EGF) or oncogenic mutations in gene encoding EGF receptor constitutively activating EGF receptor can lead to uncontrolled proliferation and cell transformation. EGF receptor overexpression appears to amplify EGF signal transduction.

In Zhu's further communication, in March 28, 2019, he consulted a patient with ulcerative laryngocarcinoma. The patient experienced the chief complaint of cough, dyspnea and severe hoarse voice for over one month duration. A harden 5x7.5cm mass was palpable in his right neck region. After being applied with topical herbal paste by a doctor, the tumor was ulcerative and hemoptysis in sputum. There was a lot of exudate emitted from ulcerative wound, and the clothing neckline was wet almost every day. Meanwhile, the patient felt that exudate of the wound penetrated the lower part of his throat. The ulcer troubling caused him to undergo ulcer healing to improve his symptoms. It is not useful to spread Yunan Baiyao on the wound for more than two months, and other erythromycin ointment was also ineffective. Moreover, the patient has used silver sulfadiazine ointment in another drug store for over one week, and the tumor tissue was bright red. When the patient was admitted to Zhu's clinic in June 8, 2019, the protocol was consisted of the prescription of traditional medicine(TCM). After obtaining his consent, in August 29, 2019, the patient was trying to topical EGF-Silvadene cream(rhEGF at initial 4 ug, then escalated to 7ug/g, twice daily) for 15 days, and then intermittent rhEGF cream use. At the third consultant, the hoarse voice of the patient was obviously improved, and the exudate of the wound was also significantly decreased. A close scrutiny uncovered granulation tissue hyperplasia and three neovascular nodules formation in ulcer wound. These data suggested that in addition to autocrine loop by tumor itself, exogenous rhEGF might accelerate the proliferative activity of malignant cells. Whether EGF can promote the growth of normal cells, accelerating the wound healing or to induce the incidence of tumor, it may depend on cell types of EGF action, internal and external environment and EGF concentration. Thus we are not yet able to draw a clear boundary between normal and abnormal cell growth(23,24,35). At present, because it is not necessary to target the normal receptor of health human cells, commercial antibodies for anti-oncogenic receptors within tumors(also anti-oncogenic receptor Abs)(Fu YX said in his

Yang-xin Fu lab in UT Southwestern Medical Center,Dallas,Texas; Icotinib-DrugBank)(36-47) have been developed available into market. Therefore,like G-CSF、GM-CSF、IGF-I、 growth hormone and sex hormones(eg.estrone and androgen),at least, use of G-CSF and GM-CSF are not without risk(Estey EH,1990). And more, in one rat,a suspicious subcutaneous nodule was noted in the continous application of rhEGF injection subcutaneously. Long term application of EGF should therefore be paid attention to this action,especially in the most destructive trauma,such as a ulcerative cancer.

In the present study,EGF administered by dorsal incision at a dose of 50 and 100ug/ml,which is 5 to 10 times higher than its use in clinical doses,was evaluated for its toxic effects for 30 days in skin. Additional toxicological tests, an injection of single 200ug or 300ug/24hours of subcutaneous and intramuscular rhEGF solution was given respectively. No adverse side effects related to rhEGF was observed. Others, a stable body weight or behaved signs in all groups indicated no obvious toxicity. In literature reports,another toxicological test was applied 6g (120ug/g) rhEGF gelatin to the skin wound for 36 days on the back of a rabbit, no adverse side effects regarding the local skin and organ toxicity was observed(data not shown). Moreover, *in vivo* overexpression of epidermal growth factor in transgenic mice could lead to growth retardation,but no tumor was observed in transgenic animals(48). In our study, we have realized in preparation of rhEGF medicine based on the experiments of an oncogenic pml/retinoic acid receptor alpha fusion (retinoid pharmacology) in APL and an aberrant androgen receptor with its methyltestosterone drug in the induction of breast tumors(29,30), and subsequently the earliest discovery of normal or proto-oncogenic receptor kinase EGF receptor in cell proliferative signalling in wound healing,and in June, 1991 patent application for rhEGF powder and its specific Shampo liquid, and its newly Band-Aids. Here,from the principle of drug action,the EGF receptor to its EGF ligand seemingly compared to 'the key in the lock' . In conclusion, in spite of a number of confounding factors, our results and preliminary clinical trials support the intention that prepared hEGF by our group is more effective therapeutic agent in improving objective parameters of wound healing,and that it may assist in wound healing time.

Conflicts of interest

The author declares that there is no conflicts of interest regarding the publication of this paper

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PBV-EGF: 100 %

10 20 30 40 50 60
MNSDSECLPS HDGYCLHDGV CMYIEALDKY ACNCVVGIG ERCQYRDLKW WELRRRRQR

KKRGY

Fig.1. Amino acid sequence of human epidermal growth factor

EGF ng/mL	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0 (对照)
OD	1.5137	1.4633	1.3657	1.3265	1.2907	1.2936	1.2836	1.2639	1.2131
	1.4446	1.4545	1.3729	1.3686	1.3486	1.3079	1.3151	1.2246	1.2779
	1.4446	1.4388	1.3581	1.3382	1.3242	1.3464	1.2877	1.2611	1.2555
	1.4354	1.4324	1.3450	1.3423	1.3278	1.2865	1.2919	1.2761	1.2278
Mean	1.4596	1.4472	1.3604	1.3439	1.3228	1.3086	1.2945	1.2564	1.2436
INCREASE % Mean±SD	117.37±3.63	116.38±1.4	109.40±1.1	108.07±1.7	106.37±2.4	105.23±2.6	104.10±1.4	101.03±2.2	100.00±2.8
		2	9	8	0	7	1	2	9

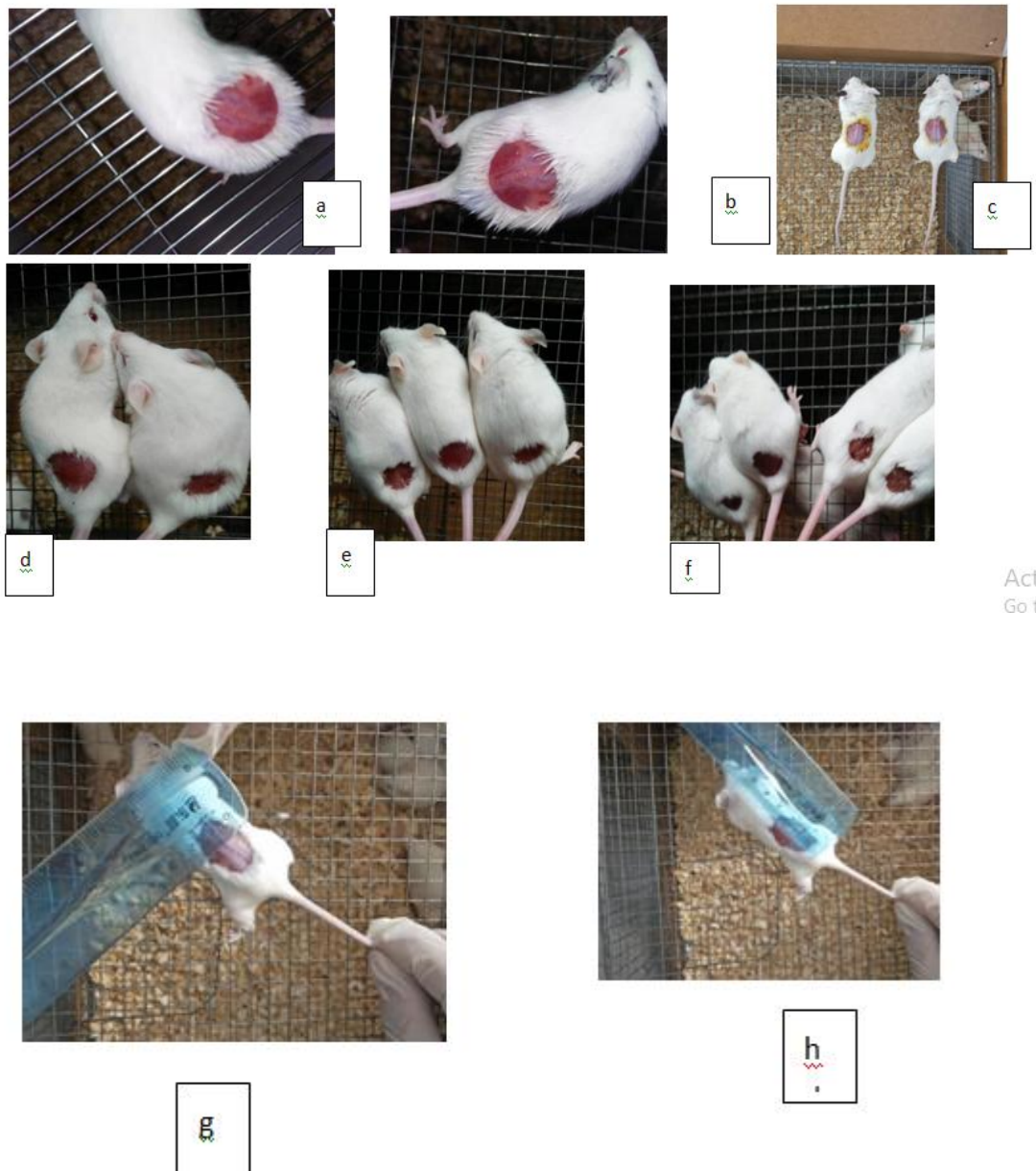
TABLE. 1A THE PROLIFERATIVE ACTIVITY OF 3T3 CULTURE CELLS FOLLOWING A VARIOUS OF RHEGF CONCENTRATION

EGF ng/mL	0	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100
OD	1.2748	1.3291	1.3300	1.3598	1.3473	1.3639	1.3639	1.4124	1.4390	1.4449	1.4692	1.4786
	1.3012	1.3149	1.3218	1.3308	1.3641	1.4027	1.3842	1.4415	1.4471	1.4707	1.4924	1.4837
	1.3123	1.3109	1.3505	1.3711	1.3689	1.3440	1.4013	1.4224	1.4556	1.4580	1.4459	1.4337
Mean	1.2961	1.3183	1.3341	1.3539	1.3601	1.3702	1.3832	1.4254	1.4473	1.4553	1.4692	1.4654
INCREASE %	100.00a	101.71a	102.93a	104.46a	104.93a	105.72a	106.72a	109.98a	111.66a	112.38a	113.35a	113.06a
Means SD	1.93	0.96	1.48	2.08	1.13	2.99	1.87	1.48	0.83	1.36	2.33	2.75

EGF ng/mL	0	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100
OD	1.2665	1.2563	1.2887	1.2848	1.3312	1.2900	1.3220	1.3969	1.4063	1.4191	1.4367	1.4738
	1.2702	1.2520	1.2909	1.3280	1.2841	1.3391	1.4107	1.3777	1.4151	1.4659	1.4879	1.4371
	1.2517	1.2803	1.2676	1.2634	1.3063	1.3308	1.3348	1.3360	1.4202	1.4854	1.4310	1.4307
Mean	1.2628	1.2662	1.2834	1.2921	1.3072	1.3200	1.3562	1.3702	1.4172	1.4301	1.4519	1.4339
INCREASE %	100.00a	100.27a	101.55a	102.31a	103.51a	104.52a	107.39a	108.50a	112.22a	113.25a	114.97a	115.13a
Means SD	0.98	1.42	1.29	3.29	2.36	2.63	4.76	3.12	1.21	3.17	3.13	2.18

EGF ng/mL	0	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100
OD	1.1371	1.1662	1.2044	1.2479	1.2914	1.3405	1.4245	1.4218	1.4245	1.4030	1.4758	1.4822
	1.1813	1.1837	1.1893	1.1549	1.2402	1.2837	1.5614	1.4353	1.4437	1.4659	1.4442	1.4467
	1.1573	1.1803	1.1729	1.2224	1.2543	1.3663	1.3711	1.3935	1.4142	1.4536	1.4575	1.4652
Mean	1.1586	1.1767	1.1889	1.2081	1.2620	1.3302	1.3856	1.4169	1.4271	1.4408	1.4592	1.4647
INCREASE %	100.00a	101.57a	102.62a	104.28a	108.93a	114.81a	119.60a	122.30a	123.18a	124.36a	125.95a	126.42a
Means SD	3.21	0.93	1.58	4.77	2.65	4.22	3.40	2.13	1.44	3.59	1.77	

FIG. 1B. THE PROLIFERATIVE ACTIVITY OF 3T3 CULTURE CELLS FOLLOWING A VARIOUS OF RHEGF CONCENTRATION



Act
Go t

Fig.2. A similar size wound before experiments(a,b,c) in control and test pair groups. After rhEGF treatment, an obvious wound closure in wound was shown at days 2(d, e) and days 5(f). From left to right in figure 1a control; figure 1b,5ug test rat; figure 1c,control(left) and 2ug(right)test rat. In figure 2d and 2e,rhEGF spray accordingly at 10ug, saline solution, or 5ug concentration. In figure 2f, a solution containing 10ug, 5ug, saline and 2ug/ml of rhEGF spray respectively. In figure 2g and 2h, wound area was measured with a ruler.

Table 2. Experimental data of the wound healing time following various doses of rhEGF

[Drug doses(ug/ml)/healing area(cm)/days]

Area(cm2)	mice No.	Days 0	1	2	3	4	7	8	10	13	15
control	2	2x1.5	1.5x1.5	1.5x1.4	1.5x1.3	1.5x1.2	1.4x1.1	1.4x1.0	1.1x0.8	0.7x0.4	0.5x0.3
			25.0%	30.0%	35.0%	40.0%	48.7%	53.3%	70.7%	90.7%	95.0%
		2.0x1.5	1.6x1.5	1.5x1.45	1.4x1.35	1.4x1.3	1.2x1.1	1.1x0.95	1.1x0.85		
			20.0%	27.3%	37.0%	39.3%	56.0%	65.2%	68.8%		
		1.8x1.8	1.7x1.4	1.5x1.2	1.5x1.15	1.5x1.1	1.15x1.0	1.1x0.9	0.9x0.8	0.65x0.5	0.5x0.4
			26.5%	44.4%	46.8%	49.1%	64.5%	69.4%	77.8%	90.0%	93.8%
2ug	1	2.5x1.5	1.5x1.4	1.3x1.3	1.3x1.1	1.3x1.0	1.2x1.0	1.0x0.8	0.7x0.5	0.5x0.3	0.3x0.2
			44.0%	55.0%	61.9%	65.3%	68.0%	78.7%	90.7%	96.0%	98.4%
5ug	2	2.5x2.0	1.8x1.5								
			46.0%								
		2.0x1.5	1.3x1.35	1.3x1.1	1.1x1.1	1.1x1.0	1.0x0.9	0.9x0.8	0.8x0.6	0.4x0.3	0.45x0.2
			41.5%	52.3%	59.7%	63.3%	70.0%	76.0%	84.0%	96.0%	97.0%
10ug	2	2.2x1.5	1.5x1.3	1.3x1.2	1.2x1.0	1.1x1.0	0.9x0.8	0.8x0.6	0.5x0.3	0.3x0.2	0.3x0.2
			40.9%	52.7%	63.6%	66.7%	78.2%	85.5%	95.5%	98.2%	98.2%
		2.0x1.5	1.3x1.45	1.1x1.05	1.1x1.0	0.9x0.9	1.0x0.9	0.7x0.65	0.7x0.6		
			37.2%	61.5%	63.3%	73.0%	70.0%	84.8%	86.0%		
		1.8x1.8	1.5x1.3	1.35x1.0	1.1x0.95	1.1x0.9	1.0x0.75	0.8x0.8	0.7x0.6	0.5x0.4	0.4x0.25
			39.8%	58.3%	67.7%	69.4%	76.9%	80.2%	87.0%	93.8%	96.9%
High Doses Test											
50ug	1	2.0x1.5	1.4x1.3	1.3x1.3	1.3x1.2	1.2x1.1	1.1x0.9	1.05x0.9	0.8x0.5	0.5x0.3	0.4x0.2
			39.3%	43.7%	48.0%	56.0%	67.0%	75.5%	86.7%	95.0%	97.3%
100ug	1	2.0x1.8	1.5x1.45	1.4x1.25	1.2x1.15	1.15x1.0	1.05x0.8	1.0x0.8	0.8x0.6		
			39.6%	51.4%	61.7%	68.1%	76.7%	77.8%	86.7%		
		1.5x1.5	1.1x1.0	1.0x0.9	0.95x0.9	0.9x0.9	0.8x0.7	0.7x0.6	0.6x0.55	0.4x0.3	0.3x0.2
			51.1%	60.0%	62.0%	64.0%	75.1%	81.3%	85.3%	94.7%	96.0%
40ug cream	1	2.0x1.5	1.3x1.1	1.1x1.0	0.8x0.7	0.3x0.3	0.7x0.6	0.8x0.5	0.7x0.4	0.4x0.3	0.3x0.2
			52.3%	63.3%	81.3%	97.0%	86.0%	86.7%	90.7%	96.0%	98.0%

Table 3.The Comparative data of wound healing rate at 2-10ug doses of rhEGF spray(Days(healing per cent)

Group	Exp. No.	Days 1	2	3	4	5	6	7	8	9	10	11	13	15
control	3	23.8	33.9	39.6	42.8	52.1 n=2	53.2 n=2	56.4	62.6	67.2	72.4	84.9 n=1	90.4	94.4
2ug	1	44.0	55.0	61.9	65.3			68.0	78.8		90.7		96.0	98.4
5ug	1	43.8 n=2	52.3	59.7	63.3	63.3	66.7	70.0	76.0	77.8	84.0	92.0	96.0	97.0
10ug	3	39.3	57.5	64.9	69.7	73.4 n=2	74.1 n=2	75.0	83.5	84.5	89.5	92.3 n=1	96.0	97.6
50ug	1	39.3	43.7	48.0	56.0			67.0	77.5		86.7		95.0	97.3
100ug	2	45.4	55.7	61.9	66.1	69.4	72.3	75.9	79.6	84.4	86.0	91.1	94.7	96.7

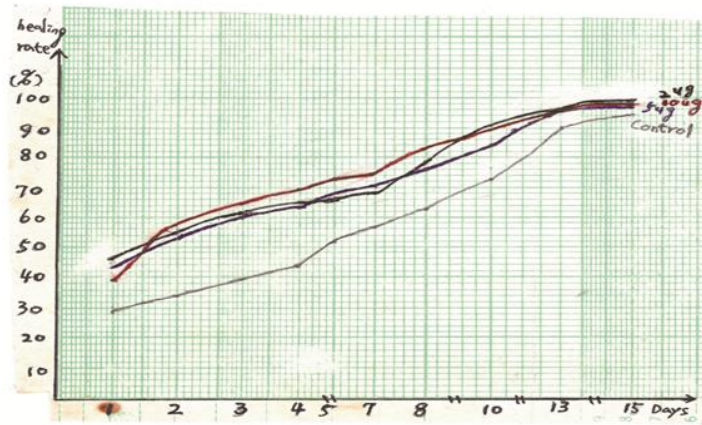


Fig.3.The wound healing rate curve was recorded in various of solution containing 2ug(black), 5ug(purple), 10ug(red) of rhEGF spray. Control received injection of saline solution(pencil color line). An obvious difference between rhEGF spray and control was distinctly shown.

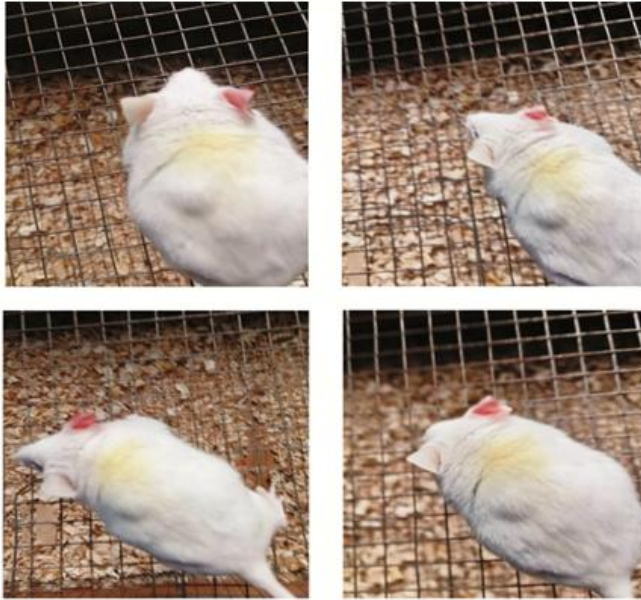


Fig. 4. Photos were taken from different angles. A subcutaneous nodule was clearly shown in the application of continuous subcutaneous rhEGF injection, whereas no sign of nodule formation was observed following intramuscular rhEGF injection in another rat within 20 days. Each rat was injected with a total amount of 1mg of rhEGF solution.

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