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# Electrical Stimulation Promotes Healing Accompanied by NOR in Keratinocytes and IGF-1 mRNA Expression in Skin Wound of Rat

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The purpose of this study was to investigate the effect of the high voltage pulsed current (HVPC) stimulation on the healing rate and the proliferative activity of keratinocytes and IGF-I mRNA expression of an incisional wound in rat skin. Twenty male Sprague-Dawley rats (265~290 g) were randomly divided into HVPC (n=10) and control group (n=10). Rats received 10 mm length of full-thickness incision wound on the back under the anesthesia. The HVPC group received electrical stimulation with a current intensity of 50 V at 100 pps for a duration of 30 minutes, while the control group was given the same treatment without electricity for a week. Polarity was negative in first three days and positive thereafter. The wound length was measured and evaluated as percentage. The mean number of nucleolar organizer regions (NORs) per nucleus and level of IGF-I mRNA expression were calculated. The mean percent of wound closure were 51.17±17.76% and 80.71±11.91%, respectively, in the sham treated control and HVPC stimulated groups ($t$=-4.308, $P$<0.001). The mean NOR number per nucleus of the keratinocytes in the control and HVPC group were 1.85±0.20 and 2.70±0.23, respectively ($t$=8.638, $P$<0.001). The IGF-I mRNA level were 0.76±0.44 and 1.32±0.41, respectively, in the control and HVPC stimulated wounds ($t$=2.906, $P$<0.01). There was a positive correlation between the mean NOR number per nucleus and IGF-1 mRNA level with a Pearson product moment correlation coefficient of 0.72 ($P$<0.05). These findings suggest that the HVPC may activate the rRNA of the basal keratinocytes and upregulate the IGF-I mRNA levels by alteration of the electrical environment, and it may increase proliferative activity of the keratinocytes in the skin wound of the rat.

**Key Words:** Electrical stimulation, Wound healing, Keratinocyte rRNA, IGF-1 mRNA, Rat

## INTRODUCTION

Wound healing is a complex and dynamic biological process of restoring cellular structures and tissue layers. It can be divided into three distinct phases: the inflammatory, the proliferative and the remodeling phase. Each phase has a well organized and coordinated series of physiologic events involving interaction between epidermal and dermal cells, the extracellular matrix, angiogenesis, cytokines and growth factors (Martin, 1997; Harding et al., 2002).

Epithelialization is the resurfacing of a wound with new stratified layer of epidermis. It is an essential step of the wound healing process. Epithelialization of wound begins within hours after injury. One to two days after injury, keratinocytes at the wound margin begin to proliferate and migrate over the wound bed (Singer and Clark, 1999). The extracellular matrix and the cytokines influenced prolifera-

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tion and migration of keratinocytes during epithelialization. Many cytokines such as epidermal growth factor (EGF) family, transforming growth factor (TGF)-β family, insulin-like growth factor (IGF)-I, interleukin (IL)-1 may stimulate the proliferation and migration of keratinocytes during the process of epithelialization (Ando and Jensen, 1993; Haase et al., 2003; Hyde et al., 2004).

In skin, IGF-1, a peptide hormone, produced by dermal fibroblasts, macrophages and epidermal melanocytes (Rappolee et al., 1988; Tavakkol et al., 1999). The IGF-1 plays an important role in epithelialization process, through its ability to stimulate proliferation, attachment, and migration of keratinocytes (Tavakkol et al., 1992). Recent study showed that the deficiency of IGF-1 may contribute to delayed wound healing in diabetic animals (Brown et al., 1997) and patients with diabetes mellitus (Blakytny et al., 2000). Systemic and local administration of IGF-1 may lead to improved wound healing. Systemic application of IGF-I can promote wound healing, although it has adverse effects such as hypoglycemia, electrolyte imbalance, and edema (Pierre et al., 1997). Local application of IGF-I to the wound site increased epithelialization and wound-healing rates (Bhora et al., 1995). Local application of IGF-I can also improve impaired wound healing associated with diabetes (Bitar et al., 1997) or corticosteroid treatment (Suh et al., 1992).

Electrical stimulation have been proposed to be involved in the epithelialization. The normal skin surface maintains negative charge with respect to the deeper epidermal layers. This results in transepidermal potential (TEP) across the skin which act as a skin battery. When skin is broken in the integrity, there will produce a current of injury through the low-resistance pathway of the injured cells and fluid exudate in the wound, creating a negative pole at the center of the wound (Nishimura et al., 1996; Ojingwa and Isseroff, 2003). The endogenous injury current flowing between the normal and injured tissue play an important role in wound healing process (Farboud et al., 2000). The exogenous electrical signals that mimic natural current of injury may regulate the wound healing process. The exogenous electrical stimulation attracts keratinocytes and facilitates proliferation and migration of keratinocytes in the wound healing process (Nishimura et al., 1996; Hinsenkamp et al., 1997). Some investigators have suggested that exogenous electrical signals can upregulate growth factors including IGF-1 in retina (Morimoto et al., 2005), IGF-II in bone cell (Fitzsimmons et al., 1992), transforming growth factor (TGF)-β1 in dermal fibroblasts (Todd et al., 2001), TGF-β1 in skeletal muscle (Ugarte and Brandan, 2006) and vascular endothelial growth factor (VEGF) in osteoblast (Kim et al., 2006). However, the effect of high voltage pulsed current (HVPC) stimulation on rRNA expression of keratinocytes and IGF-I mRNA expression in skin wound have not been demonstrated.

The purpose of this study was to investigate the effect of the HVPC stimulation on the healing rate of a full-thickness incisional wound in rat skin. We also determined the proliferative activity of keratinocytes by measuring the nucleolar organizer region (NOR) expression in the basal keratinocytes and IGF-I mRNA expression by HVPC stimulation.

## MATERIALS AND METHODS

### 1. Animal

Twenty male Sprague-Dawley rats weighing 265~290 g were used. Rats were housed one per cage in a room maintained at 22±0.5℃ with an alternating 12 hours light-dark cycle. Food and water were allowed ad libitum until they were transported to the laboratory approximately an hour before the experiments. We performed all experiments under normal room light and temperature.

### 2. Surgical procedures

Three days before the operation, the hair were shaved from back and abdominal region. Rats were anesthetized by inhalation of halothane, maintained at a concentration of 2~3%. A 10 mm length of full-thickness incision was made on the back area 20 mm from the vertical midline using a sterile scalpel #11. The incisional area was cleaned with a sterile gauze pad, and the rat was carefully observed until it had fully recovered from the anesthesia. The incision was not ligated and nor covered.

### 3. High voltage pulsed current stimulation

The animals were randomly assigned into two groups as follows: control group (no HVPCS, n=10), HVPCS group (n=10). Immediately after the skin incisions were made, HVPC stimulation was started. The carbon-silicone rubberized electrodes (3.5×3.5 cm) were placed on sterile gauze pads moistened with 0.9% sodium chloride solution. The

active electrode was placed on the incision and the passive electrode was placed distal to the incision. Electrodes were taped in place, the animals were wrapped with an elastic bandage, and then they were placed back in their cages. The HVPCS rats were received electrical stimulation with a current intensity of 50 V at 100 pps for a duration of 30 minutes using a Pulsed High-Volt Stimulator (Intelect® HV2, Chattanooga Group Inc., 4717 Adams Rd., P.O.Box 489, Hixson, TN 37343, USA). The wave form of the HVPC stimulator consists of monophasic, twin-peak pulses that have a fixed pulse duration of 65 μs. The stimulator was turned on until barely palpable contractions were felt on each rat. Voltages ranged between 35 and 50 volts. Rats were checked continuously during treatment, and current was increased or decreased as necessary. Current polarity at the active electrode was negative for the first 3 days and positive thereafter. For control group, rats were received a sham treatment without power supplied. Rats rested quietly during treatment and it was not necessary to restrain them.

**4. Wound length measurement**

Immediately after incision and at day 7 postwounding, the wound lengths were measured using a caliper accurate to 0.01 mm. Healing rate was computed for each wound. Wound length at day 7 was subtracted from the initial wound length, and the difference was characterized as a percentage of wound closure. This value was divided by the initial wound length, then multiplied by 100, and identified as the wound healing rate.

**5. Quantification of NORs in basal keratinocytes**

The rats from each group were euthanized and 10×10 mm of skin biopsies were harvested at days 7 postwounding. The harvested tissue was fixed in 10% phosphate buffered formalin and embedded in paraffin. Sections of 5 μm thickness were prepared using a microtome, then deparaffinized, hydrated, cleared using an automatic tissue processor (Citadel 1000, Shandon, Life Sciences International Ltd., Astmoor, Rumcorn, England, WA7 1PR) and stained with colloidal silver. Sections were observed using a computerized image analysis system (Image-Pro® Plus, Media Cybernetics, Inc., 8484 Georgia Avenue, Silver Spring, MD 20910, U.S.A.). Images were recorded using a colour CCD camera (IK-642K Toshiba CCD color camera, Toshiba Co., 1-1-1 Shibatori, Minato-Ku, Tokyo, Japan) attached to a light microscope (Olympus BX 50, Olympus Optical Co., Ltd., 2-43-2, Hatagaya, Shibuya-Ku, Tokyo, Japan), and processed and analyzed the images using Image-Pro® Plus (ver 3.01). The number of AgNOR dots in nucleus were counted in 100 serial fields from the randomly chosen region of the regenerated epidermis at × 400 magnification. The mean number of NORs per nucleus were calculated.

**6. RT-PCR analysis of IGF-I mRNA**

Total RNA from rat skin biopsies were prepared by the RNAzol™ B (Tel-Test Inc., TX, USA) following the instructions provided by the supplier, and stored at -80℃. 1 μg of isolated RNA was reverse transcribed to first strand cDNA with 200 U/μl of Superscript II reverse transcriptase using 5 U/μl of *Taq* DNA polymerase in the presence of 10 U/μl RNase inhibitor (Invitrogen Life Technologies, CA, USA). The cDNA synthesis reactions were performed at 42℃ for 60 min and the products were stored at -20℃. A 50 μl polymerase chain reaction (PCR) mixture containing 5 μl of the reverse transcription (RT) reaction product was carried out PCR using specific primer pairs. The primers for IGF-I (Genset Pacific Pty. Ltd., Lismore, Australia) were chosen from coding region, sense 5'-ACATCTCCCATCTCTCTGGA-3' and antisense 5'-GTGGGCCGCCCTAGGCACCA-3', generating a 481-bp fragment (Dvorak et al., 1996), and β-actin (Genset Pacific Pty. Ltd., Lismore, Australia) were also chosen from coding region, sense 5'-GTGGGCCGCCCTAGGCACCA-3' and antisense 5'-CGGTTGGCCTTAGGGTTCAG-3', generating a 245-bp fragment (Tanaka and Liang, 1996). The reaction mixtures were denatured at 94℃ for 45 s, annealed at 55℃ for 45 s and extended at 72℃ for 90 s for 41 and 37 cycles, respectively for IGF-I and β-actin. The size of amplification products was estimated in 2% agarose gel with molecular weight markers and the amount was determined using a Gel Doc system and Quantity One 1-D analysis software (Bio-Rad Laboratories Inc., Richmond, CA, USA) on the negative image of ethidium bromide staining. For the relative level of IGF-I mRNA, an OD ratio (IGF-I/β-actin) of 1.00 was designated to be 1.00 arbitrary unit.

**7. Data analysis**

For a comparison of the percent of wound closure, the mean number of NORs per nucleus and IGF-1 mRNA ratio between the HVPC stimulation and the control groups, a

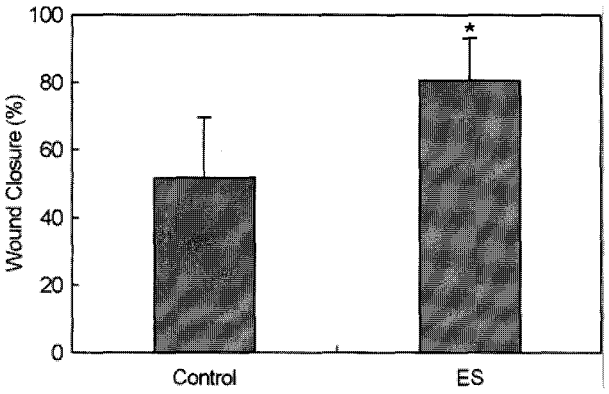



**Fig. 1.** The effect of high voltage pulse current (HVPC) stimulation on the healing rate at day 7 after electrical stimulation. The healing rate was significantly accelerated in the HVPC stimulated wound compared with sham treated control wound ($P$<0.001). Values are mean ± standard deviation; n=10.

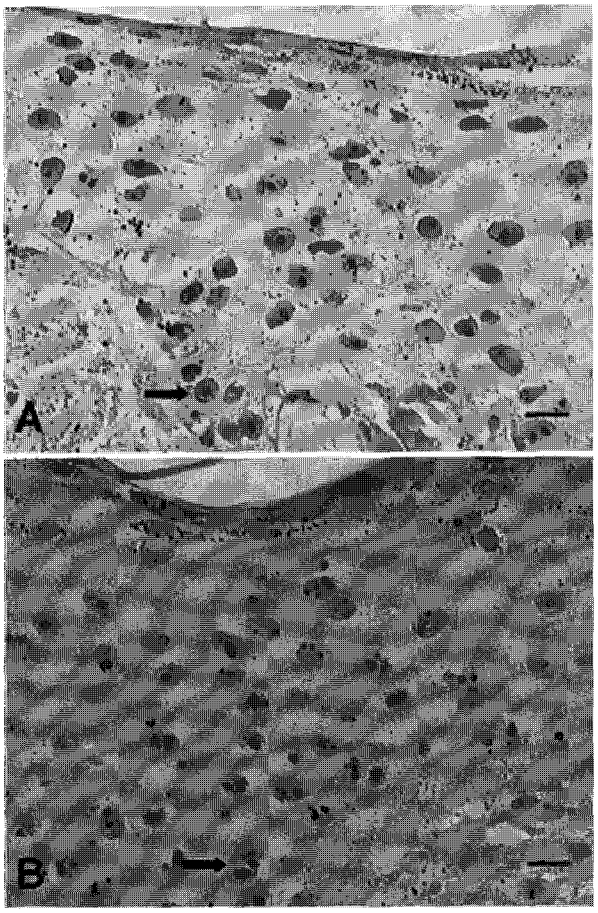
Student's $t$-test was used. The correlation between the percent of wound closure, the mean NOR number per nucleus and the IGF-1 mRNA ratio were analyzed using a Pearson product moment correlation coefficient. The statistical interpretation was based on a 0.05 significance test level. SPSS WIN (ver 10.0) software was used for the analyses.

## RESULTS

At day 7 postwounding, the wounds treated with HVPC were suppler and the surface was smoother than those control. The healing rate in the control and HVPC stimulation groups were 51.17±17.76% and 80.71±11.91%, respectively. Student's $t$-test showed a significantly higher percent of wound closure in the HVPC stimulation group than control group ($t$=-4.308, $P$<0.001). After HVPC stimulation for 7 days, the healing rate of skin wound increased 29.54% compared to the control group (Fig. 1).

Clearly defined silver stained dots were visible in all specimens. The mean NOR number per nucleus of the basal keratinocytes in the sham and HVPC stimulation groups were 1.85±0.20 and 2.70±0.23, respectively. Student's $t$-test showed a significantly higher mean NOR number in the HVPC stimulated rats than control rats ($t$=8.638, $P$<0.001). HVPC stimulation increased rRNA expression in the basal keratinocytes in the regenerated epidermis by 43.59% than control wound determined by NOR count (Fig. 2).

At day 7 postwounding, relative level of IGF-I mRNA which normalized to β-actin was 0.76±0.44 and 1.32±0.41 arbitrary unit, respectively, in control and HVPC stimulation group. Student's $t$-test showed a significantly higher the ratio of IGF-1 mRNA in the HVPC stimulated wound



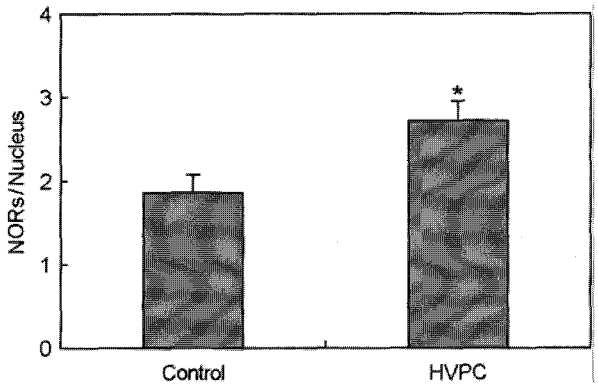




**Fig. 2.** The effect of high voltage pulse current (HVPC) stimulation on the NOR expression in the keratinocytes. NOR expression was analyzed using AgNOR count from the basal keratinocytes in regenerated epidermis at day 7 after electrical stimulation. The panels show argyrophilic stained NORs dots (arrow) in the nucleus of the basal keratinocyte from the control **(A)** and the HVPC stimulated **(B)** wound. In the figure, an increase in the expression of rRNA in the basal keratinocytes can be observed in HVPC stimulation group as compared with the control group ($P$<0.001). Magnification: ×400; Scale bar: 10 μm; Values are mean ± standard deviation; n=10.

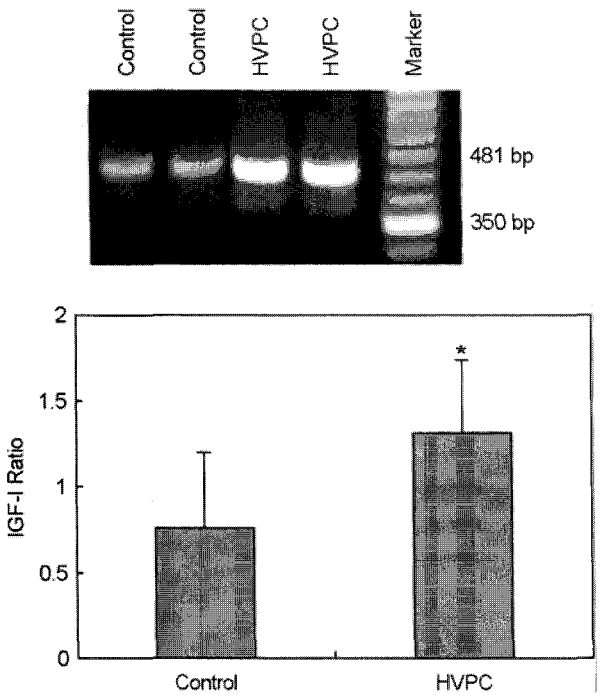



**Fig. 3.** The effect of high voltage pulse current (HVPC) stimulation on the IGF-I mRNA expression. IGF-I mRNA expression was analyzed using RT-PCR on total RNA from rat skin wound at day 7 after electrical stimulation. The expression of IGF-I mRNA was significantly increased in the HVPC stimulated wound compared with control wound ($P<0.01$). Values are mean ± standard deviation; n=10.

than sham treated wound ($t=2.906$, $P<0.01$). HVPC stimulation increased IGF-1 mRNA expression by 42.42% than control determined by RT-PCR followed by densitometry (Fig. 3).

There were a positive correlation between the percent of wound closure and the mean NOR number per nucleus of the basal keratinocyte ($r=0.74$, $P<0.05$), the percent of wound closure and the IGF-1 mRNA ratio ($r=0.87$, $P<0.05$), the mean NOR number per nucleus of the basal keratinocyte and the IGF-1 mRNA ratio ($r=0.71$, $P<0.05$) with a Pearson product moment correlation coefficient.

## DISCUSSION

Numerous studies demonstrated that pulse rate, amplitude and polarity of HVPC are important parameters for wound treatment. Bourguignon and Bourguignon (1987) demonstrated that HVPC with a pulse rate of 100 pps, amplitude at 50 and 75 V stimulate maximally the protein and DNA synthesis in human fibroblasts. Kloth and Feedar (1988) reported that HVPC stimulation with pulse rate of 100 pps, amplitude of 100~175 V accelerates the healing rate of stage IV decubitus ulcers. Griffin et al (1991) reported that HVPC stimulation with 100 pps and 200 V accelerates the healing rate of stage II-IV decubitus ulcers in patients with spinal cord injury. Some investigators have also reported that positive polarity and alternate polarity enhanced epithelialization more rapidly than the cathode. Brown et al (1987) reported that the HVPC stimulation with a negative polarity did not significantly improve wound healing rate, but epithelialization was enhanced by HVPC stimulation with a positive polarity (Brown et al., 1988) and with a negative the first three days and positive threreafter (Brown et al., 1989; Brown et al., 1995). In this study, we used an electrical stimulation using HVPC with pulse rate of 100 pps, amplitude of 50 V for a duration of 30 minutes, a negative the first three days and positive threreafter based on the previous reports. The HVPC stimulation increased percentage of wound closure by 29.54% compared to the control group. This result indicates HVPC accelerates the epithelialization during wound healing process.

NOR, the fibrillar centers of the nucleolus, are loops of rDNA that transcribe to rRNA. The argyrophilic NORs can easily be identified as black dots localized throughout the nucleolar area, and visualize the ribosomal gene activity in nucleus. A relationship between the mean number of NORs and proliferative activity of cells have been established, as determined by the degree Ki-67 immunostaining (Hall et al., 1988), BrdU immunohistochemistry (Tanaka et al., 1989) and DNA flow cytometry (Crocker et al., 1988). Therefore, NOR number in basal keratinocytes can use a marker of proliferative activity of keratinocyte. The Keratinocyte proliferation by electrical signals have been demonstrated in previous reports. In our previous study on HVPC effects in aged rat skin model, a significant increase of mean AgNOR number in basal keratinocytes with increase of epidermal thickness (Lee et al., 2004). Although types of electrical stimulation was different from high voltage pulsed current, human keratinocytes migrate towards the negative pole in physiological direct current electric fields above 50 mV/mm (Nishimura et al., 1996), migration and proliferation of human keratinocytes were significant increased by electrical stimulation using biphasic, asymmetric current with 40 Hz, a pulse width of 0.25 ms, amplitude of 20 mA for 40 min per day (Hinsenkamp et al., 1997). This study focused on the effect of HVPC stimulation on proliferative activity of

keratinocytes under alternating polarity, mean NOR numbers per nucleus of basal keratinocytes in regenerated epidermis was increased by 43.59% than control rats. In HVPC stimulated rats, many basal keratinocytes expressed more than one and two NORs in each nucleus. We also found that mean NOR number and percent of wound closure has a significant positive correlation.

IGF-I secreted from fibroblasts, macrophages and melanocytes function as a paracrine factor and stimulates proliferation and migration of keratinocytes (Rappolee et al., 1988; Bhora et al., 1995). It plays a distinct role in keratinocyte proliferation and migration in normal wound healing (Tavakkol et al., 1992) and delayed wound healing (Brown et al., 1997; Blakytny et al., 2000). IGF-I is also correlates with proliferative activity of keratinocytes (Hodak et al., 1996) but little is known of the direct effect of electrical stimulation on the IGF-I in skin wound model. Until now, effect of electrical stimulation on IGF-1 mRNA expression in wound healing has not been clearly demonstrated. There are relevant evidences, however, that the electrical stimulation can regulate growth factors. Morimoto et al (2005) demonstrated that IGF-I level was increased by electric stimulation in Muller cells. They suggested that the electrical stimulation is a new therapeutic approach to prevent or delay the degeneration of retinal neurons. Other studies demonstrated that the electrical stimulation increased bone cell proliferation associated with increased IGF-II mRNA accumulation and IGF-II secretion (Fitzsimmons et al., 1992), the electrical stimulation induces the level of TGF-β1 mRNA in osteoblastic cells (Zhuang et al., 1997), VEGF mRNA level was increased in skeletal muscle by electric stimulation in rat muscle (Hang et al., 1995), VEGF mRNA level was markedly increased by electric stimulation in rat calvarial osteoblasts (Kim et al., 2006). These previous studies support the possibility that electrical stimulation can regulate IGF-I expression in wound healing process. In this study, electrical stimulation using HVPC increases the expression of IGF-I mRNA by 42.42% in full-thickness incisional skin wound of the rat. In addition, there were significant positive correlation between the mean NOR number and the expression of IGF-I mRNA. Although the precise cellular response was not clarified, it is apparent that electrical stimulation can regulate local expression of IGF-I mRNA during wound healing process.

In conclusion, electrical stimulation using HVPC with pulse rate of 100 pps, amplitude of 50 V for a duration of 30 minutes, alternating polarity increases percentage of wound closure, NOR number in regenerated epidermis and IGF-I mRNA expression in full-thickness incisional skin wound of the rat. These findings suggest that the HVPC stimulation accelerated wound healing rate and proliferative activity of karatinocytes was probably the result of the increased transcriptional activity of rDNA and rRNA production in the basal keratinocytes and up-regulation of IGF-1 gene. Further investigations with many groups of animals and various parameters of HVPC should be performed to substantiate the optimal parameters and other cells, cytokines and matrix functions to be related to wound healing process.

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