The Effect of Human Placenta Extract in a Wound Healing Model

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Abstract: Human placenta had been used on wound healing such as burns, chronic ulcers, and skin defects. Recently, human placenta has been widely used in the form of human placental extracts (HPE) by clinical field. However, it is unclear what the effect of HPE is on wound healing. We studied the effect and mechanism of HPE on wound healing.

In this study, 10 mice (imprinting control region mice, 5 week old males, 30 g) were divided into an experimental group and a control group. An 8-mm diameter single full-thickness skin defect was made on the back by skin punch biopsy. At least 2.0×10^{-3} mL/30 g HPE was injected into the boundaries of the wound. Wound size measurements were taken by digital image every 3 days over 2 weeks. Hematoxylin and eosin (H and E), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and CD31+ immunohistochemical stains were performed on the 6th and 14th day.

The experimental group showed acceleration in the decrease of wound size compared with the control group from the third day to the ninth day. TGF-β on the 6th day showed a statistically significant increase in the experimental group. VEGF on the 14th day showed a statistically significant increase in the experimental group. CD31+ was increased in the experimental group as wound healing progressed, but this increase was not statistically significant. The total number of vessels increased in the experimental group, but this was not statistically significant.

We conclude that administering HPE directly to a wound margin promoted wound healing. This mechanism appears to be related to an increase in TGF-β in the early phase of wound healing and VEGF in the late phase.

Key Words: human placental extracts, wound healing, TGF-β, VEGF

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The use of human placenta on wounds such as burns,[1] versatile wounds,[2] ulcers,[3,4] restoration of the cornea,[5] and prevention of intestinal adhesion[6] has been reported. The use of human placenta for wound management accelerated wound healing,[7] and prevented fibrosis and acceleration of epithelization.[3] Also, due to its bacteriostatic effects on bacteria,[1,5] it has been used effectively in biologic dressing materials for large defect areas that skin grafts are incapable of covering.[8] A placental-derived tissue matrix has also been used in the treatment of abdominal fistulas.[9] Thus, human placenta is thought to prevent adhesions, protect wounds, alleviate pain, and accelerate epithelization,[5] and human placental extract (HPE) has been applied clinically for 30 years.[10–13] Kaushal et al reported studies on 120 patients with oral mucositis after radiation therapy on squamous cell carcinoma.[14] These 120 patients were divided into 2 groups as follows: an HPE injected group and a contrast group. The HPE-injected group revealed improvements in not only oral mucositis, but also dysphagia, pain, and nutritional status. Tiwary et al reported the effects of application of an HPE gel and cream on chronic, nonhealing wounds.[15] Shukla et al investigated the effectiveness of HPE on wound healing, and reported an accelerated decrease in wound surface area and neovascular progression upon histologic examination in the HPE injected group with chronic wounds.[16]

However, the mechanism by which HPE enhances wound healing has not been clarified. Therefore, we used standardized restriction in an animal wound model and investigated wound healing in an HPE-injected group to investigate the biochemical mechanism(s) underlying HPE-induced wound healing.

MATERIALS AND METHODS

Experimental Animals and Creation of a Wound

In this study, 10 imprinting control region mice supplied by the Central Laboratory Animal Institute (5 weeks GA males, 30 g) were used. All animals included in the experiment were bred in the animal laboratory of Yonsei University College of Medicine, and this study was approved by the Institutional Animal Care and Use Committee, Seoul, Korea, under the rules and regulations governing animal experiments. Laboratory animals were randomly divided into 2 groups and each 5 animals were used in the experimental group and control group.

Anesthesia was performed with inhalant isoflurane (Forane, Choongwae PharmaceuticalCo., Korea) and xylazine (0.0015 mL/100 g) peritoneal anesthesia after removal of the dorsal scalp area. Punch biopsy with an 8-mm diameter for histologic examination was used for forming the full thickness skin graft. A 2-mL ampule (Melsmon Pharmaceutical, Japan) was used for HPE injection. Generally for clinical use, 1 to 2 ample can be used at once. In this study, the proper amount for 30-g imprinting control region mouse was calculated with reference to 2 ample for 60 kg human. For easy injection, a 0.2-mL injection around the wound area was prepared by diluting a single 2-mL ampule with 200 mL 0.9% saline solution. Melsmon (2.0×10^{-3} mL) was included in the HPE diluted solution. This was injected at 8 points along the boundary of the wound. In the control group, 0.2 mL of 0.9% saline solution was injected at 8 points. The formed skin defect was maintained for 24 hours with Tegaderm (3M Health Care, St. Paul) to prevent dehydration.

Wound Size Evaluation

To evaluate changes in wound size, digital photographs were taken for 2 weeks every 3 days starting from the first experimental day. To minimize the error from the position of the camera, the camera was fixed at a certain distance using a stand. Pictures were taken with the indicator for image analysis. The digitally-imaged wound was refigured using the Image J program (NIH, Washington) so as to calculate the open area of the wound.

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HE Staining and Immunohistological Staining of TGF-β, Vascular Endothelial Growth Factor, and CD31+

On days 6 and 14 of wound healing, wound tissue was harvested and immunohistologic staining with antitransforming growth factor beta (anti-TGF-β; MAB1032, Chemicon International Inc., Temecula, CA), rabbit antivascular endothelial growth factor (VEGF; RB-222-P; Laboratory Vision, Fremont, CA), or antimouse platelet endothelial cell adhesion molecule-1 (PECAM/CD31) polyclonal antibody (M20; Santa Cruz Biotechnology, Santa Cruz, CA) was done. Using computer-assisted planimetry (Metamorph, Metamorph Ltd., Limerick, Ireland), the integrated optical density of each stained antibody on the digital image was calculated. Hematoxylin and eosin staining was also performed at these time points to investigate histologic features.

Calculation of the Amount of Neovascularization

During the analysis process, previously CD31 immunostained wounds from days 6 and 14 were randomly acquired for calculation, and the comparative analysis of the number of vessels at each high power field (×400) was performed.

Statistical Analysis

All test results are given as the average ± SD. The significance of differences between groups was evaluated using *T* tests and Mann-Whitney *U* tests. Statistical significance was set at $P < 0.05$. Statistical analyses were conducted using SPSS 13.0 (SPSS Inc., Chicago, USA).

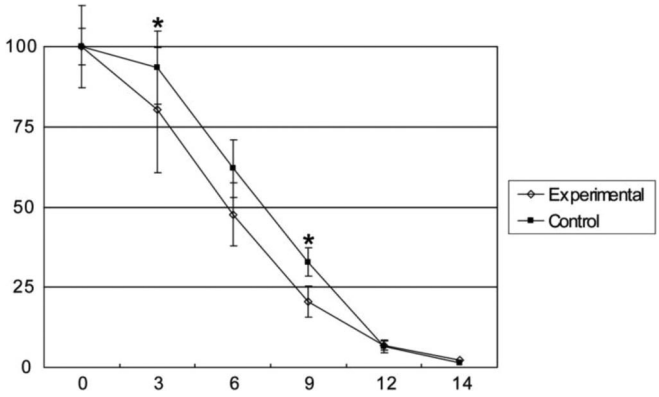

FIGURE 1. Graph of the change in wound size. The initial size of the wounds in the HPE-injected experimental and control groups is set to 100%, and the relative change in size is shown (* indicates $P < 0.05$).

RESULTS

Change in Wound Size

Application of HPE resulted in an accelerated decrease in wound size in the experimental group compared with the control group. On day 3 and 9, in particular, a statistically significant decrease in wound size was noted compared with the control group (Fig. 1). The time taken for the wound size to decrease to 50% of its original size was 3.04 days in the experimental group compared with 4.49 days for the control group. However, both groups showed similar-sized wounds on day 14.

Expression of TGF-β

On days 6 and 14, the tissues from the experimental and control groups were stained immunohistologically and integrated optical density was analyzed using computer-assisted planimetry (Metamorph, Metamorph Ltd., Limerick, Ireland). On day 6, the experimental group had a TGF-β staining value of 49,823 ± 8596 compared with 40,203 ± 4404 in the control group; on day 14, the experimental group had a value for TGF-β staining of 40,373 ± 9564 compared with 33,556 ± 7757 in the control group. Based on the immunohistological staining results, TGF-β levels were higher on day 6 than day 14 in both the experimental and control groups (Fig. 2). Notably, on day 6, levels of TGF-β were higher in the experimental group than in the control group ($P < 0.05$).

Expression of VEGF

On days 6 and 14, the tissues from the experimental and control group were stained immunohistologically for VEGF, and analysis of integrated optical density was done with computer-assisted planimetry (Metamorph, Metamorph Ltd., Limerick, Ireland). For day 6, the value for VEGF staining was 53,706 ± 8692 in the experimental group and 43,670 ± 4403 in the control group; on day 14, the experimental group had a VEGF value of 60,173 ± 9021 and the control group had a VEGF value of 44,557 ± 4216. As wound healing progressed, VEGF expression increased in the experimental group compared with the control group (Fig. 3).

Expression of CD31+ Vascular Endothelial Cell

In the experimental and control group tissues, CD31+ immunohistologic staining was performed to analyze the amount of expression. On day 6, the experimental group showed a CD31+

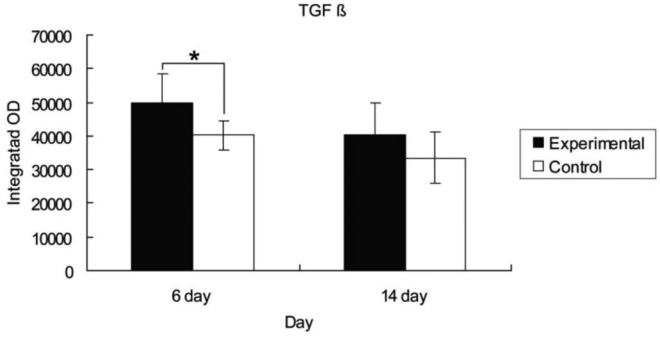

FIGURE 2. Analysis of TGF-β expression. Computer-assisted planimetry (Metamorph, Metamorph Ltd., Limerick, Ireland) was used for calculations. A significant increase in the experimental group compared with the control group was noted on day 6 (* indicates $P < 0.05$).

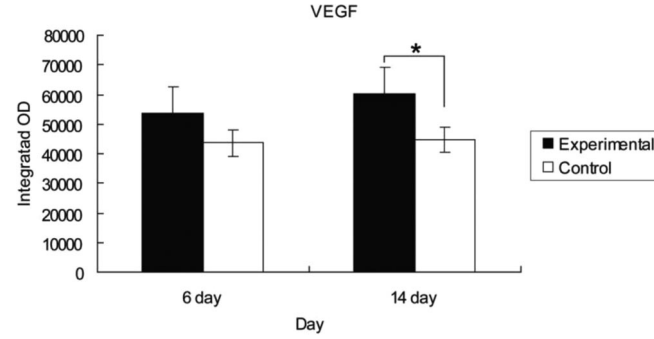

FIGURE 3. Analysis of VEGF expression. Computer-assisted planimetry (Metamorph, Metamorph Ltd., Limerick, Ireland) was used for calculations. A significant increase in the level of VEGF in the experimental group compared with the control group was noted on day 14 (* indicates $P < 0.05$).

staining value of 17,787 ± 946 compared with the control group value of 18,268 ± 1916; on day 14, the experimental group showed a CD31+ staining value of 35,444 ± 10,544, while the control group showed a value of 32,888 ± 8579. As wound healing progressed, an increase in CD31 staining was noted in endothelial cells. However, no significant differences were noted between the experimental group and the control group (Fig. 4).

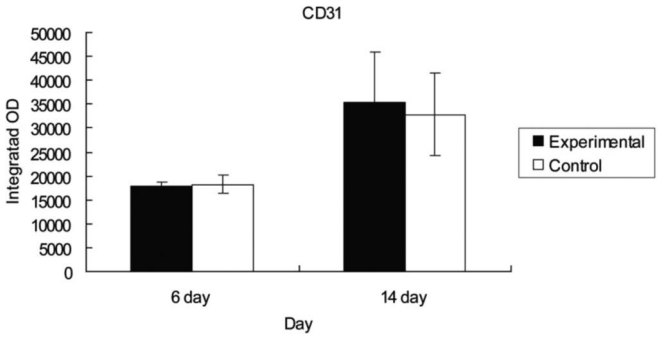

FIGURE 4. Analysis of CD31+ expression. As wound healing progressed, CD31+ positive endothelial cells also increased, this was not significant.

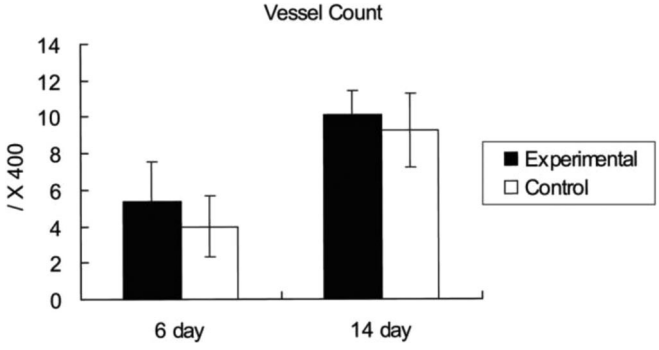

FIGURE 5. Amount of neovascularization. Neovascularization increased as wound healing progressed in both groups, with no statistical differences between the control and experimental groups.

Number of Newly Formed Vessels

In the experimental and control group tissues, CD31+ immunohistologic staining was performed to analyze if the degree of vascularization increased with wound healing. Comparative analysis was performed by counting the neovascularized vessels with a high-power field (×400). Both experimental and control groups showed an increase in vascularization as the wound healing progressed, and there was no significant differences in vascularization between the 2 groups (Fig. 5).

Changes in Histologic Features

On day 6, hematoxylin and eosin staining revealed an increase in the number of inflammatory cells in the experimental group compared with the control group. These cells were concentrated between the wound and the normal tissue. However, a similar amount of inflammatory cells was noted in the control group. On the 14th day, complete healing was noted in both groups (Fig. 6).

DISCUSSION

The placenta has long been used to treat various diseases in folk and traditional medicine. Besides its therapeutic applications, it has also been clinically applied as an anesthetic in many fields, including internal medicine, general surgery, ENT, ophthalmology, orthopedic surgery, plastic surgery, dermatology, obstetrics, and gynecology.[14]

In addition, several studies have evaluated the effect of HPE on wound healing and a considerable amount of data suggests that HPE promotes wound healing.[13,14,16] According to O'Keefe et al, keratinocytes culture far better when mixed with HPE than with insulin or EGF. In HPE cultures with keratinocytes, promotion of keratinocyte proliferation has been observed.[17,18] Muratore et al reported the promotion of fibroblast proliferation by HPE.[19]

Although the wound healing effects of HPE have been shown in vitro experiments, there are no reports explaining its mechanism

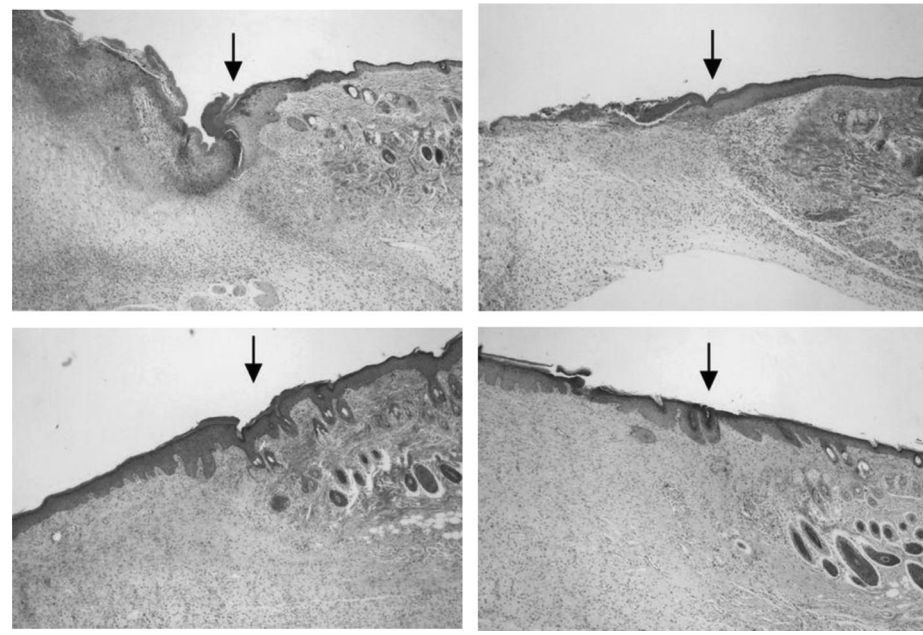

FIGURE 6. Hematoxylin and eosin staining (×100). The normal side is to the left of the arrow; the wound side is to the right of the arrow. On wound day 6, more inflammatory cells were seen concentrated around the wound margin area in the experimental group (above left) than the control group (above right), although this difference was not statistically significant. On wound day 14, no differences were seen between the experimental group (below left) and the control group (below right).

or function in vivo. Bearing this in mind, we set out to investigate the effects of HPE on wound healing in vivo and to elucidate the mechanisms of this process by establishing a wound healing model in rats.

Although there are many substances in HPE, we do not know the exact substances and their amount.[11,20,21] Therefore when we refer to the dose, we define in terms of the dose of the manufactured HPE rather than the substances. Although not clinically defined, we use 1 to 2 ample in each session. Sometimes, 32 mL of HPE were used in 8 weeks.[21] Therefore, we did a preliminary experiment using 0.1 mL assuming we administer 1 ample in each session. This is considered be the minimal dose for clinical use. There was, however, no significant difference between the experiment and the control group. In addition, we were not able to carry out the experiment when we used the 10-fold dose because of the severe inflammation around the wound. Therefore, we set our experimental model as 2 fold, which is 0.2 mL.

Wound healing consists of a complex set of processes, involving many growth factors secreted by keratinocytes, fibroblasts, leukocytes, and vascular endothelial cells.[22] Fibroblasts synthesize collagen and keratinocytes construct new epithelium. Angiogenesis also occurs to supply oxygen and nutrients to the newly formed granulation tissue.[23] The inflammatory phase of wound healing begins immediately after blood clot formation 1 day after wound formation. Epithelization also occurs during this period. The wound then enters the proliferation phase from day 4, during which neoangiogenesis and granulation tissue formation begins. From day 7, wound contraction begins, and continues for about 3 weeks.

These growth factors may influence each other or induce the expression of other factors involved in wound healing. Growth factors can be broadly categorized as those involved in granulation tissue formation (eg, TGF-β) and those involved in neoangiogenesis (eg, VEGF).[24–27] Although many growth factors are involved in the process of wound healing, adequate amount of growth factors must be secreted in a timely fashion for proper wound healing to occur.[28] Therefore, we examined TGF-β for the early wound healing phase and VEGF for the late phase in our study.

When we examined the effects of HPE on wound healing in terms of changes in wound size, the experimental group showed a greater decrease in wound size compared with the control group. The difference was noted starting on day 3 to day 9. The differences in wound size during this period were statistically significant ($P < 0.05$). Although the final size of the wounds were similar on day 14, near the end of the wound healing, the time required for the wound to reach 50% of its original size was 3.04 days for the experimental group compared with 4.49 days in the control group. Therefore, we can infer from these data that speeded-up the wound healing process.

A similar result was observed after analyzing the expression of TGF-β. In general, the level of TGF-β increases during the initial phase of wound healing and decreases as wound healing progresses. Our experiment results were consistent with these findings. A surge of TGF-β was observed in both the experimental and control groups on day 6 and a drop in the level of TGF-β was noted in both groups on day 14. TGF-β increased to a significantly greater extent in the experimental group than the control group. This indicates that HPE facilitated an increase in the expression of TGF-β, which in turn recruited more inflammatory cells thereby accelerating the initial phase of wound healing. The increased infiltration of inflammatory cells was ascertained using hematoxylin and eosin staining (Fig. 6).

New blood vessel formation is a key component of wound healing and this process accelerates as the wound healing progresses.[28] In our study, the expression of VEGF increased in both the experimental group and the control group, and this was statistically significant especially on day 14. HPE therefore appears to increase the expression of VEGF. In addition, we investigated the extent of new blood vessel formation by analyzing the expression of CD31+ positive endothelial cells. However, the neovascularization was not significantly different between the experimental and control groups.

Although we did not evaluate whether the TGF-β and VEGF were produced by the cells or were derived from the HPE, HPE influenced the process of wound healing by increasing the levels of TGF-β and VEGF.

In the 8-mm sized defect, the increase of fibroblast and contraction during the normal wound healing process resulted in almost similar size on the 14th day. Therefore, we cannot argue that the wound healing was accelerated from this experiment alone; however, we can conclude that it had a positive effect on the wound healing. For more studies in the future, the experiment with delayed wound healing model using DM mouse must be performed. Also, different effects for systemic and local application for the topical injection in the wound healing model must be clarified.

We conclude that HPE acted by increasing the level of TGF-β, thus increasing the amount of inflammatory cell infiltration in the initial phase of wound healing. It also elevated VEGF, which increased new blood vessel formation in the later stage of wound healing.

CONCLUSIONS

Locally administration of HPE directly onto wound margins promotes wound healing due to an increase in the amount of TGF-β in the early phase of wound healing and VEGF in the late phase.

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