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BURN

THE EFFECT OF ERYTHROPOIETIN ADMINISTRATION IN EXPERIMENTAL BURNS WOUND HEALING: AN ANIMAL STUDY

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ABSTRACT

Background: The hematopoietic growth factor erythropoietin (EPO) attracts attention due to its all-tissue-protective pleiotropic properties. The purpose of this study is to investigate the effect of EPO in experimental burn wounds healing.

Methods: Fifteen healthy Sprague-Dawley, strain of Rattus Novergicus weighing 300-350 grams, were prepared to achieve deep dermal burns. Animals were randomized to receive either low-dose EPO injection (600 IU/mL), high-dose EPO injection (3000 IU/mL) or nothing (control group). After 14 days of observations, quantitative and qualitative assessments of wound healing was determined.

Results: The size of the wound area and re-epithelialization rate percentage was determined on Day-0, Day-5, Day-10, and Day-14. The average of raw surface areas measurement (*p value: 0.012 in day-5; 0.009 in day-10 and 0.000 in day-14*) and healing percentage of the lesions (*p value: 0.011 in day-5; 0.016 in day-10 and 0.010 in day-14*) were significantly best in the low-dose EPO grup compared to the control group and high-dose EPO grup. The histopathology evaluation revealed that the highest score for for re-epithelialization, granulation tissue and neo-angiogenesis were achieved by the low-dose EPO injection group than in both control and high-dose EPO injection groups.

Conclusion: In this animal study using *Sprague-Dawley* rats, Recombinant Human EPO (rHuEPO) injection administration prompted the evidences of improved re-epithelialization and wound healing process of the skin caused by deep dermal burns. These findings may lead to a new therapeutic approach to improve the clinical outcomes for the management of burns wound healing.

Keywords: *burns, erythropoietin, wound healing*

Latar Belakang: Faktor pertumbuhan hematopoiesis *erythropoietin* (EPO) menarik perhatian karena kemampuannya dalam melindungi jaringan. Tujuan dari penelitian ini adalah meneyelidiki efek pemberian EPO penyembuhan luka bakar eksperimental di hewan coba.

Metodologi: Lima belas ekor tikus *Sprague-Dawley*, strain dari *Rattus novergicus* dengan berat 300-350 gram, dibuat perlakuan eksperimental luka bakar derajat 2B (dermis dalam). Hewan coba dibagi ke dalam tiga kelompok secara acak dan mendapatkan terapi injeksi EPO dosis rendah (600 IU/ml), dosis tinggi (3000 IU/ml) atau tidak mendapat perlakuan apapun (kelompok kontrol). Setelah 14 hari observasi, dilakukan penilaian penyembuhan luka secara kuantitatif dan kualitatif.

Hasil: Ukuran luka dan persentase re-epithelialisasi ditentukan pada hari-0, hari-5, hari-10 dan hari-14. Pengukuran rata-rata area permukaan luka (nilai p: 0,012 pada hari-5; 0,009 pada hari-10 dan 0,000 pada hari-14) dan persentase penyembuhan luka (nilai p: 0,011 pada hari-5; 0,016 pada hari-10 dan 0,010 pada hari-14) secara signifikan terbaik pada EPO dosis rendah dibandingkan kelompok kontrol maupun EPO dosis tinggi. Evaluasi histopatologi menunjukkan nilai paling tinggi untuk re-epitelisasi, jaringan granulasi dan neo-angiogenesis juga pada kelompok injeksi EPO dosis rendah.

Kesimpulan: Pada studi hewan coba menggunakan tikus Sprague-Dawley ini, didapatkan bahwa injeksi Recombinant Human EPO (rHuEPO) dapat mempercepat proses re-epitelisasi dan penyembuhan luka yang disebabkan oleh luka bakar derajat 2B (dermis dalam). Temuan ini diharapkan akan membuka pengetahuan baru dalam peningkatan kualitas terapi pada penyembuhan luka bakar.

Kata Kunci : *luka bakar, erythropoietin, penyembuhan luka*

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INTRODUCTION

The local care of acute burn injury can be basically be divided into three critical phase: (1) First aid in the emergency setting, (2) Prevention of burn progression, and (3) Support in wound healing, either with conservative or surgical measures. Multiple options are available to support burn wound healing. The dynamic process of burn progression therefore has a major impact on the final outcome in terms of morbidity and quality of life.[1,2]

Irreversible skin necrosis develops depending on the temperature and duration of the burn. However, the border between coagulated necrotic and surrounding healthy tissue is not clearly defined. Restoration of adequate perfusion and attenuation of inflammatory reactions may salvage this zone and decrease morbidity associated with burn injury.[3]

Wound healing is a complex process that is initiated by tissue injury and involves inflammation, proliferation, migration, angiogenesis, matrix synthesis, collagen deposition, re-epithelialization, neo-vascularization and formation of granulation tissue. The process uses interplay of cells, mediators, growth factors and cytokines. During the proliferative phase angiogenesis occurs, and the formation of new blood vessels provides oxygen and nutrient delivery. Healing is also concomitant with the release of angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF).[4]

Erythropoietin (EPO) is a 34-kDa glycoprotein and an obligatory growth factor for red blood cell proliferation, maturation and differentiation. The biological effects of EPO are mediated by its specific cell surface receptor, erythropoietin receptor (EPOR), which is a type of cytokine receptor that is present on erythroid progenitor cells as well as several non-hematopoietic cells. EPO can stimulate the first phase of angiogenesis, which includes increased cellular mitosis, motility, breakdown of the cell matrix and cellular proliferation. EPO also improves wound repair by promoting revascularization and microvascular remodeling. Moreover, the interaction between EPO and VEGF may be important in the complex phenomenon of wound healing.[5] In this study, we tried to investigate the potential beneficial of EPO injection administration in experimental burn wounds.

METHOD

This research is an experimental study on animal (rats), parallel design to compare the wound healing in experimental burn wounds in subject and control group. The study was conducted at Animal Laboratory, Faculty of Medicine, University of Indonesia from September 2014 to January 2015. The samples were 15 *Sprague-Dawley* strain of male *Rattus Novergicus* weighing 300-350 grams that has been certified and meet the inclusion and exclusion criteria. The samples were randomized into three groups: (1) Control group: the rats with burn wound that will be treated with Vaseline dressing which do not contain EPO (moist dressing only) for 14 days. (2) Low-dose EPO treated group: the rats with burn wound that will be treated with low-dose EPO injection (600 IU/mL) 1mL/day every 5 days for 14 days. (3) High-dose EPO treated group: the rats with burn wound that will be treated with high-dose EPO injection (3000 IU/mL) 1 mL/day every 5 days for 14 days.

All rats were housed on a 12-hour light/dark schedule and received stock diet and water ad libitum for 14 days. Burn model procedures were performed with aseptic and antiseptic techniques. Anesthesia is provided by intramuscular injection of Ketamine 10% (dose: 35 mg/kg) and Xylazine (dose: 5 mg/kg). For burns model preparation, the rats were placed in prone position, hair on the dorsal skin was shaved and skin washed with povidone iodine and wiped with sterile water. The areas to be burned were outlined with a marking pen (Figure 1) by 3x3cm ($9cm^2$; which correspondence to 10% of TBSA in rat).[5] The shaved skin on the dorsal will be burned by a chromium nickel steel template (3x3cm) which was immersed in hot boiling (98-100^0C) water for 15 minutes until equilibration of the temperature. The template was then applied for 60 seconds to the previously prepared area on the back, perpendicular to the skin surface and parallel to the spine.

Disclosure: *The authors have no financial interest to disclose.*

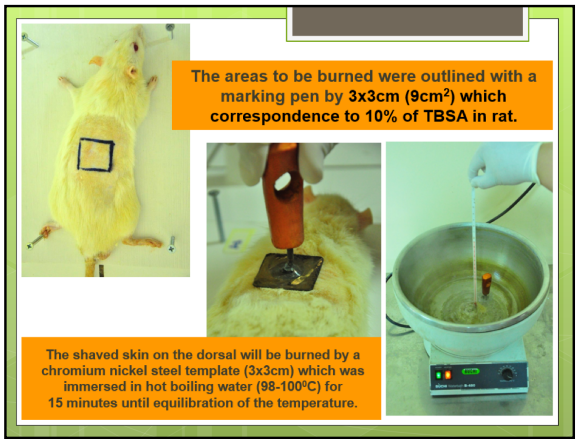

Figure 1. Outline of the area to be burned and application of hot nickel steel template to create burns model.

Assessments to be evaluated were the epithelialization (quantitative) of the wound and score of histological changes (qualitative).

Cooling was performed immediately after burn induction with cold tap water (17-20^{0}C) soaked gauze applied directly to the burn area for 20 minutes. Each group were treated every five days (day 0, day 5, and day 10) and be observed for 14 days.

RESULTS

Quantitative assessment of wound closure was measured in day-0, day-5, day-10 and day-14 after burn induction and treatment. The rate of wound healing and epithelialization was observed using digital photographs of the burned area. The photographs were obtained by a digital camera (Nikon® D-90) from a distance of 30 cm. The images were analyzed by Analyzing Digital Images® software in computer, and percentages of wound healing areas will be measured as square in millimeters. The macroscopic presentations of the three groups are presented below (Figure 2, 3 and 4).

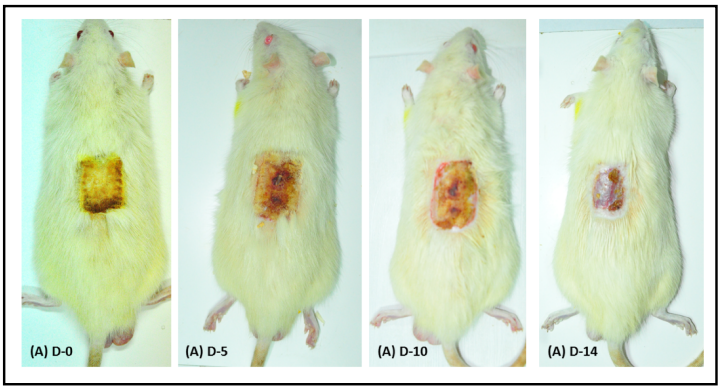

Figure 2. Macroscopic presentation of Group A (Control Group)

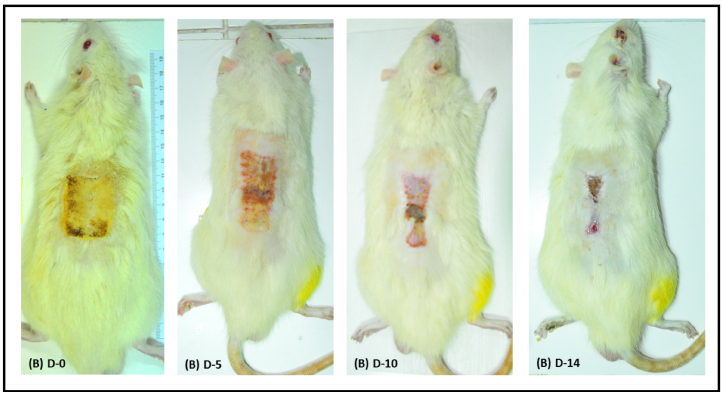

Figure 3. Macroscopic presentation of Group B (Low-Dose EPO Injection)

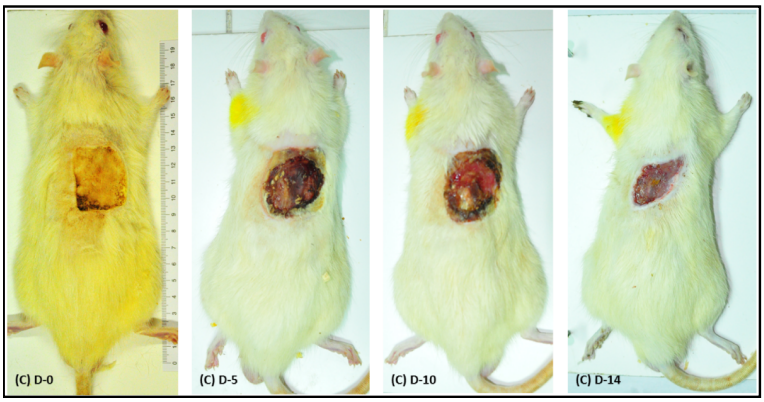

Figure 4. Macroscopic presentation of Group C (High-Dose EPO Injection)

The size of the wound area and re-epithelialization rate percentage was determined on Day-0, Day-5, Day-10 and Day-14; by digital photographs and analyzed using Analyzing Digital Images® Software and the p-value are calculated by SPSS® 20.0 Software (Table 1 and 2).

Table 1. Wound Area Measurements and Healing Percentage

Group	Wound Area in mm2				Healing Percentage			
Sample	**Day-0**	**Day-5**	**Day-10**	**Day-14**	**Day-0**	**Day-5**	**Day-10**	**Day-14**
A-1	1157.0	950.4	843.6	199.5	0.00%	17.86%	27.09%	82.67%
A-2	860.9	400.9	121.9	176.4	0.00%	53.43%	85.84%	79.51%
A-3	1391.0	963.8	368.6	189.2	0.00%	30.71%	73.50%	86.40%
A-4	1013.0	554.9	106.4	140.8	0.00%	45.22%	89.50%	86.10%
A-5	1096.0	967.4	234.0	242.4	0.00%	11.73%	78.65%	77.88%
Average	**1103.6**	**767.5**	**334.9**	**189.7**	**0.00%**	**31.79%**	**70.91%**	**82.53%**
B-1	970.7	499.6	171.5	43.7	0.00%	48.53%	82.33%	95.50%
B-2	1056.8	714.7	484.3	266.7	0.00%	32.37%	54.17%	74.76%
B-3	986.4	635.2	599.7	190.5	0.00%	35.60%	39.20%	80.69%
B-4	822.9	209.4	421.7	Died	0.00%	74.55%	48.75%	Died
B-5	834.5	255.1	94.0	49.0	0.00%	69.43%	88.73%	94.13%
Average	**934.3**	**462.8**	**354.2**	**137.5**	**0.00%**	**52.10%**	**62.64%**	**86.27%**
C-1	1275.0	980.4	819.0	406.3	0.00%	23.11%	35.76%	68.13%
C-2	942.0	891.0	712.9	434.5	0.00%	5.41%	24.32%	53.87%
C-3	1212.0	998.7	887.5	669.1	0.00%	17.60%	26.77%	44.79%
C-4	1250.0	940.0	684.4	522.2	0.00%	24.80%	45.25%	58.22%
C-5	986.8	960.2	947.8	Died	0.00%	2.70%	3.95%	Died
Average	**1133.2**	**954.1**	**810.3**	**508.0**	**0.00%**	**14.72%**	**27.21%**	**56.26%**

Note: Sample B-4 and C-5 died in day-14

Table 2. Mean (SD) of Wound Area Measurement and Healing Percentage (SPSS Analysis)

	Group A (Control)	Group B (Low-Dose EPO Inj)	Group C (High-Dose EPO Inj)	p value
Healing Percentage (%)				
Day-5	31.79(17.65)	52.09(19.22)	14.72(10.14)	0.011
Day-10	70.91(25.27)	62.63(21.69)	27.21(15.39)	0.016
Day-14	82.49(3.84)	86.27(10.17)	56.25(9.69)	0.001
Wound Area Measurement (mm^2)				
Day-5	767.48(269.97)	462.80(224.65)	954.06(41.52)	0.012
Day-10	334.90(303.18)	354.24(213.79)	810.32(112.11)	0.009
Day-14	189.66(36.88)	137.47(109.74)	508.02(118.18)	0.000

Table 3. Individual and Cumulative Score of Histological Changes in Wound Healing

Grup Sample	Re-Epithelialization	Granulation Tissue	Inflammatory Cells	Angiogenesis	Cumulative Score
A-1	2	3	1	3	8
A-2	2	3	1	3	8
A-3	2	2	1	4	9
A-4	3	3	0	4	10
A-5	2	3	0	3	8
Average	**2**	**2.8**	**0.6**	**3**	**8.6**
B-1	3	3	0	3	9
B-2	3	3	3	4	13
B-3	3	2	0	4	9
B-4	Died	Died	Died	Died	Died
B-5	3	3	0	3	9
Average	**3**	**2.75**	**0.75**	**4**	**10**
C-1		2	0	3	7
C-2	2	2	1	4	9
C-3	2	1	1	4	7
C-4	2	1	0	2	5
C-5	2	Died	Died	Died	Died
Average	Died	1.5	**0.5**	**3**	**7**

The analysis by One Way ANOVA showed that the average of healing percentage and wound area measurement in group A, B and C in day-5, day-10 and day-14 are significant (*p value*<0.05). In day-5 we can see that there was a faster healing rate (52.09%) in Group B (Low-Dose EPO Injection) compared to Group A (Control) and C (High-Dose EPO Injection). And this phenomenon is in line until the end of the observation in day-14, the fastest healing rate (86.27%) was also achieved by Group B (Low-Dose EPO Injection).

After 14 days of observation, the rats were anesthetized and the wound area was excised for histopathology examination as the qualitative assessment. Photomicrographs of the slides are captured in 2.5x magnification for 'Re-Epithelialization Assessment" (Figure 5); in 10x magnification for 'Granulation Tissue Assessment" (Figure 6) and in 40x magnification for "Inflammatory Cells and Angiogenesis Assessment". The qualitative aspects of the score were evaluated by assessment of the cells and tissues presenting the specific qualitative features indicated in the table "Score of Histological Changes in Wound Healing". The results of the qualitative assessment and statistical analysis are described below (Table 3 and 4).

Table 4. Statistical Analysis of Cumulative Score of Histological Changes

Group	Mean(Median)	*p value*
A (Control)	8.6(8)	
B (Low-Dose EPO Injection)	10(9)	0.062
C (High-Dose EPO Injection)	7(7)	

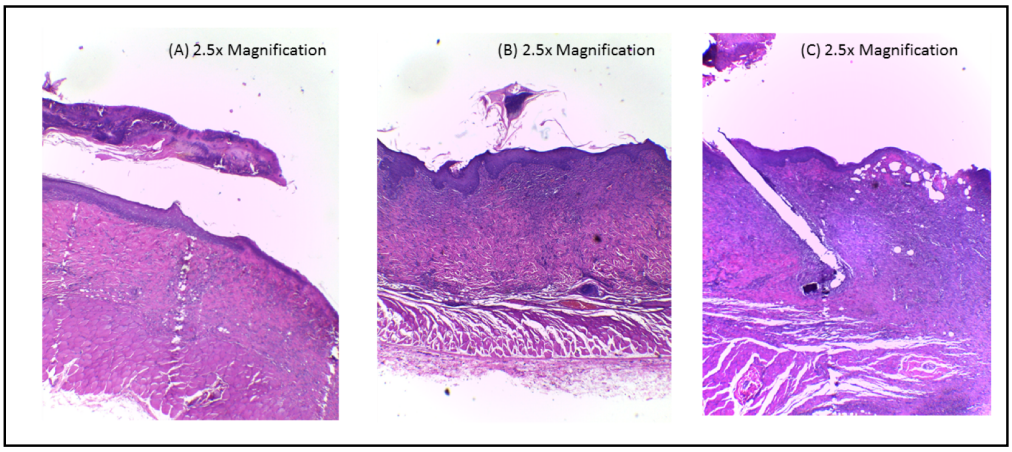

Figure 5. Photomicrographs in 2.5x HPF for re-epithelialization assessment in Group A, B and C.

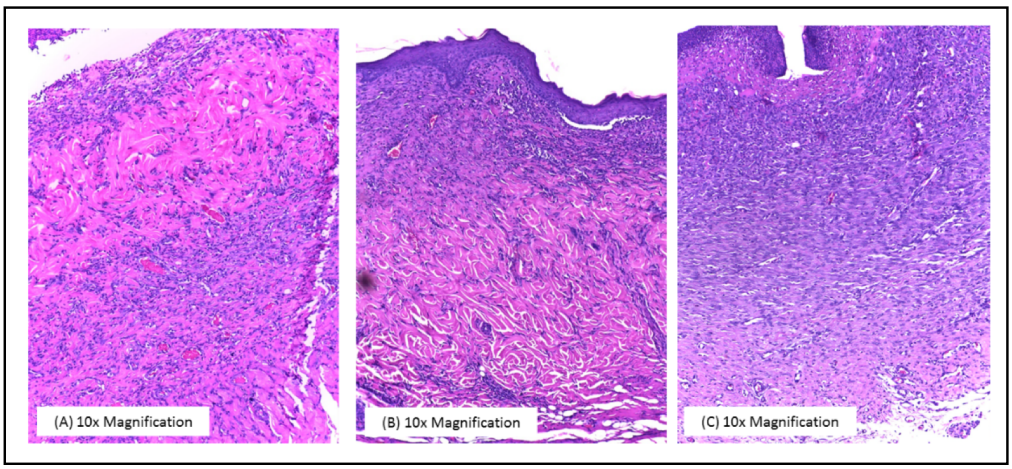

Figure 6. Photomicrographs in 10x HPF for Granulation Tissue Assessment in Group A, B and C.

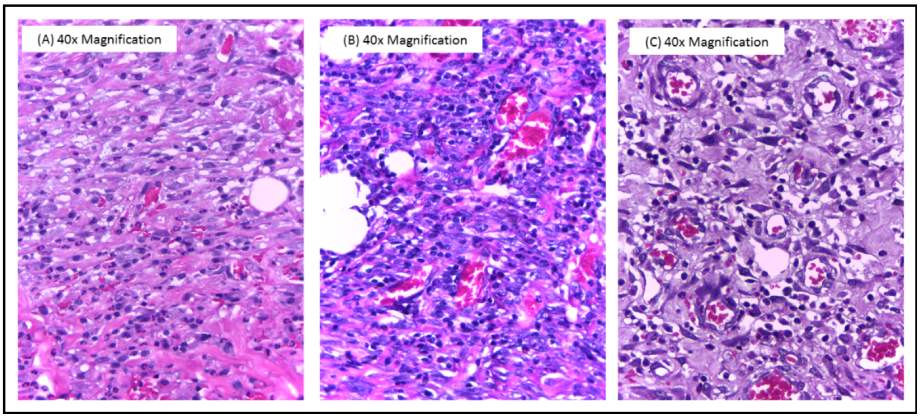

Figure 7. Photomicrographs in 40x HPF for inflammatory cells and angiogenesis assessment in Group A, B and C.

DISCUSSION

Erythropoetin is a glycoproteic hormone that possesses several biological effects. These effects are mediated by a specific binding with its cell surface receptor (EPOR), a type-1 cytokine receptor that is expressed in erythroid progenitor cells and in several non-hematopoeietic cells. EPO stimulates the mitosis and induced the differentiation and activation of numerous cell lines, such as the endothelial cells. EPO acts as a growth factor and the recent discovery of an EPO receptor in human endothelial cells, and the synergy between VEGF and EPO, indicate that EPO may act as a direct, as well as an indirect, angiogenic factor.[5]

Many evidences prompted us to study the effects of rHuEPO (Recombinant Human Erythropoetin) in experimental burn wounds. In this study, low-dose rHuEPO (600 IU/mL) that we administeredby subcutaneous injection, was successfully to improve wound healing in burn injury. In particular, the hematopoietic factor in EPO increased the area of re-epithelialization and the time needed for the complete wound closure. Furthermore, the increase healing of burn wounds after the administration of rHuEPO also accompanied by marked increased of the angiogenesis in the healing site.

In this study using deep-dermal burns wound model, we can demonstrate statistically significant faster wound healing and re-epithelialization after low-dose EPO injection administration. In addition, the extracellular matrix proliferation is much abundant and increased neo-angiogenesis could be shown in histopathological slides review. This findings is in accordance with the experiments conducted by Galeano et al and Bader et al.[5, 13]

The molecular effects of rHuEPO were correlated well with histological findings. EPO enhanced burn wound repair by reducing inflammatory cells infiltration and stimulating dermal and epidermal regeneration, proliferation of fibroblasts, and formation of new well-structured capillary vessels (neo-angiogenesis). Angiogenesis in fact is central to granulation tissue formation because the in-growth of newly formed vessels is needed to ensure the supply and delivery of oxygen and nutrients to the regenerating tissue. The marked effect of rHuEPO on angiogenesis was confirmed by enhancement and the robust increase of newly-formed vessels in histopathology assessment.

CONCLUSIONS

In this study, low-dose rHuEPO (600 IU/mL) that we administered by subcutaneous injection in burns model, was successfully to improve wound healing in burn injury. In particular, the hematopoietic factor in EPO increased the area of re-epithelialization and the time needed for the complete wound closure. Furthermore, the increase healing of burn wounds after the administration of rHuEPO also accompanied by marked increased of the angiogenesis in the healing site.

Recombinant Human EPO (rHuEPO) injection administration prompted the evidences of improved re-epithelialization and angiogenesis in wound healing process of the skin caused by deep dermal burns. These findings may lead to a new therapeutic approach to improve the clinical outcomes for the management of burns wound healing.

Suggestion for an easier clinical usage, it may be considered to develop the modification of rHuEPO by topical administration. And clinical trial in patients for the pro-regenerative agents in conjunction with debridement and skin resurfacing technique by skin grafting is amenable to be conducted in the care of wound management in Burns Unit.

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