THE EFFECTS OF NEGATIVE PRESSURE WOUND THERAPY ON VEGF AND ANGIOGENESIS IN DEEP DERMAL BURN INJURY: AN EXPERIMENTAL STUDY

EFFET DE LE THÉRAPIE À PRESSION NÉGATIVE SUR LE FACTEUR VASCULAIRE DE CROISSANCE ENDOTHÉLIALE ET L'ANGIOGÉNÈSE DANS LES BRÛLURES PROFONDES : ÉTUDE EXPÉRIMENTALE

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SUMMARY. Burn injuries are the fourth most common type of trauma worldwide, after traffic injuries, falls and interpersonal violence. Vascular endothelial growth factor (VEGF) is one of the most critical proangiogenic factors. Failure in angiogenesis is often associated with chronic, non-healing wounds. This study aimed to compare the effect of sterile gauze with normal saline (NaCl) 0.9%, intermittent negative pressure wound therapy (NPWT), continuous NPWT, and silver sulfadiazine dressing on increasing VEGF and angiogenesis in deep dermal burn injury. This experimental laboratory study involved six Yorkshire pigs. Twenty burns were made on each pig's flank and dorsum areas, which were divided into four treatment groups: sterile gauze with NaCl 0.9%, intermittent NPWT, continuous NPWT, and silver sulfadiazine dressing. Skin biopsies were done on days 1, 3, 7, 14 and 21 to evaluate VEGF histoscore and mean microvascular density (MVD). We used immunohistochemical staining of VEGF-165 as VEGF's protein marker and hematoxylin-eosin (HE) to count the MVD. There was a significant difference in mean VEGF histoscore on evaluation day 14, in which continuous NPWT had the highest score compared to sterile gauze with NaCl 0.9%, intermittent NPWT, and silver sulfadiazine. The elevated VEGF histoscore could significantly increase the MVD.

Keywords: negative pressure wound therapy, angiogenesis, vascular endothelial growth factor, wound healing, deep dermal burn injury, microvascular density

RÉSUMÉ. *Les brûlures représentent la 4ème cause mondiale de traumatisme, après les accidents de la voie publique, les chutes et les violences interhumaines. Le facteur vasculaire de croissance endothéliale (FVCE) est un des principaux facteurs de l'angiogénèse qui, lorsqu'elle dysfonctionne, fait passer les plaies à la chronicité. Cette étude compare les effets de pansements au sérum physiologique (NaCl), des thérapies à pression négative (TPN) continue ou intermittente et de la sulfadiazine argentique (SFDA) sur l'augmentation du FVCE et l'angiogénèse dans les brûlures de 2ème degré profond. Cette étude expérimentale a été conduite sur 6 porcs Yorkshire. Vingt brûlures ont été réalisées sur les flancs et régions dorsales de chacun d'eux, réparties en 4 groupes selon leur traitement : NaCl, TPN intermittente, TPN continue et SFDA. Des biopsies cutanées ont été réalisées à J1, 3, 7, 14 et 21 afin d'évaluer histologiquement le score FVCE (par mesure colorimétrique de FVCE-165) et la densité microvasculaire (par coloration hématoxyline- éosine). A j14, la TPN continue permettait d'obtenir le score FVCE le plus élevé, comparativement aux 3 autres pansements et pourrait augmenter la densité microvasculaire.*

Mots-clés: *thérapie à pression négative, angiogénèse, facteur vasculaire de croissance endothéliale, cicatrisation, brûlure profonde, densité microvasculaire*

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Introduction

Burn injuries are one of the most common traumas in everyday life. It is the fourth most common type of trauma worldwide, following traffic injuries, falls and interpersonal violence.[1-3] Over 95% of burn-associated mortality occurs in low-and middle-income countries. It is more likely for people from lower socioeconomic status to be cast into further poverty due to personal injury, making it a significant public health problem.[4] In Indonesia, the overall mortality rate in Cipto Mangunkusumo Hospital was 24%, with children under five years old at the highest risk for suffering burn injuries.[5]

The four phases of wound healing are hemostasis, inflammation, proliferative, and tissue remodeling. In the proliferative phase, keratinocytes migrate to the injured dermis, forming new blood vessels.[6] The process of developing new blood vessels is called angiogenesis. It plays an essential part in the healing process because tissues rely on the oxygen and nutrients carried by the blood to survive and grow; failure in angiogenesis is often associated with chronic, non-healing wounds.[7] Vascular endothelial growth factor (VEGF) is one of the most important regulators of angiogenesis as a proangiogenic factor. It also influences wound closure, repairs the epidermal barrier and the underlying dermis, granulation tissue formation, and the healing quality.[6-9] Epidermal keratinocytes typically produce VEGF at low levels. When the skin is injured, the VEGF expression is upregulated by epidermal keratinocytes and activated fibroblasts, mast cells and macrophages.[7,9]

Based on the medical literature, the ideal dressing that can adapt to any type of burn in any situation has not been found.[10] The most commonly used material for partial and full-thickness burn injury with <15-20% total body surface area (TBSA) treatment is silver sulfadiazine.[11] Silver sulfadiazine 1% is a topical agent with a silver component that could decrease bacterial contamination and subsequently enhance wound healing; however, prolonged application for more than three weeks could induce the formation of hypertrophic scars.[12]

Negative pressure wound therapy (NPWT) is a non-invasive therapy that uses negative pressure in a closed system to create a suction force and enables wound drainage to promote healing.[13,14] NPWT is an effective wound bed preparation modality that could accelerate acute and chronic wound healing and reduce hospital length of stay by promoting re-epithelialization, increasing blood flow and nutrients, reducing infectious complications and mortality rate, and providing optimal wound healing moisture.[13,15-20] NPWT is divided into continuous NPWT, in which the negative pressure is kept constant, and intermittent NPWT, in which the negative pressure is repeatedly switched on and off.[21]

This study aimed to compare the effect of sterile gauze with normal saline (NaCl) 0.9%, intermittent NPWT, continuous NPWT, and silver sulfadiazine dressing on increasing VEGF and angiogenesis in deep dermal burn injury.

Materials and methods

This was an experimental laboratory study that was conducted at Prof. Soeparwi Veterinary Hospital, Faculty of Veterinary Medicine, Universitas Gadjah Mada, and has been approved by the Institutional Review Board of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (KE/FK/0729/EC/2019) for the care and use of laboratory animals.

Animal model

We used 2-3 month-old healthy male Yorkshire pigs (*Sus scrofa domesticus)* weighing 10 kilograms and well-nourished with no skin continuity disturbances.[19] The pig was used as an experimental animal because the anatomical structure and physiological function of its skin mimic that of human skin.[13] We used six Yorkshire pigs based on the degrees of freedom sampling method. Pigs were allowed to acclimate for seven days before the experiment began, and given a standard diet twice a day and water *ad libitum.*[20]

Burn wound design

Based on a prior study, pigs were sedated by IM injection of atropine 0.06/kg, Zoletil® 4.4 mg/kg, and Xylazine 2.2 mg/kg, followed by endotracheal intubation.[18] Anesthesia was maintained using isoflurane (0.5-2.5%) inhalation. Next, the pig's flank and dor-

sum regions were shaved and disinfected three times using povidone-iodine. Deep dermal burn wound models were made by attaching a 20 mm stainless-steel round plate heated to 92°C, with one kilogram-force (kgf) for 20 seconds with a push-pull force gauge (AK-2, Algol Instrument Co., Taoyuan, Taiwan) on the pig's back.[18,20] One technician made burn wounds to prevent bias. Burns that fit deep dermal criteria reveal delays or capillary return loss when applied pressure[18,20,22] (*Fig. 1*).

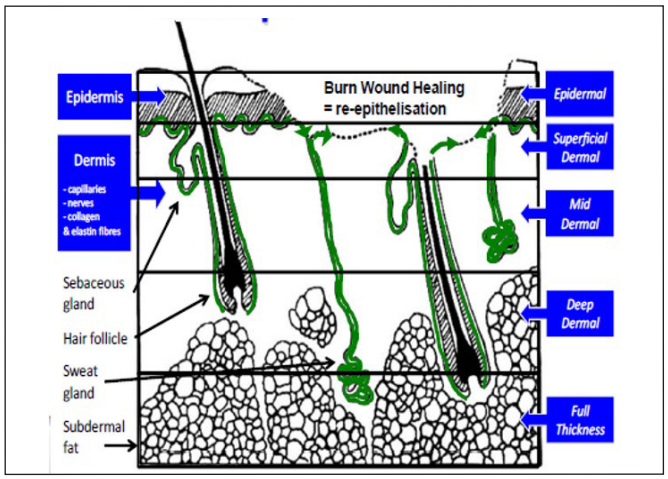

Fig. 1 - Wound depth illustration[22]

On each pig, 20 burns were made (*Fig. 2*). We differentiated them into four different treatment groups,

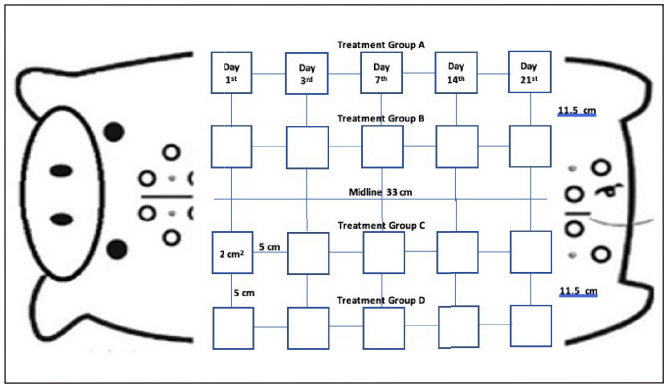

Fig. 2 - Burns map illustration on the male Yorkshire pig's back. The burn wound's width was 2 cm^2, and the distance between each wound was 5 cm. (Group A) sterile gauze with normal saline (NaCl) 0.9% group, (Group B) continuous negative pressure wound (NPWT) group, (Group C) intermittent NPWT group, and (Group D) silver sulfadiazine dressing group

which were: sterile gauze with normal saline (NaCl) 0.9% group (A), continuous negative pressure wound (NPWT) group (B), intermittent NPWT group (C), and silver sulfadiazine dressing group (D) (*Fig. 3*).

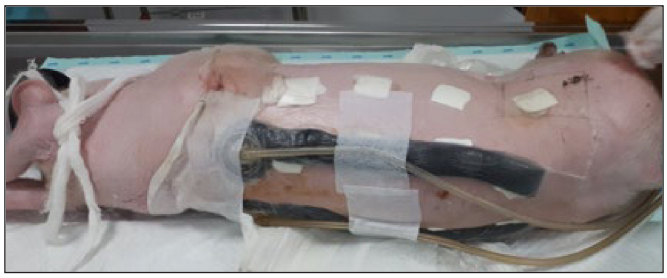

Fig. 3 - Application of dressing to the pig

We applied sterile gauze with NaCl 0.9% in group A, as the control group, and changed the gauze every 24 hours. In group B, the wounds were treated with vacuum-assisted closure (VAC) device with 125mmHg negative pressure continuously for 24 hours (V.A.C Original Veraflow® by KCI-USA). In group C, the wounds were treated with a VAC device with 125mmHg negative pressure; the machine was set to stay on for 3 minutes and off for 9 minutes (V.A.C Original Veraflow® by KCI-USA). In group D, the wounds were treated with silver sulfadiazine (*Burnazine®*) with a thickness of 1/16 inch for 24 hours.

Each treatment group was evaluated on days 1, 3, 7, 14 and 21 (*Fig. 2*). We took a sample and then sutured the wound each time we finished evaluating the wound on the specific days.[18] Total samples in every treatment group were 6; therefore, we made 120 burn wounds. The maximum pressure used on the NPWT was -125mmHg.[18]

Microscopic examination

Skin biopsies were excised under anesthesia; then, the wound was treated with sterile gauze. The samples were sunk in formalin 10% and sent to the pathological anatomy laboratory in Dr. Sardjito Hospital for 2x24 hours following the excision time.

VEGF examination

Samples were stained with immunohistochemistry (IHC) VEGF-165 as the protein marker (VEGF Rabbit Polyclonal, Wuhan Fine Biotech Co., Ltd.). Two observers examined each sample under a binocular microscope (Olympus Cx 30) in three different fields of view at 400x magnification. The VEGF expression was rated based on staining intensity and VEGF histoscore. The score for staining intensity was divided into a score of 1 for weak expression of VEGF, a score of 2 for moderate expression, and a

score of 3 for strong expression of VEGF. The VEGF expression on every sample was then calculated using the Histoscore formula [(total number of weak expressions x 1) + (total number of strong expressions x 2)]. After that, we calculated the mean VEGF histoscore on every sample by averaging VEGF histoscore from three different fields. An intraclass correlation coefficient test was done to measure the consistency of the two observers.

Angiogenesis examination

Samples were stained with hematoxylin-eosin (HE) staining and examined under a binocular microscope (Olympus Cx 30) at 100x magnification by two observers in three fields of view. We counted the microvascular density (MVD) by measuring the capillaries per high power field. After that, we calculated the mean MVD for every sample by averaging the MVD from three different fields of view and compared the mean MVD results of every group. An intraclass correlation coefficient test was done to measure the consistency of the two observers.

Statistical examination

The data obtained were analyzed by IBM SPSS version 23 (IBM Corp., Armonk, NY). The Shapiro-Wilk test was used as a normal distribution test. Since the distribution was normal, the ANOVA test was used as a mean differences test. Results were expressed as the mean ± standard deviation (SD) and compared by analysis of variance. A multiple linear regression test was done to find the causal relationship between those variables. A value of $p < 0.05$ was considered a statistically significant difference.

Results

Intraclass correlation coefficient tests on VEGF and angiogenesis examination were 0.996 and 0.996 ($p<0.05$), which means the data examined by the observers have excellent reliability. On the first day of evaluation, it was found that the silver sulfadiazine dressing group showed the highest mean VEGF histoscore and mean MVD, which were 27.84 ± 22.79 and 30.5 ± 11.57, respectively. The intermittent NPWT group had the lowest mean VEGF histoscore, with 13.33 ± 15.75 (*Table I, Fig. 4*), and the sterile gauze with NaCl 0.9% group had the lowest mean MVD, which was 30.5 ± 11.57 (*Table II, Fig. 5*). However, the differences in mean VEGF histoscore and mean MVD between those treatment groups were insignificant, $p=0.756$ and $p=0.760$, respectively (*Tables I & II*).

On evaluation day 3, the group of continuous NPWT's mean VEGF histoscore and mean MVD were the highest scores, with 22.13 ± 38.35 and 331.67 ± 44.1, respectively. The group of intermittent NPWT's mean VEGF histoscore was 8.25 ± 4.51, which was the lowest score (*Table I, Fig. 4*), and the mean MVD of the group of sterile gauze with NaCl 0.9% was 14.33 ± 8.96, which was the

Table I - Mean VEGF histoscore

Evaluation Day	Intervention				*p*-value
	Sterile gauze with NaCl 0.9%	Intermittent NPWT	Continuous NPWT	Silver sulfadiazine dressing	
Day 1	18.95 ± 26.24	13.33 ± 15.75	24.72 ± 27.22	27.84 ± 22.79	0.756
Day 3	11.39 ± 13.03	8.25 ± 4.51	22.13 ± 38.35	16.73 ± 15.61	0.771
Day 7	51.94 ± 76.24	35 ± 43.48	26.33 ± 22.81	15.93 ± 3.95	0.623
Day 14	34.72 ± 19.49	66.27 ± 16.79	167 ± 139.13	74.83 ± 40.69	0.039
Day 21	59.61 ± 42.88	73.17 ± 48.37	82.27 ± 83.94	97.61 ± 114.07	0.858

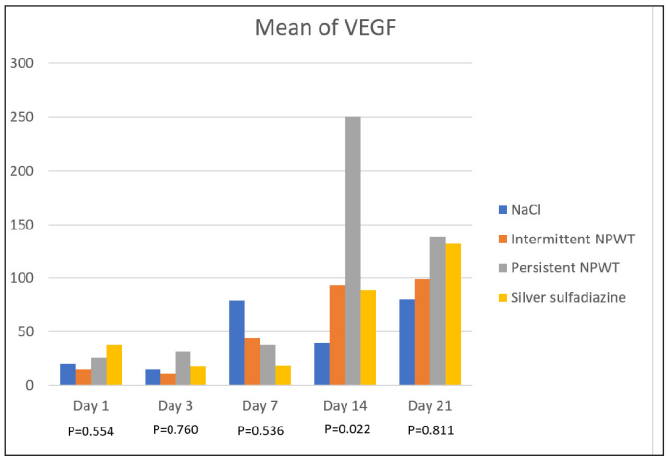

Fig. 4 - Mean VEGF histoscore in 5 examination days

lowest score (*Table II, Fig. 5*). However, the differences in mean VEGF histoscore and mean MVD between those treatment groups were insignificant, p=0.771 and p=0.678, respectively (*Tables I & II*).

On the seventh evaluation day, the sterile gauze with NaCl 0.9% group showed the highest mean VEGF histoscore, with 51.94 ± 76.24. The silver sulfadiazine dressing group showed the lowest mean VEGF histoscore, which was 15.93 ± 3.95 (*Table I, Fig. 4*), but on the contrary, it showed the highest mean MVD, 96 ± 195.67. The continuous NPWT group showed the lowest mean MVD, with 22.17 ± 10.41 (*Table II, Fig. 5*). However, the differences in mean VEGF histoscore and mean MVD between those treatment groups were insignificant, p=0.623 and p=0.975, respectively (*Tables I & II*).

On the 14th day of evaluation, the highest mean VEGF histoscore and mean MVD were in the continuous NPWT group with 167 ± 139.13 and 247.33 ± 251.98, respectively. The lowest mean VEGF histoscore and mean MVD were in the sterile gauze with NaCl 0.9% group with the scores of 34.72 ± 19.49 and 91 ± 84.82, respectively. The difference in mean VEGF histoscore between those groups of treatments was significant, p=0.039 (*Table I*); however, the difference in mean MVD between those treatment groups was not significant, p=0.455 (*Table II*).

On evaluation day 21, the continuous NPWT group still had the highest mean VEGF histoscore with 97.61 ± 114.07. But the highest mean MVD score was in the silver sulfadiazine dressing group with 119.83 ± 77.63. The lowest mean VEGF histoscore and mean MVD were in the sterile gauze with NaCl 0.9% group with the scores of 59.61 ± 42.88

Table II - Mean MVD

Evaluation Day	Intervention				*p*-value
	Sterile gauze with NaCl 0.9%	Intermittent NPWT	Continuous NPWT	Silver sulfadiazine dressing	
Day 1	14.17 ± 8.32	16.5 ± 8.59	28.5 ± 18.46	30.5 ± 11.57	0.760
Day 3	14.33 ± 8.96	23.17 ± 27.2	331.67 ± 44.1	19.83 ± 5.84	0.678
Day 7	37 ± 34.65	26.67 ± 13.83	22.17 ± 10.41	96 ± 195.67	0.975
Day 14	91 ± 84.82	158.16 ± 107.32	247.33 ± 251.98	189.17 ± 173.12	0.455
Day 21	58 ± 60.75	88.17 ± 68.47	75.17 ± 87.25	119.83 ± 77.63	0.396

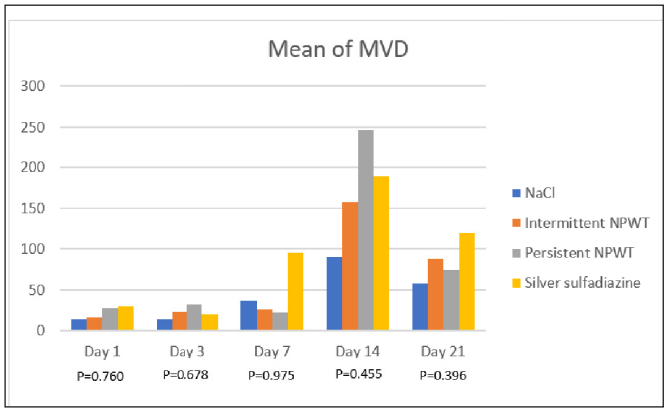

Fig. V - Mean MVD histoscore on five examination days

and 58 ± 60.75, respectively. However, the differences in mean VEGF histoscore and mean MVD between those treatment groups were not significant, p=0.858 (*Table I*) and p=0.396, respectively (*Table II*).

Based on the multiple linear regression test analysis, it was found that treatment, length of evaluation, and mean VEGF histoscore affect the angiogenesis as much as 6.1%. Only the relationship of mean VEGF histoscore on angiogenesis was statistically significant, p=0.024. There was a negative interaction between evaluation day and angiogenesis; however, it was not statistically significant, p=0.180 (*Table III*).

Table III - The relationship of treatment, length of evaluation, and VEGF histoscore on angiogenesis

Model	Unstandardized Coefficients	Standardized Coefficients	*p-value*
	B	B	
Mean VEGF histoscore	.980	.247	.024
Treatment	15.417	.074	.444
Evaluation day	-4.596	-.144	.180

R square value: 0.505
R square value: 0.061

Discussion

The rate of wound healing is determined by the formation of new capillaries, matrix formation and the availability of vascular supply.[23] VEGF is a proangiogenic factor that stimulates in vitro endothelial cell migration, activation, and in vivo angiogenesis during the proliferative phase of wound healing.[24] The proliferative phase starts from 3 days up to 2-4 weeks following the injury.[25] Prior studies stated that VEGF contributes to vascular permeability at the early stages of healing.[7,9] Our study found that the mean VEGF histoscore was statistically significantly associated with the mean MVD representing its impact on angiogenesis. Angiogenesis is central to granulation tissue formation because the in-growth of newly formed vessels is needed to ensure oxygen and nutrient supply to regenerate tissue.

Our research showed no significant differences in the mean VEGF histoscore on days 1, 3, 7 and 21 and mean MVD on days 1, 3, 7, 14 and 21 between four different wound treatments.[26] However, there was a significant difference in the mean VEGF histoscore on day 14. The continuous NPWT treatment group had the highest mean VEGF histoscore, and sterile gauze with NaCl 0.9% had the lowest score. On the same day, the mean MVD of continuous NPWT and sterile gauze with NaCl 0.9% treatment groups were also the highest and the lowest, respectively, among the other groups; however, the results were not statistically significant. Previous studies stated that NPWT therapy could increase the number of interleukin-6 (IL-6), VEGF, fibroblast growth factor-2 (FGF-2), and neovascularization in wounds.[27,28] Based on a prior systematic review, NPWT was more effective than other local wound treatments.[29] Sterile gauze with NaCl 0.9% is a conventional dressing with a lower cost than NPWT and silver sulfadiazine. A study by Malmsjö et al. showed that wound contraction and granulation tissue formation in intermittent NPWT is more pronounced than in continuous NPWT.[19] Yet, we did not discuss the macroscopic progression of the sample's wound healing.

There was a decline in mean VEGF histoscore and mean MVD on evaluation day 21. We supposed that the wound healing process was already in the remodeling stage; therefore, the mean VEGF histoscore and mean MVD decreased. In the remodeling and delivery stage, matrix metalloproteinase (MMP) mainly degrades collagen and other extracellular matrix components.[30]

This was the first experimental study that compared VEGF and angiogenesis following NaCl 0.9%, NPWT, and silver sulfadiazine treatment in deep dermal burn injury. The limitation of this study was that the manual scoring systems in detecting strong immunostaining within each slide could be influenced by subjectivity, and there were confounding factors that we could not control, such as immunological factors, host-pathogen response, and behaviors in pigs.

Conclusions

In the evaluation of day 14, continuous NPWT had a significantly higher VEGF histoscore compared to treatments using sterile gauze using NaCl 0.9%,

intermittent NPWT, and silver sulfadiazine. The elevated VEGF histoscore is usually associated with higher microvascular density and wound healing responses.

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Availability of data and materials. The datasets used during the study are available from the corresponding author on reasonable request.
Registration of research studies. This trial was registered on Research Registry: researchregistry7796 on April 8, 2022.
Guarantor. Muhammad Rosadi Seswandhana, MD, PhD
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