

The Effect of PDRN
(Polydeoxyribonuceotide, Placentex[®])
on Rat Skin Flap Survival

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on Rat Skin Flap Survival

Directed by Professor Won Jai Lee

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This certifies that the Master's Thesis of
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ABSTRACT

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Polydeoxyribonuceotide (PDRN) has multiple vascular actions such as angiogenesis and production of a vascular endothelial growth factor (VEGF) through the adenosine A2 receptor stimulation. We applied PDRN on the ischemic flap of rat back and investigated its effects on the flap survival by calculating the area of viable flap and blood perfusion. We think that inadequate perfusion, which is the most common cause of distal flap necrosis, may well be overcome through the neovascularization and VEGF production of the PDRN.

A total of 20 Sprague-Dawley rats were divided into two groups: PDRN group and control group. On the distally based flap of 3×9 cm in size, it was subdermally injected with PDRN and Phosphate Buffered Saline (PBS), which were administered 48 hours prior and immediately after flap elevation. The PDRN group was daily maintained with PDRN (8mg/kg) from postoperative 1st day to 10th day, while same dose of saline was injected in the control group. A survival area of a flap and the amount of blood flow were measured using laser doppler flowmetry. On postoperative day 10, the CD31 positively stained vessels and VEGF protein expression were examined using immunohistochemistry.

There was a significant increase in the survival area of the flap in the PDRN group. The mean survival rates of flap in PDRN group ($79.5\pm 6.3\%$) are significantly larger than control groups ($51.7\pm 6.7\%$) on postoperative day 10 ($p=0.0009$). The blood flow measurement also showed significantly increased blood flow immediately after the operation and on postoperative days 7 and 10. The number of CD31 positively stained vessels (6.70 ± 1.88 in samples from control group and 19.40 ± 5.58 in PDRN group, $p=0.001$) and VEGF protein expression were significantly higher in the PDRN group.

Conclusively, we think that administration of PDRN into the ischemic skin flaps increased VEGF expression, number of capillaries, and blood flow to the flap, thereby improving the rat skin flap survival.

Key words : PDRN, Rat flap, Survival, Blood perfusion, VEGF

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I. INTRODUCTION

The cutaneous flaps have been used to repair defects resulting from trauma, congenital malformations, head and neck tumor excision, or other causes. However, partial or complete skin flap necrosis is a well-known postoperative complication. If flap necrosis occurs, another management often includes time consuming and repetitive dressing changes or even secondary flap reconstruction. The causes of this complication are infection, hematoma, and ischemic tissue necrosis resulting from vasospasm, thrombosis, and insufficient vascularity. Among them, insufficient blood perfusion is thought to be the major factor that causes several detrimental changes in the tissue and vasculature, resulting in flap necrosis. Therefore, augmentation of perfusion in the ischemic flap has long been experimental and clinical challenges.¹

Many reports on augmenting the surviving areas of the random-pattern skin flaps are also directed on preventing ischemic injury to the flaps.²⁻⁷ The surgical delay method², local hyperbaric oxygen treatment^{5,6}, and many pharmacological agents such as sympatholytics, vasodilators, calcium channel blockers, hemorheological

agents, anticoagulants, and free radical scavengers were introduced.^{2,7} However their effects are limited, and there is a risk of systemic side effects. Recently, the concept of therapeutic local angiogenesis using exogenous growth factors such as vascular endothelial growth factor (VEGF) has emerged to augment blood flow and viability of the skin flap^{3,4}.

Polydeoxyribonuceotide (PDRN) is acquired from trout sperm by extraction process with purifying and high temperature sterilizing procedures to obtain an 95% pure active principle without pharmacologically active proteins and peptides (Registration Dossier, Italian Ministry of Health) and acting through the adenosine A2 receptors. An increase in VEGF has been shown as a result of adenosine A2 receptor stimulation.⁸⁻¹¹ This seems to be mediated by increasing endothelial cell migration, microvascular endothelial cell VEGF production, and also promotion of VEGF production in macrophages. Stimulation of the adenosine receptor has been shown to result in fibroblast differentiation and maturation, increasing the rate of granulation tissue and consequently accelerating the repair process.¹⁶⁻¹⁸ Also previous reports revealed effects of neovascularization in the experimental model of lower leg ischemic disease, delayed wound healing and burn wound¹²⁻¹⁵.

Inadequate perfusion which is the most common cause of distal flap necrosis may well be overcome through the neovascularization and VEGF production of the PDRN. Therefore, we applied injection of PDRN into the ischemic skin flap of rat and investigated the effects of flap survival by calculating the area of viable flap and blood perfusion by laser Doppler flowmetry. Also, immunohistologic studies were performed to examine changes of the CD 31 positively stained vessels and

expression level of VEGF protein.

II. MATERIALS AND METHODS

1. Animal Preparations

The Institutional Animal Care and Use Committee of Yonsei University approved all animal protocols used in this study. 20 male Sprague-Dawley rats weighing 300 to 350 g were used. Rats were managed in a regulated airflow room, where temperature, humidity, and light were controlled. The rats were divided into two groups. The experimental group (n=10) was treated with PDRN (Placentex Integro[®], 5.625/3mL, Mastelli Farmaceutica, Italy) by a subdermal injection and intraperitoneal injection. The control group (n=10) was treated with Phosphate Buffered Saline (PBS).

2. Experimental protocol

First, the rats underwent general anesthesia with isoflurane (Aerane[®]; Ilsung Pharmaceuticals, Seoul, Korea) and an intraperitoneal injection of zolazepam-tiletamine mixture (30 mg/kg, Zoletil[®]; Virbac, Carros, France) and Xylazine (10 mg/kg, Rompun[®]; Bayer, Seoul, Korea). Body hair was removed from the entire dorsal area with a depilatory cream. From the dorsum of the prepared rats, a 3x9 cm flap including the panniculus carnosus muscle was designed and was vertically elevated with its base on the caudal portion. To block the new blood supply from the bed, a silicone sheath (Bioplexus Corporation, Saticoy, CA) was placed on the flap bed, and the flap was sutured with a 4-0 nylon suture to its original location. To prevent the rats from biting the flaps on others after recovery from anesthesia, each rat was placed in a separate cage.

The experimental group (n=10) was pretreated with 1.2mL of PDRN (8mg/kg, Placentex Integro[®], 5.625mg/3mL, Mastelli Farmaceutica, Italy) by a subdermal injection distributed evenly in the 12 areas at proximal, middle, and distal areas of the flap (0.1mL each) two days before surgery and immediately after the flap elevation, while control group (n=10) was pretreated with PBS. The experimental group was daily maintained with PDRN (8mg/kg) from postoperative 1st day to 10th day, while same dose of saline was injected in the control group. (Fig. 1)

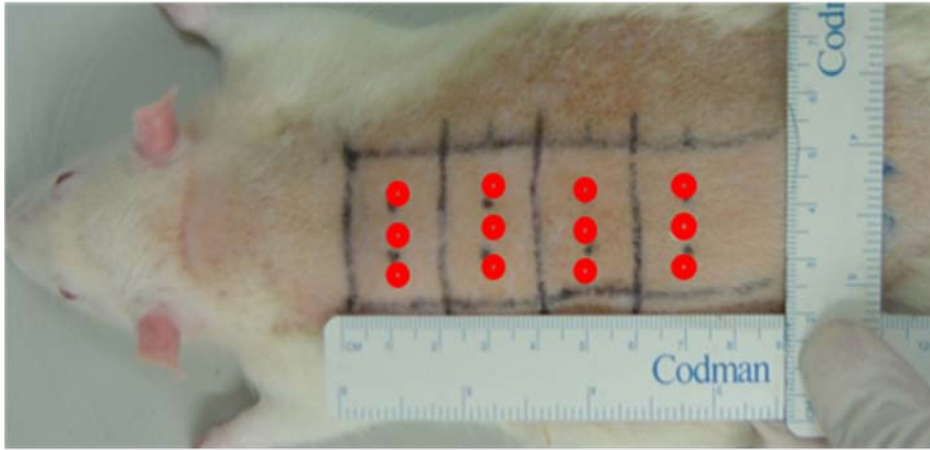


Figure 1. The design of flap and location of PDRN injection. The flap was designed on the back of rat sized 3×9cm. In the experimental group, 1.2 ml PDRN was injected into the whole areas of the mapped skin flap of rat. The 12 red dotted area in Figure 1 was injected 0.1 mL each subdermally. The timing of PDRN injection was 48 hrs before surgery and immediately after flap elevation.

3. Evaluation of skin flap survival rate

The elevated flaps returned to their original position and flap survival was checked on postoperative days 3, 7, and 10. On each day, a digital photograph was taken, and a Scion image program (NIH-Scion Corporation, Frederick, MD) was run. Using the program, the length of the image was converted to the actual length and the surface area was calculated. The survival area was determined by subtracting the demarcated area of necrosis from the total surface area. The flap survival was calculated by the percentage of viable surface to the total surface area.

4. In vivo measurement of microcirculation in skin flap

To assess the changes in blood flow in the flap, the Periflux system 5000 (Periflux[®] system 5000, Perimed AB, Jarfalla, Sweden) was used. The serial measurements of skin vascularity were taken from four areas (proximal, mid-proximal, mid-distal, and distal) using laser Doppler flowmetry on the initial status before injection of PDRN, immediately before flap elevation, immediately after the flap elevation, and 3, 7, and 10 days after repositioning of the flap. At the four points of the flap, data were measured at 1-minute intervals, and the mean value was obtained. Simultaneously, data were collected from the control group for comparison.

5. Immunohistochemistry

Samples (1x1 cm) were taken along the longitudinal midline 5 cm from the pedicle of the skin flap 10 days after surgery in the experimental and control group and fixed with 10% formaldehyde. Formaldehyde-fixed tissues were transferred into a paraffin-embedded block, mounted on a slide, and stained with hematoxylin and eosin for histological examination. Tissue sections were pretreated with a 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase, and were treated with protein block serum-free reagent (DAKO, Carpinteria, CA; X0909) for 30 minutes to prevent non-specific reactions. Sections were incubated at 4°C overnight with primary antibodies (rabbit anti-vascular endothelial growth factor (VEGF); RB-222-P; Laboratory Vision, Fremont, CA, antimouse platelet endothelial cell adhesion molecule-1 (PECAM/CD31) polyclonal antibody; M20, Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated at room temperature for 20 minutes with DAKO Envision Kit (DAKO) as secondary antibodies.

To calculate the amount of neovascularization, the CD31 positively stained vessels were counted, and the comparative analysis of the number of vessels at each high power field ($\times 200$) was performed. The expression level of VEGF was semi-quantitatively analyzed using the MetaMorph[®] image analysis software (Universal Image Corp.) Results are expressed as the mean optical density for eight different digital images.

6. Statistic Analysis

Each measurement is shown as mean \pm standard deviation. All pair wise differences between the measurements of two groups were examined by a student *t*-test. A *p* value of < 0.05 was considered significant.

III. RESULTS

1. Efficacy of PDRN for augmentation of flap viability

Upon examination of the flap survival 3 days after flap elevation, $83.7\pm 9.7\%$ mean flap survivals was noted in the PDRN group and $84.5\pm 13.2\%$ mean flap survivals in the control group (Figure 2). On the postoperative day 7, the PDRN group ($78.8\pm 8.9\%$) revealed increased flap survival compared the control group ($63.9\pm 13.3\%$) with statistical significance ($p < 0.05$). On digital photograph obtained on postoperative day 10, ischemic necrosis occurred in half of the distal portion of the flap in control group ($51.7\pm 6.7\%$). However, local subdermal injection of 1.2 ml of PDRN and daily intraperitoneal injection increased skin flap viability with significance ($79.5\pm 6.3\%$, $p < 0.05$) (Figure 3).

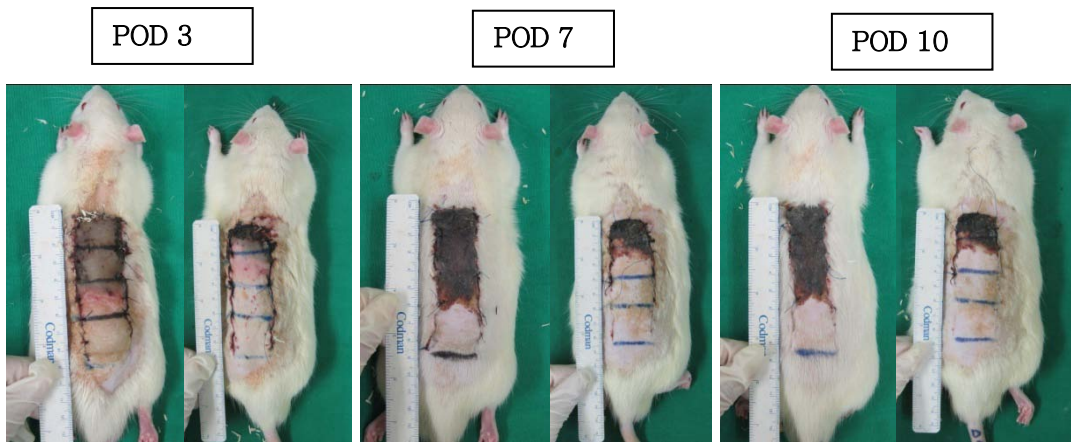


Figure 2. Comparison of flap survival area. Representative photographs of skin flaps at postoperatively day 3, 7, and 10. The survival flaps in PDRN group is significantly larger than control group in the course of time.

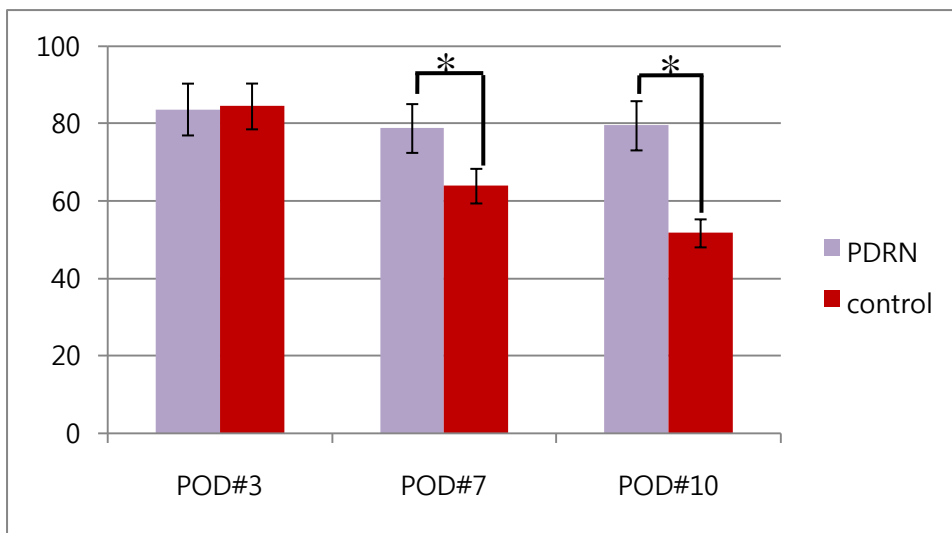
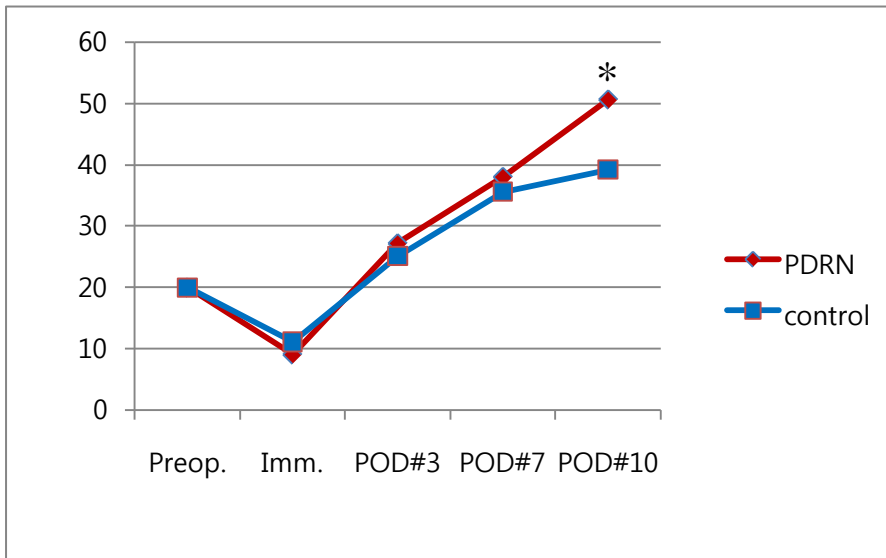


Figure 3. The mean survival rates of flap. The mean survival rates of flap in PDRN group are significantly larger than control groups on postoperative day 7 and 10 (* $p < 0.05$).

2. Effect of PDRN on skin flap blood flow

Before flap elevation, the initial vascular flow measured in the experimental groups and control group showed no statistical differences. Immediately after the elevation of the flap, there were statistically significant differences of vascular flow in the mid-proximal area and mid-distal area (Figure 4 and 5). In proximal area of the flap, the blood flow of PDRN group was significantly increased on postoperative day 10. In mid-proximall part of the flap, blood flow was significantly increased than other group at immediate postoperative period and on postoperative day 10. In the mid-distal part of the flap, the blood flow of PDRN group was significantly increased on day 3, 7, and 10 (Figure 4). In distal area of the flap, blood flow was significantly increased than other group on postoperative day 10 ($p<0.05$). Therefore, vascular flow of the flap in the PDRN group had significantly increased the amount of flow at all portion of the flap compared to control groups on postoperative day 10 ($p<0.05$) (Figure 5).

Proximal



Mid-proximal

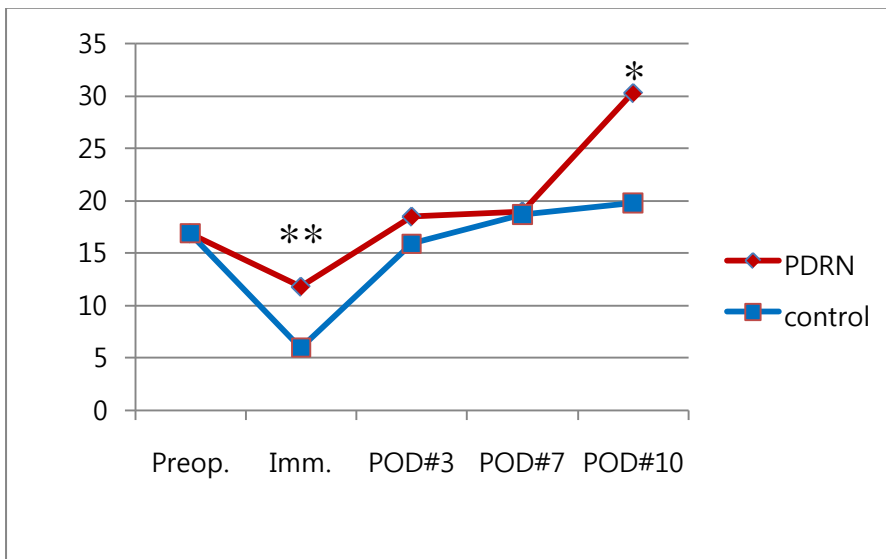
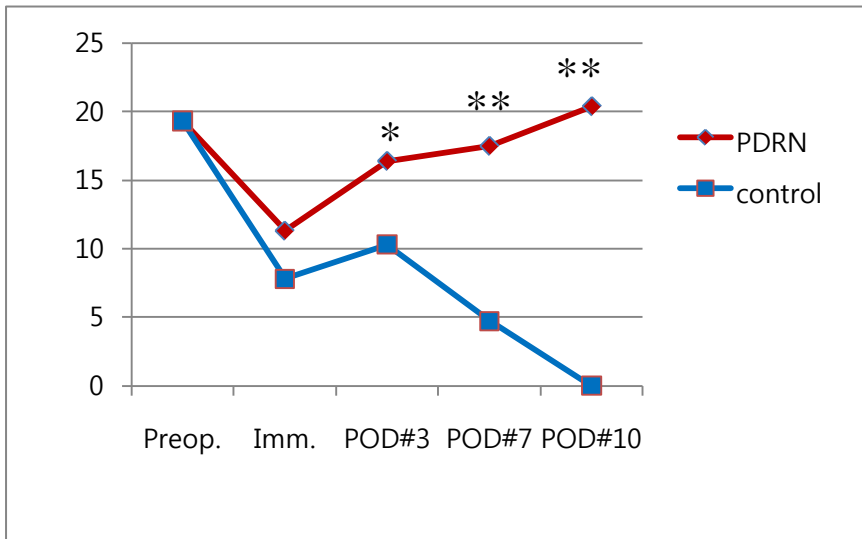


Figure 4. The result of blood flow measuring in proximal area of flap. In proximal part of the flap, the blood flow of PDRN group was significantly increased on postoperative day 10. In mid-proximal part of the flap, blood flow of PDRN group was significantly increased than control group at immediate postoperative period and on postoperative day 10. (* $p < 0.05$, ** $p < 0.01$)

Mid-distal



Distal

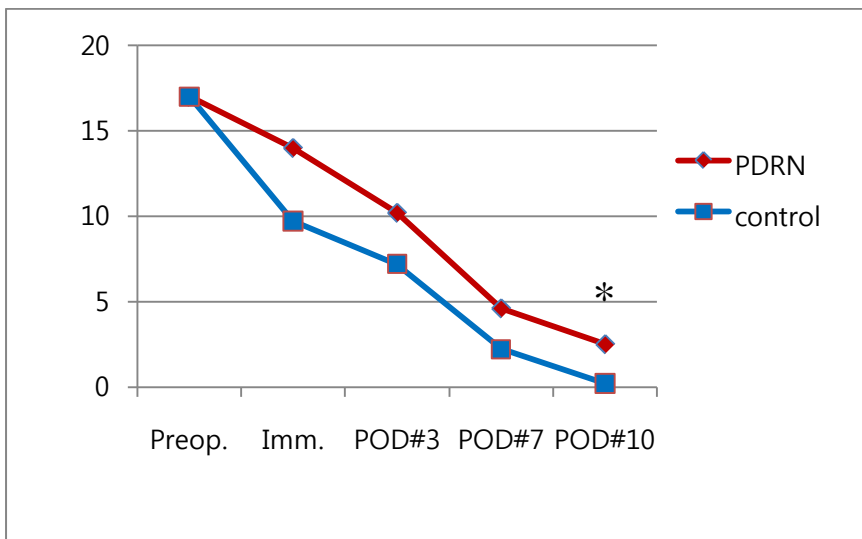


Figure 5. The result of blood flow measuring in the distal part of flap. In the mid-distal part of the flap, the blood flow of PDRN group was significantly increased on postoperative on day 3, 7, and 10. In distal part of the flap, blood flow of PDRN group was significantly increased than control group on postoperative day 10.

(* $p < 0.05$, ** $p < 0.01$)

3. Effect of PDRN on capillary density in skin flaps

To calculate the amount of neovascularization, CD31 positively stained vessels were counted, and the comparative analysis of the number of vessels at each high power field (x200) was performed on postoperative day 10 (Figure 6). The number of CD31 positively stained vessel was 6.70 ± 1.88 in samples from control group. Meanwhile, the average number of the blood vessels in PDRN group was 19.40 ± 5.58 , which was higher than control groups. The differences between the PDRN group and control group were very significant ($p < 0.001$) (Figure 7).

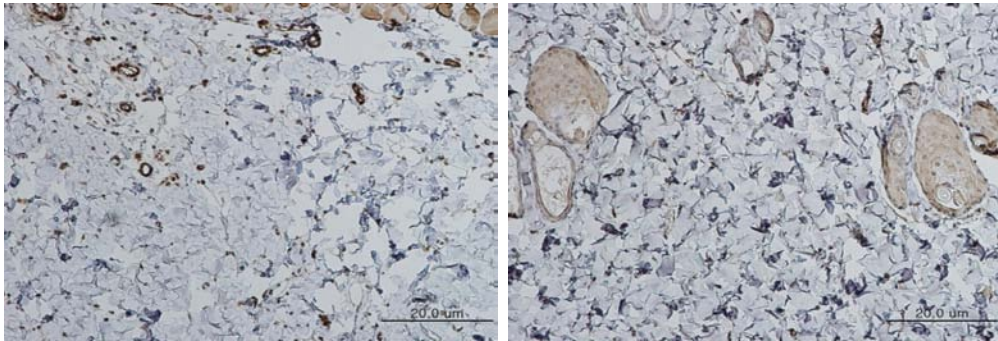


Figure 6. The microscopic image for counting blood vessels in flap ($\times 200$). In PDRN group (left), more CD31 positively stained vessels in the tissue were noted than control group (right).

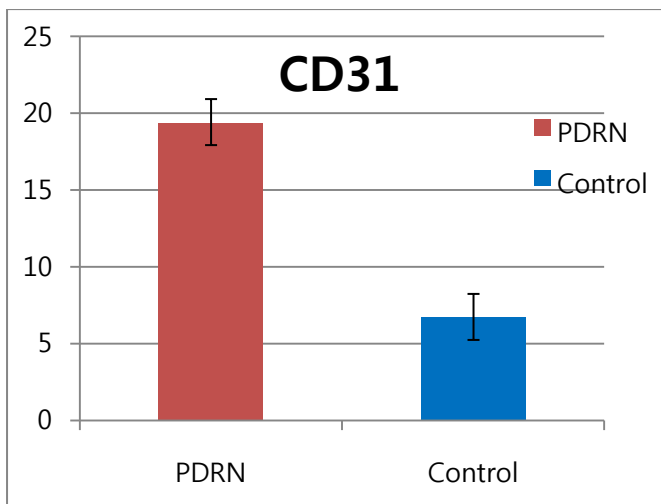


Figure 7. The comparison of CD31 positively stained vessel count. The mean number of the blood vessel in PDRN group was 19.40 ± 5.58 , while $6.70 (\pm 1.88)$ in control group. PDRN group showed higher mean number of blood vessels than control group with statistically significance ($p < 0.001$).

4. Effect of PDRN on VEGF expression in skin flaps

The expression level of VEGF was semi-quantitatively analyzed using the MetaMorph[®] image analysis software (Universal Image Corp.) The VEGF expression was 12398 ± 6996 in samples from control group (Figure 6). However, local subdermal and intraperitoneal injection of PDRN increased VEGF expression (22056 ± 13016) with significance ($p < 0.05$) (Figure 8 and 9).

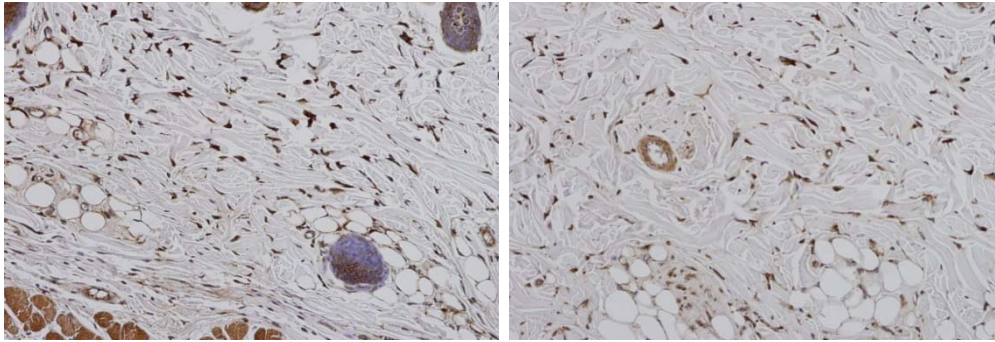


Figure 8. Microscopic image of VEGF immunohistochemistry ($\times 200$). The expression of VEGF was increased in PDRN group (left) than the control group (right). (brown area; the site of VEGF protein expression)

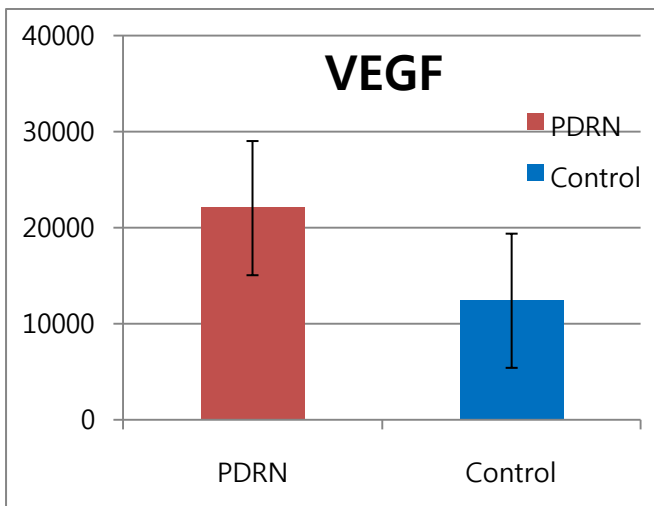


Figure 9. The quantitative analysis of VEGF expression. The expression of VEGF in PDRN was significantly increased compared to the control group. ($p < 0.05$).

IV. DISCUSSION

In this study, we demonstrated that PDRN augmented the blood perfusion of ischemic skin flap, and therefore increased the survival rates of rat skin flaps. Additionally, significantly increased VEGF expression levels and capillary density were observed in the rat skin flaps receiving PDRN treatment.

Immediately after skin flap elevation, sympathectomy and catecholamine release all direct toward constriction of blood vessel, which ultimately leads to decreased blood flow. Also, arteriovenous shunt forms at the distal part of the flap followed by decreased blood pressure. If this whole process goes beyond the tolerable threshold of the flap, partial flap necrosis develops at the distal region¹.

Therapeutic angiogenesis using angiogenic growth factors, particularly vascular endothelial growth factor (VEGF), is expected to be a valuable treatment.¹³⁻¹⁵ The most used approaches are based on VEGF local delivery or gene therapy, but they failed to meet the expected primary goals of therapy. Adenosine receptor stimulation can induce VEGF expression in many types of cells and this may be achieved by stimulating the A_{2A} or A_{2B} receptor or both, following the signaling pathways activated by hypoxia. Polydeoxyribonucleotide (PDRN), through the adenosine A₂ receptors, has been known to accelerate production of Vascular Endothelial Growth Factor (VEGF) in DM and burn patients through adenosine A₂ receptors.^{11,12}

Several experimental studies demonstrated the successful use of PDRN for therapeutic angiogenesis. Bitto et al investigated the effects of PDRN in an experimental model of hind limb ischemia (HLI) in rats to stimulate vascular

endothelial growth factor (VEGF)-A production and to avoid critical ischemia. As a result, administration of PDRN dramatically increased VEGF mRNA throughout the study¹³. Recently, Altavilla advocated PDRN as a safe pharmaceutical agent to induce therapeutic angiogenesis in peripheral artery occlusive disease and in diabetic foot ulcers.¹⁴

Stimulation of the adenosine receptor has been shown to result in fibroblast differentiation and maturation, increasing the rate of granulation tissue and consequently accelerating the repair process¹⁶⁻¹⁸. Previous observations from studies by Bitto et al strongly suggest that PDRN acting through adenosine receptors is able to stimulate VEGF production as well as fibroblast maturation in incisional skin wounds with a concrete improvement in the healing process.¹⁰ From the studies on thermal injury, polydeoxyribonucleotide is thought to be an effective therapeutic approach to improve clinical outcomes.

It has been shown previously that PDRN is a tissue repair-stimulating agent in some pathologies including radiodermatitis¹⁹, skin graft donor sites²⁰, and photorefractive keratectomy²¹. It is likely that PDRN is cleaved by active cell membrane enzymes, providing a source for deoxyribonucleotides and deoxyribonucleosides that can increase the proliferation and activity of cells of different tissues.²² Overall, PDRN derivatives could act as growth promoters for fibroblasts,⁸ osteoblasts, ECs, and neuroglia^{23,24} and could stimulate nucleic acid synthesis through the salvage pathways¹⁷ and/or binding to the purinergic receptors in response to a variety of stimuli, including hypoxia.^{22,25}

On our result, the flap survival area was larger in the PDRN treated group on

postoperative day 7 and 10 than the control group (Figure 3). However, greater flap survival was noted in the control than the PDRN group on the postoperative day 3 without statistical significance. This result is thought to be from the limited amount of study and control group in our experiment. Serial measurements of blood perfusion before injection of PDRN, 2 days after injection, immediately after flap elevation, and 3, 7, and 10 days after flap elevation demonstrated that the blood flow was increased on the proximal, middle, and distal area and that there was statistical significance throughout all area on the 10 days after PDRN injection. In addition, we observed greater amount of blood flow in PDRN group compared to the control group immediately after flap elevation, especially in the mid-proximal and mid-distal portion of flap (Figure 4,5). Increased blood flow by PDRN was evident until postoperative day 10, and the differences between PDRN treated group and other groups seemed to increase gradually with time. The mid-distal and distal portion of flap survival in the control group decrease in flap survival with time and this can possibly be explained by irreversible ischemic changes in the distal portion which eventually leads to partial flap necrosis even after blood flow increase if a critical time point has been reached. PDRN is a potent stimulator known to accelerate production of VEGF through the adenosine A2 receptors and in our results, we think that augmentation of rat skin flap viability was mediated by overexpression of the VEGF to influence the perfusion of ischemic skin flaps.

Also, on the immunohistochemical examination, we have known that the number of blood vessels and the VEGF expression in tissue samples were significantly increased in the PDRN treated group (Figure 7 and 9). We think that enhancement

of the survival rate of skin flaps was mediated by new vessel formation induced by increased production of VEGF by PDRN.

V. CONCLUSION

We have introduced PDRN into a rat ischemic skin flap and resulted in increased VEGF expression, number of capillaries, and blood flow to the flap, thereby enhancing flap survival rates.

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ABSTRACT(IN KOREAN)

PDRN(Polydeoxyribonucleotide, Placentex[®])이

피관의 생존에 미치는 영향

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악종종양 절제술, 외상, 선천성 기형 등으로 인한 연부 조직의 부족이나 결손 등을 재건하기 위해서 피관이 효율적이며 최근 각종 피관술의 빈도 및 적응증이 증가하고 있다. 피관술 실패의 대표적 원인으로는 허혈성 괴사, 감염, 혈종 등이 있으며 이중 혈액 순환의 장애로 괴사가 일어나는 경우가 제일 흔하며 이러한 허혈성 괴사를 줄이거나 막기 위해 많은 노력들이 있어 왔다. Polydeoxyribonucleotide(PDRN)는 송어 정자에서 추출 과정을 통해서 얻을 수 있으며 아데노신(A2) 수용체를 통해서 작용하여 당뇨나 화상에서 VEGF의 생산을 촉진시키는 것으로 알려져 있다. 본 연구에서는 원위부 피관의 괴사의 가장 큰 원인인 혈행 장애를 이러한 PDRN의 효과들이 피부 피관의 생존율을 증가시킬 수 있다는 가설 하에 PDRN의 직접적 투여를 이용하여 이를 국소피관 거상 시 투여하여 피관의 생존율의 차이를 비교 관찰하였다.

Male Sprague-Dawley rat(300-400g) 20 마리를 준비하였다. 이들을 두 그룹으로 나누는데 한 그룹(n=10)은 PDRN (Placentex Integro[®], 5.625mg/3ml, Mastelli Farmaceutica, Italy) 투여하는 실험군이며 다른 그룹(n=10)은 생리 식염수를 투여하는 대조군이다. 백서의 등에 3 X 9 cm 크기의 꼬리쪽에 기저를 두고 있는 피관을 작도하였다. 실험군에는 PDRN 8mg/kg 를 피관의 12 곳의 정해진 위치에 0.1ml 씩 피내 및 복강 주입하였다. PDRN의 피내 투여 시기는 피관 수술 48 시간 전과 피관 수술 직후로 하며 술 후 1일부터 10일까지는 동량을 매일 복강 내로 주입하고 대조군에는 동량의 생리식염수를 투여하였다. 전처치 전, 수술 직후 및 수술 후 3일, 7일, 10일 후에 피관 생존 면적 및 혈류의 측정하고 수술 후 10일에 생존한 피관의 가장 원위부 중양에서 조직을

얻어서 H&E 염색과 VEGF 면역조직 화학염색(immunohistochemistry)을 시행하여 VEGF 를 측정하였다. 또한, CD31 을 측정하여 얻어진 결과를 비교, 분석하였다.

피관 생존 면적을 3 일째 확인한 결과, 실험군에서는 83.7%, 대조군은 84.5% 생존율을 보여 통계적으로 차이가 없었으며, 수술 후 7 일째 ($78.8 \pm 9\%$ $63.9 \pm 13\%$, $p=0.004$) 와 10 일째는($79.5 \pm 6\%$, $51.7 \pm 7\%$, $p=0.0009$)는 실험군이 통계적으로 유의하게 생존율이 증가된 것을 확인할 수 있었다. 피관의 혈류량을 측정한 결과에서는 수술 직 후, 피관의 중근위부와 중원위부에서 유의한 혈류량의 증가 소견이 확인되었으며, 근위부와 원위부에서는 수술 후 10 일째 실험군에서 혈류량이 증가되었다. 신생혈관의 생성을 측정하기 위하여 CD 31 양성인 혈관을 확인한 결과에서는 실험군에서 대조군보다 유의하게 증가된 수를 확인할 수 있었으며, VEGF 발현량의 평가에서는 PDRN 군이 실험군에 비하여 통계적으로 유의한 발현량을 나타내었다.

본 실험의 결과로 PDRN을 쥐의 국소 피관에 주입하였을 때 VEGF의 발현이 증가되고 모세혈관의 수가 증가하며, 피관의 혈류가 증가하여 피관의 생존률이 높아지는 것으로 나타났다.

핵심되는 말 : PDRN, 백서 피관, 생존율, 혈류량, VEGF