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동물모델에서 *Dendropanax morbifera* 추출물의 상처 치유 효과

조선대학교 대학원

의 학 과

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ABSTRACT

동물모델에서 *Dendropanax morbifera* 추출물의 상처 치유 효과

Wound-healing effect of *Dendropanax morbifera* extracts in animal models

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PGE_2는 상처치유의 중요한 매개체로 알려져 있는데 NAD^+ 의존 15-hydroxyprostaglandin dehydrogenase (15-PGDH)에 의해 분해되기 때문에 15-PGDH 억제제는 PGE_2의 농도를 높일 수 있다. 두릅나무과에 속하는 황칠나무(*Dendropanax morbifera* Leveille)는 우리나라의 남부 해안지역과 제주도에서 자생하는 상록활엽교목으로 최근 황칠나무 헥산 추출물(*Dendropanax morbifera* hexane extracts, DMHE)이 15-PGDH를 억제하여 PGE_2를 증가시키고 *in vitro*에서 상처치유에 효과가 있는 것으로 밝혀졌다. 하지만 아직 상처치유에 대한 DMHE의 동물 모델에서의 결과는 없는 상태이다. 따라서 본 연구에서는 두 가지 동물모델에서 DMHE의 상처치유 효과를 연구하였다.

마우스에서 급성상처와 만성상처를 재현할 수 있는 피부전층 상처모델과 하지하혈 마우스에서 발 상처 모델을 만들어 실험했다. 피부전층 상처모델은 마우스의 등에 5 mm 직경의 원형상처를 유발하고 대조군, TGF-β1 20 ng/day 군, DMHE 16.5 μg/day 군, DMHE 66 μg/day 군의 네 가지 군으로 나누어 각각의 약물을 일주일 동안 매일 처리했다. 상처 사이의 거리를 측정하여 대조군과 비교하고 그 비율을 상처치유 효능을 구했다. 하지허혈 마우스에서의 발 상처 모델을 만들기 위해 마우스의 좌측 대퇴동맥의 근위부와 원위부를

결찰하고 발등에 2x5 mm의 상처를 유발했다. 마우스는 연고 대조군, 3% *Centella Asiatica* 연고 투여군, 3% DMHE 연고 투여군, 경구 대조군, 1mg/mL/kg DMHE 경구 투여군, 10 mg/mL/kg DMHE 경구 투여군으로 나누고 각각을 1주일 동안 처리했다. 분자 수준에서 상처치유에 대한 DMHE의 영향을 알아보기 위하여 4주째 발 상처조직과 하지의 반박양근과 비장근을 채취하고 상처치유 및 혈관신생과 관련된 connective tissue growth factor (CTGF), vascular endothelial growth factor-A (VEGF-A), CD 31, α-smooth muscle actin (α-SMA), 15-PGDH의 mRNA 발현 확인을 real-time PCR 방법으로 측정하였다.

피부전층 상처모델에서 DMHE를 16.5 μg으로 처리한 경우 상처간 거리는 2.02 mm으로 59.5%가 치유되었고, 66 μg을 처리한 경우에는 0.13 mm로서 97.4%의 치유력을 보여 주었다. DMHE 66 μg/day 군에서 음성 및 양성 대조군에 비하여 198%와 148%의 향상된 상처 치유 효능을 보였다. 정상 상처에서 3% DMHE 연고투여가 2주째 98%의 상처 치유능으로 다른 군과 비교하여 통계학적으로 의미 있는 차이를 보였으나 (p=0.040) 허혈 상처에서 연고치료는 각 치료군 사이에 의미 있는 차이를 보이지는 않았다. 허혈 상처에서는 DMHE 10 mg/mL/kg 경구치료 군에서 90.7%의 상처 치유능을 보였으며, 다른 군과 비교하여 통계학적으로 유의한 상처치유 효과를 보였다 (p=0.025). Real time PCR 결과, 경구와 국소 DMHE 투여 후 상처와 하지근육 모두에서 상처치유에 관계하는 CTGF의 발현은 증가하였으나 나머지 VEGF-A, CD31, α-SMA, 15-PGDH의 발현양상과 상처치유와의 상관관계는 없었다.

이상의 결과는 DMHE가 마우스의 피부 전증 상처모델과 하지허혈 발 상처모델에서 상처 치유에 효과가 있음을 보여주었다. 특히 하지허혈 발 상처 모델에서 정상 상처는 DMHE 연고치료가, 허혈 상처는 DMHE 경구치료가 상처치유에 효과가 있었다. 또한 DMHE 투여 후 상처치유에 CTGF의 과발현이 관여하는 것으로 보인다. 그러므로 DMHE는 허혈성 상처에서는 국소보다는 경구투여 또는 국소와 경구 투여를 병합하는 것이 좋을 것으로 사료된다.

I. Introduction

Korea's adoption of westernized diets and its aging society have led to increases in cases of obesity and diabetes, and chronic diseases, such as hypertension, hyperlipidemia, and arteriosclerosis, have become a serious social issue. Consequently, chronic wounds, such as ischemic ulcers due to peripheral arterial diseases and diabetic ulcers due to diabetic complications, are becoming more frequent [1]. Severe conditions that do not respond to surgical or endovascular treatment require expensive long-term treatment and may lead to amputation or even death, which negatively affects individuals' quality of life and causes various social and economic problems,. Main causes of non-healing wounds include ischemic ulcers resulting from peripheral arterial diseases and diabetic ulcers [2, 3].

1. Ischemic ulcers are caused by peripheral arterial diseases

Peripheral arterial diseases occur when atherosclerosis causes the occlusion of peripheral blood vessels. This most commonly occurs in patients exhibiting risk factors, such as old age, hypertension, diabetes, and hyperlipidemia. When ischemic wounds are not managed with appropriate revascularization, approximately 25-40% of such cases will result in amputation [4, 5]. Even if the patient has received surgical treatment to improve blood flow, infection or amputation may follow if wounds are managed inappropriately. Management of wound-healing is therefore essential, and although surgical treatment methods have been established to some extent, adjuvant treatment methods that promote wound-healing have not been established. Studies of methods promoting wound-healing are essential because appropriate wound management will enable the limbs of even high-risk patients, who cannot be surgically treated, to be saved.

2. Diabetic ulcers

According to the 2012 Korean Diabetes Research Report published by the Korean Diabetes Association, the number of diabetics in 2050 is predicted to be approximately twice the present number because both life expectancy and the prevalence of diabetes are increasing every year [6]. Unfortunately, there are no domestic data on the subject, but approximately 25% of patients with diabetes in the USA eventually require treatment for diabetic ulcers, and the hospitalization and treatment costs of these patients account for up to 20-40% of diabetes-related treatment costs [7]. Since active insulin usage extends life expectancy of patients with diabetes, attention to chronic complications has become increasingly important.

Diabetic ulcers commonly result in infection, which rarely occurs in the absence of any wound. Along with adjuvant treatment for wounds that have already formed, strict regulation of blood glucose, diabetic neuropathy, and vasculopathy is critical. Importantly, treatment of diabetic ulcers is more difficult in patients who also suffer from peripheral arterial diseases, which accounts for 20-58% of all diabetics [8].

3. Wound-healing

Wound-healing consists of a series of complicated processes that restores the structure of the original tissues and their ability to function [9, 10]. It normally proceeds through phases of hemostasis, inflammation, proliferation, and remodeling. These phases include a series of complicated processes involving diverse cells, mediators, and the extracellular matrix. Normal interactions between blood platelets, neutrophils, macrophages and their generation of growth factors, cytokines, and proteinases are necessary for normal wound-healing.

Acute wounds achieve restorated structure and function by an orderly and timely reparative process. In contrast, chronic wounds fail to restore their functional integrity and remain in the inflammatory phase without proceeding to closure. Failure to recover the structural durability of tissue during wound-healing results mainly from the inability of fibroblasts to synthesize or recompose the extracellular matrix to the same extent as the original tissue. In conditions like peripheral arterial diseases and diabetes, normal healing processes do not occur in either the inflammatory or the proliferative stages [11].

In such chronic wounds, inflammatory conditions persist abnormally, and inflammatory lyases such as matrix metalloproteinase (MMP) increase [12]. Thus the reduced ability of fibroblasts or keratinocytes to synthesize DNA and cause cell division does not recover. In cases of peripheral arterial diseases and diabetic ulcers, inflammatory states continue, and the expression of angiogenesis factors decreases, which further delays wound-healing [13]. Currently, our incomplete understanding of mechanisms of wound-healing often leads to the disappointing results of modern therapies. Therefore, new therapeutic strategies for abnormal wound-healing should be designed to treat these pathologic conditions.

4. Wound-healing models

Chronic non-healing wounds presented at clinics are heterogeneous according to etiology, duration, and other factors that delay wound-healing. In addition, it is difficult to collect repeated biopsies from patients for the purpose of understanding the mechanistic underpinnings of chronic wounds. Because the complexity of wound-healing *in vivo* cannot be fully reproduced in *in vitro* models, a preclinical model of wound-healing is necessary. *In vivo* wound experiments remain the most predictive models for human wound-healing

because they can accurately present complete wound-healing [14]. Small animals such as mice or rats are especially useful in these studies because they are economic, reproducible, and easy to handle and maintain for the investigation of specific mechanisms of wound-healing and regeneration.

The splinted excisional wound model, ischemia reperfusion model, and ischemic flap model are well-known, reproducible murine wound-healing models [15]. It is reasonable to induce the wound on the foot rather than any other part of the body to produce the same wound condition as peripheral arterial disease and diabetic foot in humans. Additionally, producing an ischemic limb condition is necessary to investigate the process of ischemia-induced wound formation and subsequent revascularization. The ischemic hind-limb foot wound animal model, which is generated by unilateral ligation of the femoral artery, has been widely used as a model of human peripheral arterial disease [16-20].

5. The present state of wound-healing adjuvant therapy studies

Many chronic wounds fail to heal with conventional therapy. Identifying therapeutic targets for poor wound-healing has been difficult due to the condition's complexity. In addition to general wound management, including continuous wet dressing of wounds, debridement, infection control, and reduction of pressure on the wound, studies relating to adjuvant wound management are currently being conducted for both ischemic ulcers caused by peripheral arterial disease and diabetic ulcers. Most of these therapies focus on increasing the expression of growth and angiogenesis factors.

Reports have indicated that injecting vascular endothelial growth factor (VEGF) into ischemic wounds improves angiogenesis and tissue

perfusion in many animal models [21-23]. However, VEGF injection still has limited clinical application for humans because of the possibility of tumor occurrence, rapid growth, and metastasis, and the FDA has not approved any angiogenic factor therapies for ischemic wound treatment.

Although a number of studies have been conducted concerning the use of various growth factors for adjuvant treatment of diabetic ulcers, platelet derived growth factor (PDGF) is the only therapeutic agent to be approved by the FDA thus far [24]. PDGF promotes angiogenesis, fibroblasts activity, and epithelial cell movements by secreting VEGF, and it plays an important role in wound-healing. However, since the FDA has recently recommended using PDGF with care because of its carcinogenic potency, it should only be used on wounds that do not respond to conventional treatment.

Eicosanoids such as prostaglandin E_2 (PGE_2) have been known to heal wounds effectively [25]. When wounds occur, inflammatory reactions and fibrosis are controlled by the upregulation of cyclooxygenase 2 (COX-2), which increases PGE_2 (Figure 1). The various biological effects of PGE_2 are mediated by four EP receptors, which show differential patterns of tissue distribution (Figure 2). Reports have indicated that PGE_2 suppresses leukocytosis and induces the proliferation of fibroblasts to promote wound-healing [26]. In addition, Kamoshita [27] reported that PGE_2 promotes angiogenesis in surgical wound-healing via the upregulation of VEGF. However, similar to VEGF and PDGF, PGE_2-related pharmaceutical preparations have not yet been approved by the FDA because the results of related clinical studies were insignificant.

6. PGE_2-related studies of wound-healing are necessary

When COX-2 is upregulated after damage, PGE_2 regulates the cell

migration and proliferation that underlie re-epithelialization and angiogenesis. PGE_2 is also an important mediator of dermal wound-healing. It exerts specific effects on fibroblasts and prevents excessive scarring by inhibiting the differentiation of fibroblast into myofibroblast [28]. That being said, PGE_2 is known to have a very short half-life *in vivo* because it is metabolized quickly by the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH), an enzyme that decomposes PG [29] (Figure 3). 15-PGDH catalyzes the oxidation of 15-hydroxyl group to keto-derivatives (Figure 4). These keto-derivatives exhibit greately reduced biological activities. Thus, PG is elevated in tissues where 15-PGDH is not expressed and is decreased in tissues where 15-PGDH is present. If 15-PGDH is suppressed, the concentration and pharmacological action of PGE_2 is to be expected to increase. Accordingly, 15-PGDH inhibition may be a valuable target for wound-healing.

Although many studies have reported the effectiveness of PGE_2 in wound-healing [26, 30], its use in clinics has been limited because of its cost, required intravenous administration, and frequency of adverse side effects [31]. Therefore, identifying substances that can increase the activity of PGE_2 is an advantageous alternative to administering PGE_2 directly.

7. *Dendropanax morbifera* Leveille

Dendropanax morbifera Leveille (Figure 5) belongs to the Araliaceae family and grows naturally on the southern coast of Korea and Jeju Island. When the bark of this tree is wounded, resin called Hwangchil is released. Various studies have shown that the *Dendropanax morbifera* extracts possess antidiabetic[32], antiarteriosclerosis[33], antioxidation, and anticancer properties [34]. Recently, *Dendropanax morbifera* extracts were also found to effectively suppress 15-PGDH and

increase PGE_2 [29].

In a study exploring the biological effects of *Dendropanax morbifera* extract [35], the *Dendropanax morbifera* hexane extract (DMHE) was shown to strongly increase PGE_2 by suppressing 15-PGDH. In A549 cells, PGE_2 concentration increased dose-dependently in response to all extract fractions, and DMHE was the most potent fraction. In addition, the time-dependent change in extracellular PGE_2 in HaCaT cells was confirmed, and the intracellular and extracellular levels of PGE_2 significantly increased compared to the control group.

DMHE significantly increased the expression of COX-1 and MRP4 mRNA, while it decreased the expression of PGT and 15-PGDH in HaCaT cells [35]. Increased expression of COX-1 mRNA by DMHE in addition to 15-PGDH inhibition could result in a huge elevation in intracellular PGE_2 levels. Increased levels of MRP4 and decreased levels of PGT seem to result in extracellular PGE_2 levels and sustain the activity of PGE_2 over a longer period in pericellular space.

In an *in vitro* scratch assay, DMHE was confirmed to a similar effect as TGF-β1 on keratinocyte migration, although it might differ in its capacity [35]. It was found to minimize scar formation and promptly proceed with wound-healing. Moreover, the wound-healing ability of DMHE was found to be equivalent to or exceed that of TGF-β1, which is one of the cytokines secreted from various types of cells (platelets, macrophages, and fibroblasts) and is traditionally known to be effective in wound-healing. Since its discovery in 1980, TGF-β1 has been widely researched in various wound-healing models [36, 37]. It can be used as wound-healing agent that increases monocyte and fibroblast inflow and collagen accumulation [38, 39].

Additionally, COX-1 expression plays a greater role in wound-healing

than COX-2. Hence, when the selective inhibitor of COX-1 SC560 and non-selective inhibitor of COX-1/2 naproxen have been used, DMHE-induced wound-healing was delayed [35]. When the COX-2 inhibitor celecoxib was used, no effect on wound-healing was observed. These findings were also consistent with the mRNA gene expression profile, affected by DMHE. Together, these results suggest that COX-1 contributes more to the rise of PGE_2 and wound-healing than COX-2.

In vitro scratch analysis in HaCaT cells confirmed that DMHE accelerates wound-healing [35]. These effects were not observed when 15-PGDH and supplementary NAD^+ were injected at the same time, implying that the wound-healing effect of DMHE is mediated directly by PGE_2. Thus, the potent inhibitory activity of DMHE might effectively treat diseases requiring high PGE_2 levels as well as wound-healing.

The exact molecular mechanism underlying the DMHE's ability to control 15-PGDH and PGE_2 in wound-healing has not yet been identified; there have been no reports of these extracts for the treatment of diseases by using molecular mechanisms associated with wound-healing. In addition, no studies have demonstrated the wound-healing effect of DMHE *in vivo*. Therefore, the purpose of this study was to determine whether DMHE effectively heals wounds in *in vivo* mouse models of wound-healing.

II. Materials and methods

1. Preparation of the *Dendropanax morbifera* leaf extract

(1) Extracts

At room temperature, the leaves of *Dendropanax morbifera* were dried in the shade, and the dried leaves were extracted three times by using methanol. The methanol extracts were filtered through Whatman No. 1 filter paper, and the combined methanol extracts were condensed in vacuum, using a rotary evaporator.

The methanol extracts were suspended in air and then fractioned according to their polarity from hexane, diethyl ether, ethyl acetate, n-butanol, and water. Each extract was condensed using a rotary vacuum evaporator (Figure 6).

(2) Fractionation and separation process

Using the gradient solvent system [$CHCl_3$ (0.1% HCOOH) 100% through $CHCl_3$ (0.1% HCOOH):MeOH (0.1% HCOOH)/55:45 for 120 min] Isolera Flash Purification System (BIOTAGE, Sweden), I performed activity-guided fractionation of hexane extracts at 254 nm and 280 nm to make 10 fractions (DEE01-DEE10). Each fraction was monitored and detected using HPLC in the gradient MeCN-H_2O (0.1% HCOOH) solvent system. In addition, spots were detected by thin-layered chromatography under UV light (254 nm) or heated to 110 ° C after being sprayed with vanillin-H_2SO_4.

Using a Sunfire TM Prep C18 (10 × 250 mm and 19 × 150 mm, 5 μm) column, which is a gradient solvent system of MeCN/H_2O that contains 0.1% formic acid (30:70 → 100% MeCN), repeated semi-preparative HPLC experiments were performed for 40 minutes. From these fractions, compounds were finally separated.

A previous study by Moon [35] revealed that the hexane extract is the most potent inhibitor of 15-PGDH, so we selected DMHE as a agent for wound-healing in animal experiments.

2. *In vivo* wound-healing effect

To investigate *in vivo* wound-healing effect of DMHE, an experiment was performed in the following manner. Firstly, 7-week-old ICR male mice (average weight: 25 g) were purchased from Damul Science Co. (Daejuen, Korea). After an adaptation period of 1 week, they were anesthetized through intraperitoneal administration of the anesthetic drug (Zoletil, 50 μg), and a circular wound of 5 mm in diameter was made on the back of mice. Animals were divided into four different groups, and treatment was applied to the wound every day after the injury (Table 1). Each drug (10 μL) was applied to wounded area, and the degree of healing was observed through tissue staining at the same time. Tissue staining was performed by fragmenting wounded tissue in the mice of each group by using hematoxylin-eosin (H&E). The recovered distance in the stained tissue was measured using a microscope. The end-point of cellular re-epithelialization during wound-healing was marked with an arrow, and the distance between two arrows was measured. In addition, the measured distance between wounds was compared to that of the control group, which was calculated as a wound-healing efficiency.

Animal experiments were performed in accordance with Chonnam National University Animal Experiment Ethics Committiee (IRB) approval (CNU IACUC-YB-R-2012-12).

3. Foot wound-healing in a mouse model of hind-limb ischemia

Experiments were conducted on mouse models of hind-limb ischemia to identify the wound-healing effects of DMHE on ischemic ulcers in peripheral arterial diseases. No animal models for foot ulcers in

peripheral arterial disease have been established for the study of non-healing wounds. To establish this kind of animal model, we made a foot wound in a mouse model of hind-limb ischemia.

The animal model of hind-limb ischemia described here is an elaboration of a previously published model [18, 40]. We combined the hind-limb ischemia models with foot wound models, as described by Lau [41].

Seven-week-old male C57BL/6 mice were subjected to inhalation anesthesia by using isoflurane after undergoing an adaptation period of one week. An incision was made in the left inguinal region of each mouse to expose the proximal and distal femoral artery, which was ligated and cauterized using an electrocautery device. The incision site was sutured using Nylon 5-0 threads. A site to be incised was marked on the dorsum of the foot on the same side by using a pre-prepared 2 × 5 mm grid, and the entire layer of the skin on the site was exfoliated and removed to make a wound. Another wound was made using the same method on the opposite side, where protraction was not made. Mice were divided into six groups (Table 2): an ointment vehicle control, 3% *Centella Asiatica* ointment application, 3% DMHE ointment application, oral control, 1 mg/mL/kg DMHE (oral administration) group, and 10 mg/mL/kg DMHE (oral administration). They were treated with their respective drugs from the day of surgery for 7 days. The weight of each mouse was measured on the day of surgery, and on the 3rd, 7th, 14th, and 21st day after surgery, while the images of the two wounds were taken using a dissecting microscope (SZ61, Olympus, Japan) and the area of the wound was calculated using the Image J program (NIH, Bethesda, MD). After surgery, the areas of the wound on individual days of measurement were divided by the area of the wound on the day of surgery, to calculate the wound-healing efficiency. On the 28th day,

tissues including the foot wound, gastrocnemius and semimembranosus muscles were collected to identify the mRNA expression of connective tissue growth factor (CTGF), VEGF, CD 31, α-SMA and 15-PGDH.

Animal experiments were performed in accordance with Chosun University Institutional Animal Care and Use Committee approval (CIACUC2014-A0010).

4. Quantitative real-time PCR

To find out the influence of *D. Morbifera* on growth of connective tissue, angiogenesis and myofibroblast formation, mRNA expression of CTGF, VEGF-A, CD31 and α-SMA was measured in tissue samples from foot wound, gastrocnemius and semimembranosus muscles. In addition, to determine the influence of DMHE on inhibition of 15-PGDH activity, mRNA expression of 15-PGDH was measured.

After the tissues were disrupted and homogenized, total cellular RNA was isolated from cells using TRI reagent (RNAiso Plus, Takara) according to manufacturer's instructions.

cDNA for each RNA sample was synthesized in 20 μL reactions using the superscript first strand synthesis system for reverse transcription-PCR (Invitrogen, USA) following manufacturer's instructions. PCR reaction contained 4 μL of 1:5 diluted cDNA, 4 mM $MgCl_2$, 10 p mole of each primer and 4 μL of Fast Starter Mix buffer (dNTPs, SYBR Green dye and Tag polymerase). The primers and conditions used for RT-PCR are as shown in Table 3, 4.

5. Statistical analysis

The experimental results were expressed as average ± standard deviation. The paired Student's t-test was used to determine statistical significance between two groups, where $p < 0.05$ was

considered as being statistically significant. The Kruskal-Wallis test and Mann-Whitney test were used for non-parametric analyses.

III. Results

1. The *Dendropanax morbifera* leaf extract

The yield rate for each extracting solvent of the *Dendropanax morbifera* leaf extracts was determined and found to be highest for the methanol extract (13.6%) and relatively low for the ethyl acetate extract (1.9%; Table 5).

2. Wound-healing in the full-thickness wound mouse model

In the experiments used to determine the wound-healing effect of DMHE, the degree of wound-healing in each experiment group was measured by tissue staining. The results indicate that the groups that processed DMHE (A3 and A4) had a shorter distance between the wounds than the other groups (Figure 7). Four days after drug treatment, the distance between the wounds was measured to be 2.55 mm in the control group, indicating 49% recovery from the original diameter of the wound (5 mm). In the positive control group, which used TGF-β1 (20 ng), the distance was 1.72 mm, indicating 65.6% recovery (Figure 8). In addition, when wounds were treated with 16.5 μg DMHE, the distance was 2.02 mm, indicating 59.5% recovery, whereas 97.4% recovery was observed when 66 μg DMHE treatment was used. In DMHE (66 μg) treated animals, the healing efficacy was 198% and 148%, compared to the negative and positive control groups, respectively (Figure 9).

3. Wound-healing of foot wounds in a mouse model of hind-limb ischemia

Wound closure in foot wounds of ischemic limbs was significantly reduced compared to non-ischemic limbs (Figure 10). The mean percent of remaining wound area at postoperative day 14 was 21.8% in normal and 42.89% in ischemic wounds.

In non-ischemic wounds, groups treated with 3% DMHE ointment showed a

significantly higher wound-healing rate over 14 days than other ointment treatment groups (Table 6, Figure 11). There was no significant difference between the wound-healing rates of ischemic wounds receiving ointment treatments.

In ischemic wounds, groups treated with DMHE (10 mg/mL/kg) showed a significantly higher wound-healing rate over 14 days than other ointment treatment groups (Table 7, Figure 12). However, there was no significant difference in wound-healing rates between non-ischemic wounds receiving oral treatments.

Overall, 3% DMHE ointment showed a 98.0% wound-healing rate in normal wounds, and 10mg/mL/kg DMHE (oral administration) showed a 90.7% wound-healing rate in ischemic wounds (Figure 13). All wounds, regardless of treatment, were healed within 28 days.

4. Effects of *Dendropanax morbifera* on gene expression affecting wound-healing

Real time PCR assay showed that DMHE increased the mRNA expression of CTGF in foot wound and all muscles treated with topical and oral administration, except for gastrocnemius muscles treated with ointment (Figure 14).

However, DMHE treatment did not affect the significant mRNA expression of VEGF-A and CD 31 regardless of administration route in both normal and ischemic wounds (Figure 15, 16). The mRNA expression of α-SMA was significantly decreased in normal and ischemic wounds in foot wounds treated with oral administration of DMHE (Figure 17).

In addition, the mRNA expression of 15-PGDH after DMHE treatment was not inhibited significantly in all wounds and muscles compared with control treatment (Figure 18).

When compared the normal and ischemic wound according to treatment, the expression of CTGF, VEGF-A, CD31, α-SMA and 15-PGDH did not show a consistent trend for each tissue samples.

IV. Discussion

Prostaglandins (PGs) are lipid mediators known as eicosanoids. They are produced from arachidonic acid liberated from the cell membrane through the cyclooxygenase pathway by certain cytokines, growth factors, and other stimuli. Among those, PGE_2, which is a major PG in human and rat skin, is known to be an important mediator of wound-healing [42].

Typically, when the dermis under the skin is damaged, COX-2 increases momentarily, resulting in an increase in skin PGE_2 to stimulate early phase cell spreading and migration [43]. It controls wound-healing processes, including inflammation and fibrogenesis, and accelerates wound-healing [43-45].

Recent reports claim that PGE_2 suppresses fibroblast proliferation and collagen synthesis, while it increases the expression of MMPs [46]. Furthermore, Kolodsick et al. [28] reported that PGE_2 has specific effects on fibroblast behavior and can inhibit the differentiation of fibroblast into myofibroblast. Therefore, using medication that raises PGE_2 levels will potentially increase wound-healing and prevent excessive scarring.

However, local administration of PGE_2 is still a limited therapeutic option because it is biologically unstable and has not been well characterized. Furthermore, PGs have very short half-lives because they are rapidly metabolized by 15-PGDH. Ding et al. [47] reported that 15-PGDH decreases the level of proliferative PGE_2 and induces apoptosis. Therefore, agents that can inhibit 15-PGDH activity may be useful for the management of diseases requiring elevated PGE_2 levels.

Previously, it was reported that the antidiabetic drug ciglitazone and its analogue thiazolidine-2,4-dione were potent inhibitors of 15-PGDH,

and the inhibition of 15-PGDH may lead to increased levels of PGE_2 [48, 49]. Recently, different thiazolidinedione analogues of 15-PGDH inhibitors were synthesized and tested for their influence on wound-healing in a scratch wound test [50]. That being said, the effect of thiazolidine-2,4-dione derivatives on wound-healing *in vivo* is still unclear. Even though several studies have documented the effect of various derivatives on wound-healing, no derivatives are known to have the same wound-healing effect as 15-PGDH inhibitors. In a recent study, it was reported that *Dendropanax morbifera* extract exerts its wound-healing effect by inhibiting 15-PGDH to effectively increase PGE_2 [29].

The roots and stems of *Dendropanax morbifera* have been used in traditional medicine to treat headache, infectious diseases, and skin diseases [51]. More recent evidence suggested that *D. morbifera* essential oil has a significant lipid lowering effect in high-cholesterol diet rats [33]. Furthermore, dendropanoxide, which is present in the leaves of *D. morbifera* Leveille, has significant hypoglycemic activity in streptozocin-induced-diabetic rats [32]. Polyacetylene compounds isolated from *D. morbifera* showed significant anticomplement activity on the classical pathway complement system in a hemolytic assay [52]. Furthermore, rutin from *D. morbifera* significantly decreased rotenone-induced generation of reactive oxygen species in SH-SY5Y cells and protected SH-SY5Y cells from rotenone-induced caspase-9 and caspase-3 activation and apoptotic cell death [53]. These results suggest that rutin has therapeutic potential as a treatment for neurodegenerative disorders associated with oxidative stress. As a whole, these findings may provide some ideas for the development of agents used to treat cardiovascular, inflammatory, and degenerative diseases in humans.

The biologic activity of *D. morbifera* extracts has not been investigated in detail, especially in regards to wound-healing. In this study, aqueous extracts of *D. morbifera* were fractionated in methanol, hexane, ethyl acetate, butanol, and water. It has been demonstrated that DMHE in particular has a strong ability to inhibit 15-PGDH and significantly increase intracellular and extracellular PGE_2 levels [35]. Accordingly, we selected DMHE as our material of interest and verified its wound-healing effect *in vivo*.

Previously, DMHE was found to increase the expression of COX-1 and MRP4 mRNA, while decreasing the mRNA expression of PGT (prostaglandin transporter) and 15-PGDH [35]. MRP4, which is a member of the multidrug resistance proteins, functions as an energy-dependent, transmembrane efflux transporter and mediates the efflux of PGE_2 and subsequent increase of extracellular PGE_2 [54]. On the contrary, because PGE_2 undergoes reuptake by PGT after release from cells, decreased expression of PGT increases extracellular levels of PGE_2 [55]. Such mRNA expression after DMHE treatment demonstrates that DMHE potently increases PGE_2 levels.

The *in vitro* scratch assay, which is done using a sterile pipette tip in an HaCaT cell line is an easy, economic, and well-developed method to measure cell migration *in vitro* [56, 57]. It is particularly suitable for studies mimicking cell migration during wound-healing *in vivo* and for studies showing the regulation of cell migration by cell interactions with the extracellular matrix and cell-cell interactions.

Although *in vitro* experiments have many advantages, animal models of wound-healing provide a greater understanding of the mechanism and pathophysiology of wound-healing. Selection of the most appropriate wound-healing animal model is important to the success and significance of experiments. We adopted a full-thickness excisional wound model to

recapitulate the state of an acute wound. In this model, the wound bed can be easily accessed to investigate the repair process. In addition, wound closure can be visualized, measured, and documented at different time points, and tissue samples can be harvested for molecular and cellular analysis [58]. Because the basic mechanism of wound-healing is contracture in mouse, a splinted wound model using silicone rings can be used to prevent wound margin contracture and create a wound-healing model that closely parallels human wound-healing [59].

With regard to the wound-healing properties of DMHE, these results indicate that groups treated with DHME had shorter distances between the wounds than the other groups, especially animals treated with 66 μg DMHE. The full-thickness excisional wound model in mice was suitable for *in vivo* studies of acute wound-healing in response to DMHE treatment.

That being said, there are no standardized animal models that reflect the complexity of the ischemic wound bed in humans. To closely mimic the clinical condition of chronic non-healing wounds such as the ischemic ulcer condition in human peripheral arterial disease, it is necessary to induce the wound on the foot rather than on other parts of the body. Therefore, we adopted the foot wound model in a mouse ischemic hind-limb to recapitulate the condition of a chronic wound. The foot wound model was first described by Lau et al. [41] to investigate the wound-healing effect of medicinal herbs on a diabetic rat model. Standard 10 mm^2 wounds were created on the feet, and wound areas were analyzed by image analysis software.

The hind-limb ischemia model involving acute interruption of arterial supply is currently considered to be the most effective preclinical in vivo model for recapitulating the human condition of critical limb ischemia [18]. However, the method used to ligate the femoral artery

with a single ligature spares most of the collaterals to the lower limb, allowing blood flow to the limb to be restored within 7 days [60]. To overcome this and obtain an effective therapeutic window, different patterns of perfusion restoration and levels of vascular occlusion were compared [20]. Among the various surgical approaches, the double coagulation mouse model offers a therapeutic window in which improvements can be monitored efficiently. Therefore, we performed double ligation of femoral artery, and foot wounds were produced on the dorsum of both ischemic and non-ischemic limbs of mice.

On postoperative day 14 in the control group, the wound closure was significantly faster in non-ischemic wounds (78.1%) than ischemic wounds (57.1%), which validated the hind-limb ischemia model with foot wounds as an effective ischemic wound model.

In non-ischemic limbs, wound area steeply decreased up to the 7th day and then decreased more slowly to 14th day. In contrast, in ischemic limbs, wound area was most effectively decreased around the 10th day. We found that the 7th and 10th days were the best cut-off times for wound assessment in non-ischemic and ischemic-limbs, respectively. In other studies, the 8th day was usually accepted as a suitable time for wound-healing assessment [61, 62].

In non-ischemic wounds, the 3% DMHE ointment was found to have a significant wound-healing effect, and in ischemic wounds, 10 mg/mL/kg DMHE (oral administration group) was found to have the greatest effect. These findings demonstrate the wound-healing effect of DMHE in ischemic and non-ischemic foot wound models. Local DMHE application showed wound-healing effect in normal wound but not in ischemic wound, suggesting the importance of blood flow in its wound-healing effect. Oral DMHE administration facilitated wound-healing in both of normal and ischemic wounds in a dose-dependent manner. It is thus thought that

local or systemic administration of DMHE could help wound-healing in the presence or enhancement of blood flow.

We expected that main molecular mechanism of wound-healing in DMHE treatment is 15-PGDH inhibition and angiogenesis by PGE_2 increment. However, we failed to prove decreased expression of 15-PGDH and increased expression of VEGF-A and CD31 in real-time PCR analysis. In addition, the expression of α-SMA mRNA which is known as a marker of myofibroblasts and known to enhance fibroblast contractile activity was also decreased [63]. It may be due to wound tissue and muscles were harvested two weeks after the last treatment. Accordingly, it is suggested that the expression of molecular markers for wound-healing was not reflected in results of real-time PCR.

However, the expression of CTGF, which is regarded as a central mediator of tissue remodeling and fibrosis was increased after DMHE treatment, especially in oral administration. It is well known that CTGF participates in early wound-healing and promotes hypertrophic scar formation by activation of myofibroblast [64]. Alfaro et al. [65] reported that CTGF has a physiologic role during cutaneous wound repair. They suggested two possibilities: regulation of fibroblast population and vascular cell populations. However, in spite of the evidence implicating CTGF in dermal wound-healing and scarring, its molecular mechanisms *in vivo* has not been well defined.

That being said, there are several limitations to foot ulcer models in ischemic hind-limbs. The severity of ischemic damage cannot be uniform, and substantial biologic variation exists in wound-healing, even among the same animal strains. Therefore, a consistent surgical procedure with minimal technical variations should be performed to produce reliable and objective results and to yield significant conclusions by wound analysis. For this, laser doppler perfusion image (LDPI) should

be performed to verify the consistency of the ischemic condition after the surgical procedure [66, 67].

In some cases, limb edema occurred, and the wound area was larger than the initial wound area, possibly resulting from vein and lymphatic injury during surgical procedure. Accordingly, it is important not to injure vein and lymphatic structure during dissection of the femoral artery.

Generally, wounds healed within 28 days regardless of treatment. This may be due to the fact that the basic mechanism of wound-healing in rodents is contracture. Therefore, in addition to the ligation of femoral artery, it necessary to delay wound-healing to test the therapeutic window of adjuvant treatment. Kalka *et al.* [68] reported that the ligation of the femoral artery in immune-deficient athymic mice results in impaired blood flow restoration compared to wild type mice. Furthermore, double coagulated hind limb ischemia in immune-deficient mice has a larger therapeutic window for blood flow stimulation [20]. These results suggest that double coagulation in immune-deficient mice can be a useful wound model for testing more severe models of ischemia and more delayed wound-healing.

Although there were some drawback, the animal model, which has feet ulcer in ischemic hind-limb, was probably suitable for *in vivo* studies of wound-healing in response to different adjuvant treatments. This study provides a valuable reference tool for elucidating the biology of ischemic wounds and an objective rationale towards novel adjuvant treatments for wound-healing.

V. Summary and conclusions

Wound healing is the effort of injured tissue to restore their normal function and structural integrity after injury. Prostaglandin E_2 (PGE_2) is an important mediator of wound healing with specific effect on fibroblast behavior. Since PGE_2 is catalyzed by NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), 15-PGDH inhibitor could significantly increase PGE_2 levels. Recently, it was suggested that the *Dendropanax morbifera* hexane extract (DMHE) has an wound-healing effect by suppressing 15-PGDH activity and then increasing PGE_2 levels. So far, there is no *in-vivo* result of DMHE on wound healing. In this study, we investigated the in-vivo wound-healing effect of DMHE.

We used two *in-vivo* wound models to verify the wound healing effect of DMHE, full-thickness wound model and hind-limb ischemic wound model. In full-thickness wound model, treatment was applied to the wound differently for each group every day from the next day for seven days. The measured distance between wounds was compared to that of the control group, which was calculated as a wound healing efficiency. For creation of foot wound in mouse model of hind-limb ischemia, left proximal and distal femoral artery was ligated and 2x5 mm wound was created on both feet of mouse. The areas of the wound on individual days of measurement after surgery were divided by the areas of the wounds on the day of surgery to calculate the wound healing efficiency.

In full-thickness wound model in mouse, when the wound was treated with 16.5 μg/day and 66 μg, wound healing rate was 59.5% and 97.4%. 16.5 μg/day DMHE treatmtnt showed wound healing efficacy of 198% and 148%, compared to the negative and positive control group. In hind-limb ischemic wound model in mouse, DMHE 10 mg/mL/kg oral administration showed significant wound healing effect in ischemic limb. 3% DMHE

ointment showed 98.0% of wound healing rate in normal wound (p=0.040) and 10 mg/mL/kg oral administration showed 90.7% of wound healing rate in ischemic wound (p=0.025). In real-time PCR analysis, DMHE treatment increased the expression of CTGF which is invovled in tissue wound repair. Howerver, there was no correlation of DMHE treatment in wound healing with expression of VEGF-A, CD31, α-SMA and 15-PGDH.

These results suggest that DMHE is considered as an effective agent for wound healing in full-thickness wound model and hind-limb ischemic wound model in mouse. In particular, DMHE ointment treatment was effective in normal wound healing and DMHE oral treatment was effective in ischemic wound healing in hind-limb ischemic wound model. In addition, it is suggested that the over-expression of CTGF is involved in wound healing after DMHE treatment. Therefore, it is considered that oral treatment rather than ointment treatment or combination of ointment and oral treatment is better for wound healing in ischemic wound.

Table 1. Drug treatment for investigating the wound-healing effect of DMHE in vivo

Group	Drug treatment	Mark drawings (Figure 15)
1	Vehicle control group	A1
2	TGF-β1 (20 ng/day), a positive control	A2
3	DMHE (16.5 μg/day)	A3
4	DMHE (66 μg/day)	A4

DMHE, *Dendropanax morbifera* hexane extract

Table 2. Subgroups for investigating the wound-healing effect of DMHE in foot wounds of hind-limb ischemia of mouse models

	Treatment	Number (n)
Group 1	Ointment vehicle control	3
Group 2	3% Centella Asiatica ointment	4
Group 3	3% DMHE ointment	4
Group 4	Oral control	3
Group 5	DMHE 1 mg/mL/kg PO	2
Group 6	DMHE 10 mg/mL/kg PO	4

DMHE, *Dendropanax morbifera* hexane extract

Table 3. Primers of real-time PCR

Gene	Primer	
CTGF	Sense	5'-AATGCACTTGCCTGGAT-3'
	Antisense	5'-AAATGTGTCTTCCAGTCGGT-3'
VEGF-A	Sense	5'-TACTGCCGTCCGTATTGAG-3'
	Antisense	5'-ATGATCTGCATGGTGATGTT-3'
CD31	Sense	5'-GCCAGGGTTTTCCCAGTCACGAC-3'
	Antisense	5'-GAGCGGATAACAATTTCACACAGG-3'
α-SMA	Sense	5'-CAGGGAGTAATGGTTGGAAT-3'
	Antisense	5'-TCTCAAACATAATCTGGGTCA-3'
15-PGDH	Sense	5'-TGCTTCAAAGCATGGCATAG-3'
	Antisense	5'-AACAAAGCCTGGACAAATGG-3'
GAPDH	Sense	5'-CGGTGCTGAGTATGTCG-3'
	Antisense	5'-TGAGTCAGTTGTCATATTTC-3'

CTGF, connective tissue growth factor; VEGF-A, vascular growth factor-A; CD31, cluster of differentiation 31; α-SMA, α-smmooth muscle actin; 15-PGDH, 15-prostagnaldin dehydrogenase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

Table 4. Conditions for real time PCR

Gene	Hot start	Denaturation	Annealing	Extension
CTGF	95 ℃, 1 min	95 ℃, 15 sec	60 ℃, 5 sec	72 ℃, 10 sec
VEGF-A			60 ℃, 5 sec	
CD31			57 ℃, 5 sec	
a-SMA			57 ℃, 5 sec	
15 - PGDH			60 ℃, 5 sec	
GAPDH			60 ℃, 5 sec	

Other legends are same as in the Table 3.

Table 5. Yield rates of D. morbifera Lev. extracts according to each extracting solvent

Solvent	Final product (g)	Yield (%)
Methanol	230.1	13.6
n-Hexane	34.6	2.1
Ethyl acetate	31.0	1.9
n-Butanol	46.6	2.8
Water	98.4	5.8

Table 6. Comparison of wound area and wound-healing rate between the ointment treatment groups

	Group 1 (Ointment vehicle control)	Group 2 (3% Centella Asiatica ointment)	Group 3 (3% DMHE ointment)	p-value
Mean wound area (mm^2) (Normal wound)				
Day 1	12.56 ± 3.33	9.12 ± 0.85	9.20 ± 1.14	0.258
Day 14	1.47 ± 0.68	1.27 ± 0.41	0.18 ± 0.37	0.128
Mean wound area (mm^2) (Ischemic wound)				
Day 1	12.31 ± 0.98	9.96 ± 1.15	11.44 ± 2.31	0.183
Day 14	2.21 ± 2.10	2.45 ± 1.95	2.68 ± 1.30	0.628
Wound-healing rate (%)				
Normal wound	88.3 ± 3.74	86.1 ± 4.79	98.0 ± 3.63	0.025*
Ischemic wound	82.0 ± 19.36	75.2 ± 18.25	76.6 ± 6.35	0.700

** Group 2 vs Group 3: p = 0.029*

Table 7. Comparison of wound area and wound-healing rate between the oral treatment groups

	Group 4 (Oral control)	Group 5 (DMHE 1 mg/mL/kg PO)	Group 6 (DMHE 10 mg/mL/kg PO)	p-value
Mean wound area (mm^2) (Normal wound)				
Day 1	11.09 ± 0.72	10.26 ± 0.58	10.71 ± 1.62	0.574
Day 14	2.43 ± 1.52	0.60 ± 0.15	0.46 ± 0.37	0.064
Mean wound area (mm^2) (Ischemic wound)				
Day 1	11.39 ± 1.42	10.62 ± 0.93	10.44 ± 1.87	0.705
Day 14	4.88 ± 1.30	3.08 ± 0.63	0.97 ± 0.36	0.043*
Wound-healing rate (%)				
Normal wound	78.1 ± 15.65	94.1 ± 1.93	95.7 ± 4.21	0.064
Ischemic wound	57.1 ± 11.61	71.0 ± 12.81	90.7 ± 4.35	0.040†

** Group 4 vs Group 6: p=0.036*

† Group 4 vs Group 6: p=0.041

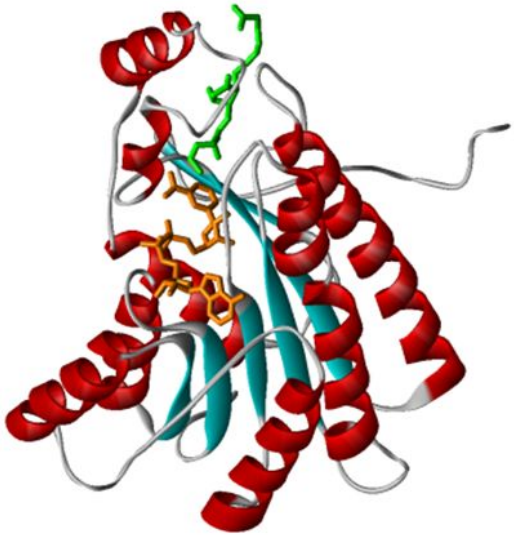


Figure 1. A stereoview of 3D structure of 15-PGDH-NAD^+-PGE_2 complex. The green molecular is substrate PGE_2, the orange color molecular is cofactor NAD^+.

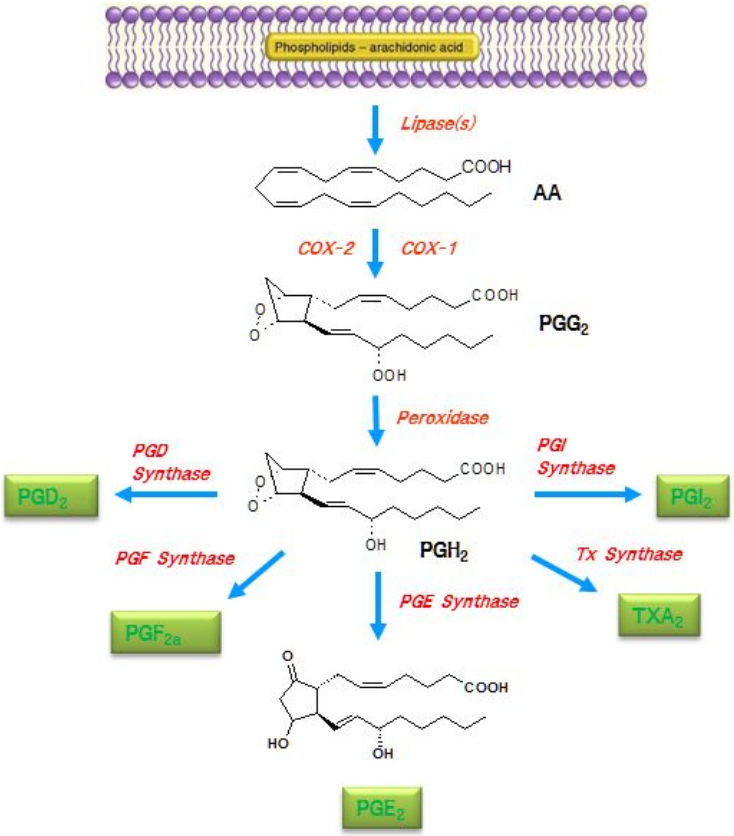


Figure 2. Biosynthesis of prostaglandins.

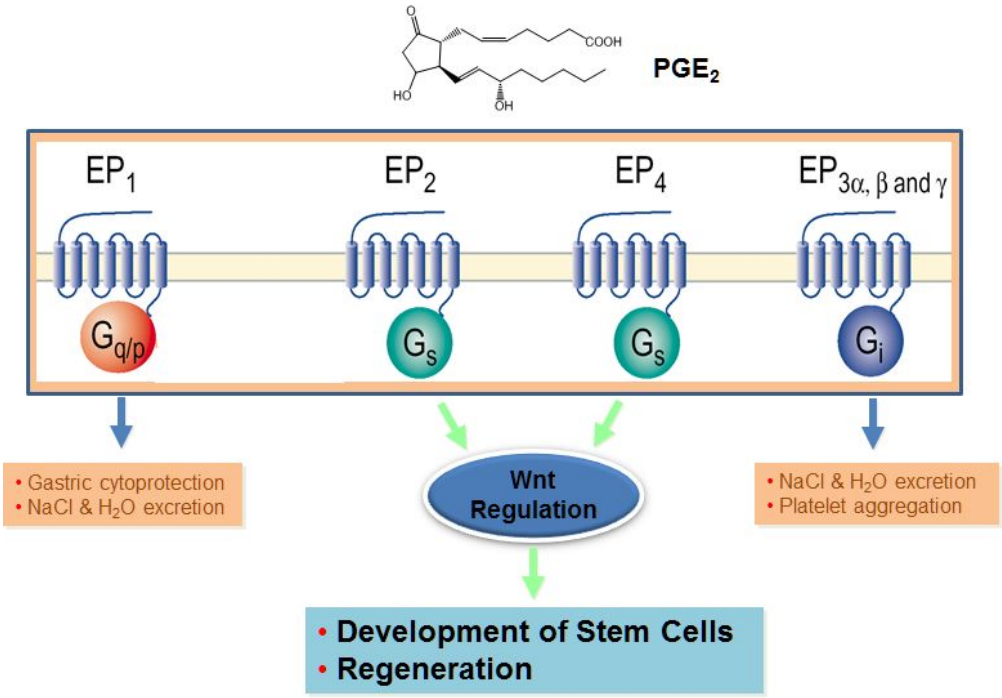
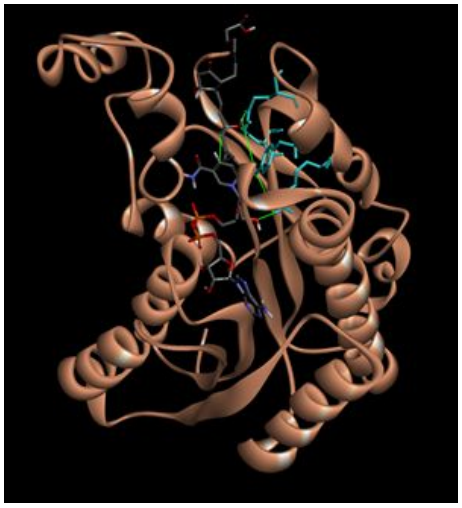


Figure 3. Biological actions of PGE_2 through four receptors.



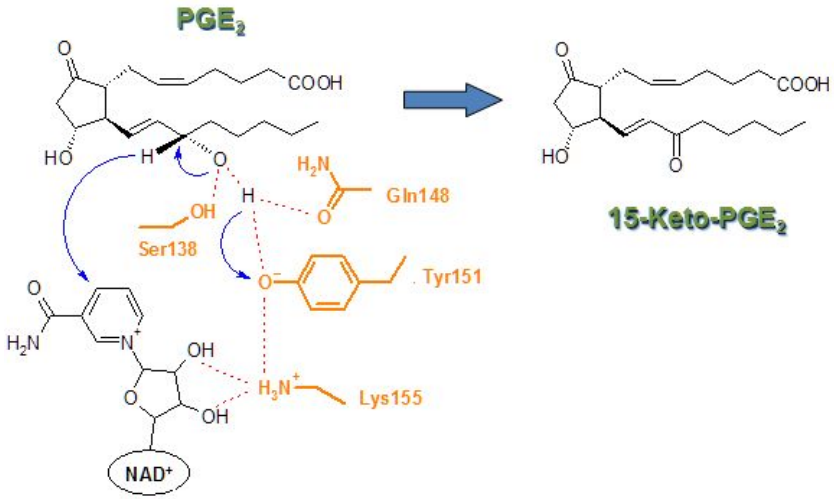


Figure 4. Catalytic mechanism of PGE_2 oxidation.

Figure 5. Photograph of a *Dendropanax morbifera*.

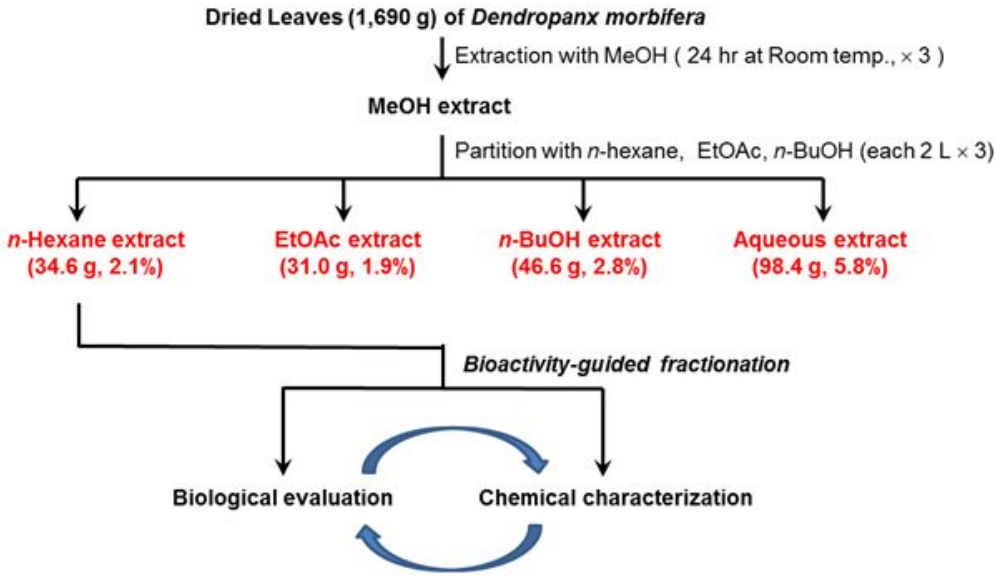


Figure 6. A diagram showing the work process of preparing *Dendropanax morbifera* extracts.

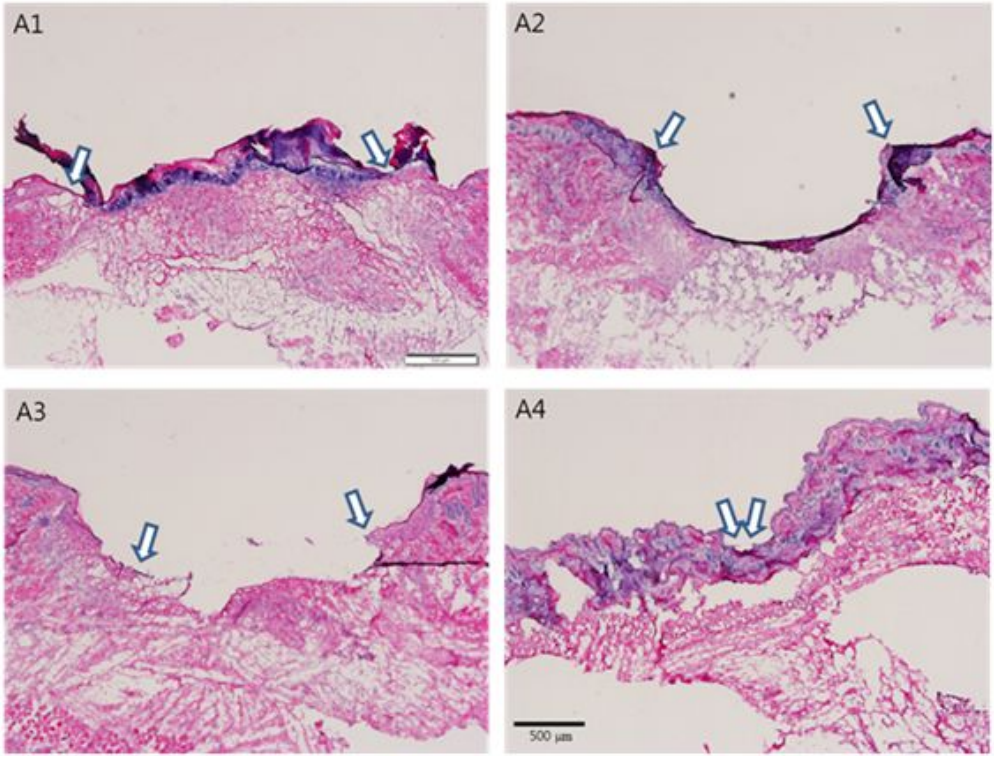


Figure 7. Hematoxylin & eosin staining of 5 mm round wounds in mouse models after treatment with (A1) 0.1% BSA (control), (A2) TGF-β1 (20 ng/day), (A3) DMHE (16.5 μg/day), and (A4) DMHE (66 μg/day).

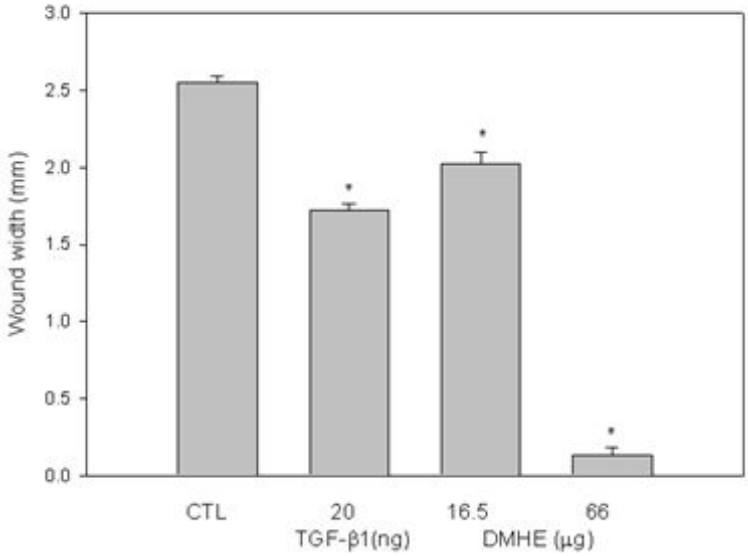


Figure 8. Effect of DMHE on wound-healing in full-thickness wound model;* $p < 0.05$ *versus* the control. CTL, control; TGF-β1, transforming growth factor-β1;DMHE, *Dendropanax morbifera* hexane extract.

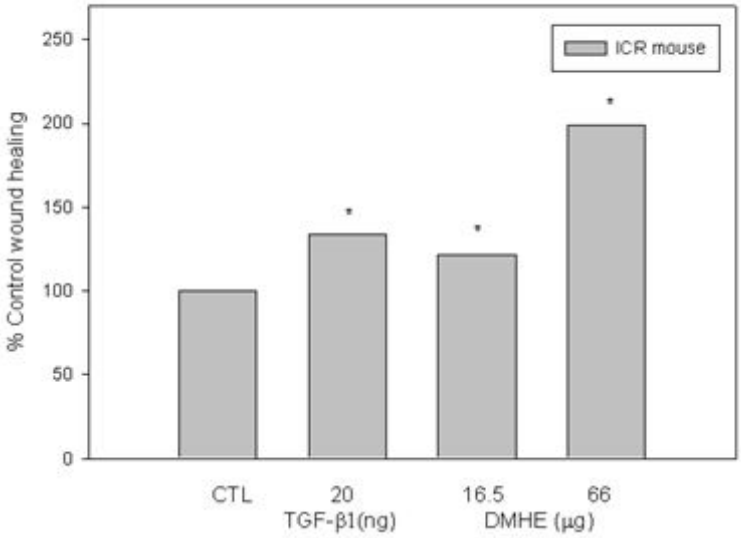


Figure 9. Wound-healing efficacy, as calculated by comparing the distance between wounds with that of the control group; * $p < 0.05$ *versus* the control. CTL, control; TGF-β1, transforming growth factor-β1;DMHE, *Dendropanax morbifera* hexane extract.

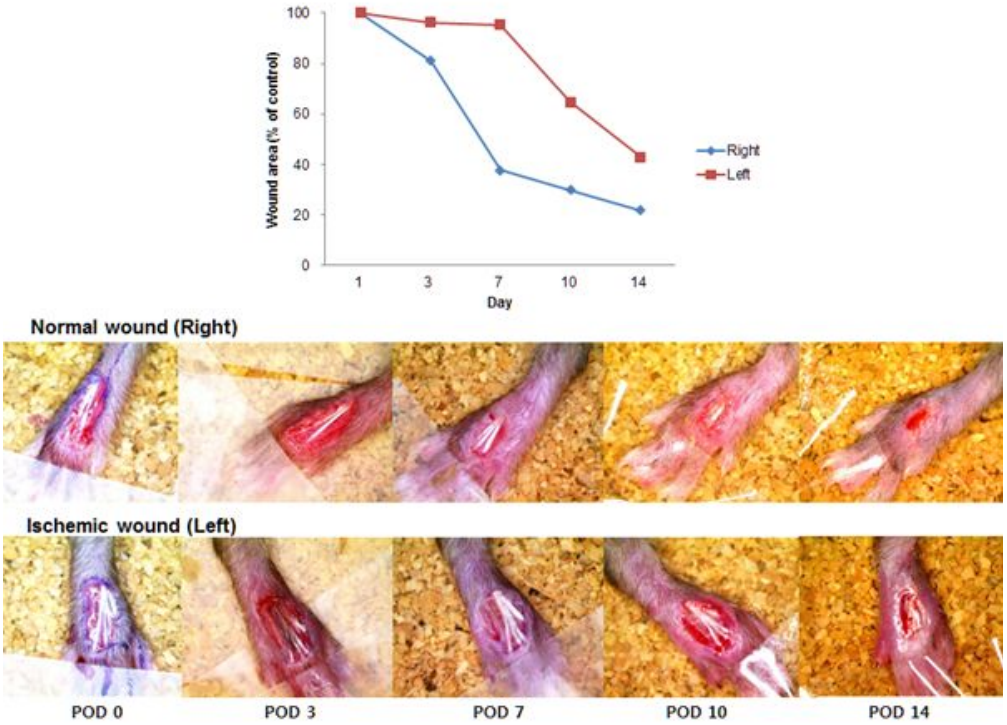


Figure 10. Effect of hind-limb ischemia in delayed wound healing (percent wound area from post-operative day 0-14 in the control group).

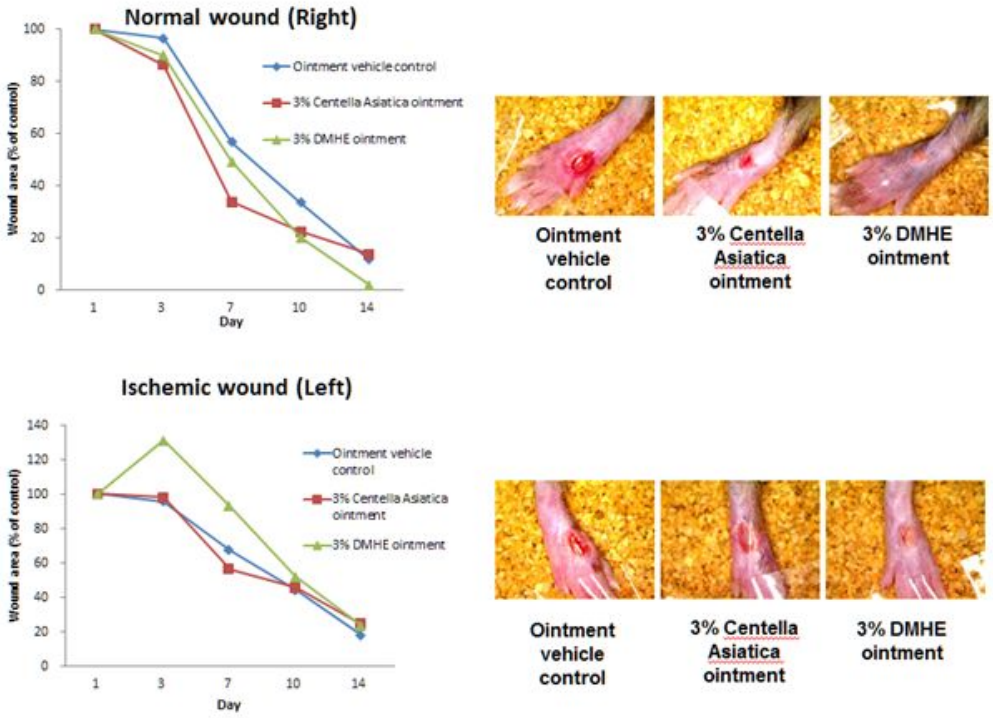


Figure 11. Different effect of topical DMHE treatment on normmal and ischemic wound in wound healing (percent wound area from post-operative day 0-14 in the ointment treatment group and photographs of wounds at post-operative day 14).

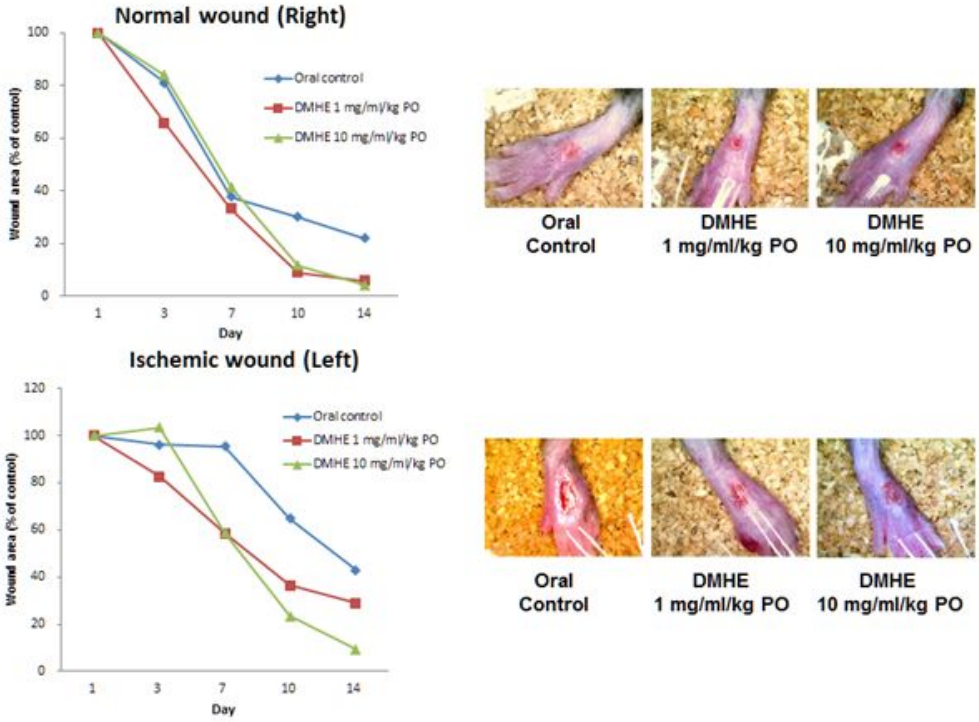


Figure 12. Different effect of oral DMHE treatment on normmal and ischemic wound in wound healing (percent wound area from post-operative day 0-14 in the ointment treatment group and photographs of wounds at post-operative day 14).

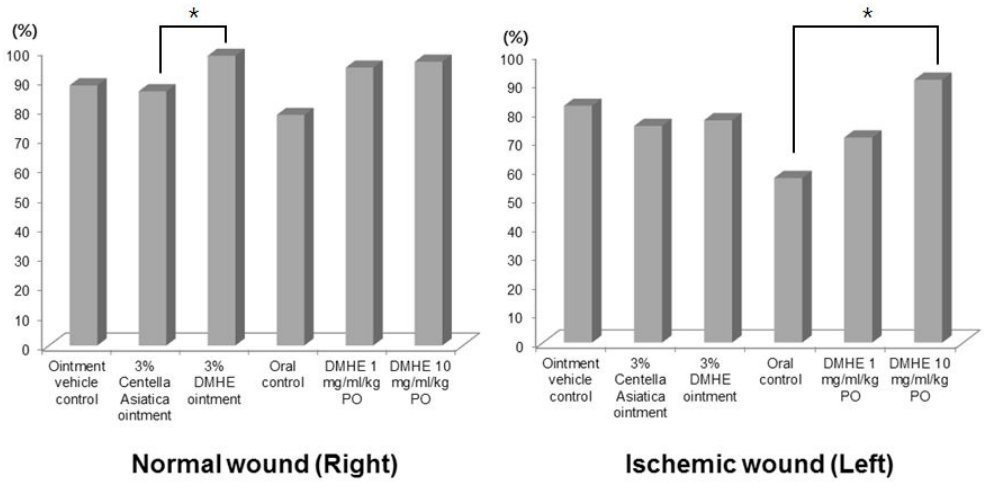


Figure 13. Overall wound-healing rate of DMHE treatment in normal and ischemic wounds. * $p < 0.05$.

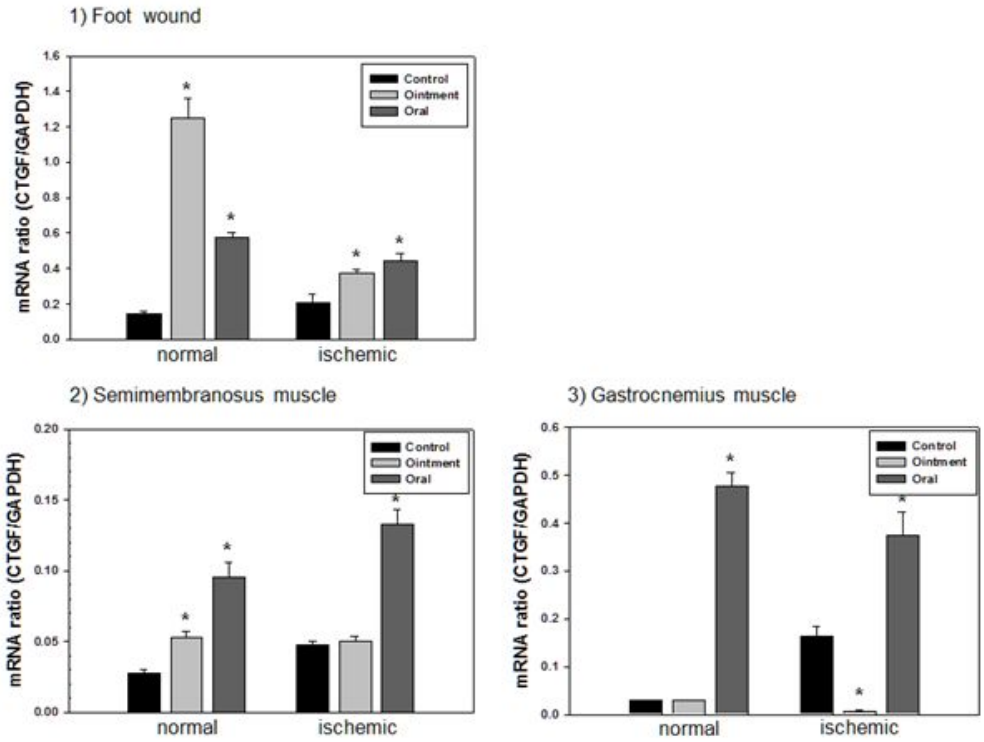


Figure 14. Effect of topical and oral administration of DMHE on CTGF mRNA expression in 1) foot wound, 2) semimembranosus and 3) gastrocnemius muscles. * $p < 0.05$ *versus* the control.

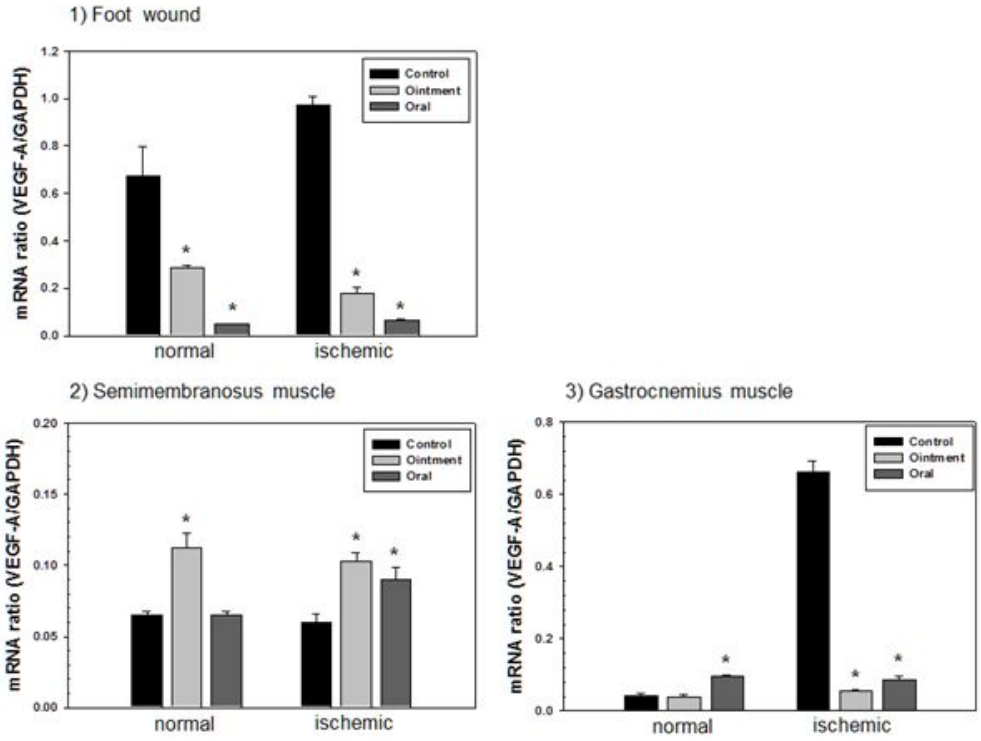


Figure 15. Effect of topical and oral administration of DMHE on VEGF-A mRNA expression in 1) foot wound, 2) semimembranosus and 3) gastrocnemius muscles. * $p < 0.05$ *versus* the control.

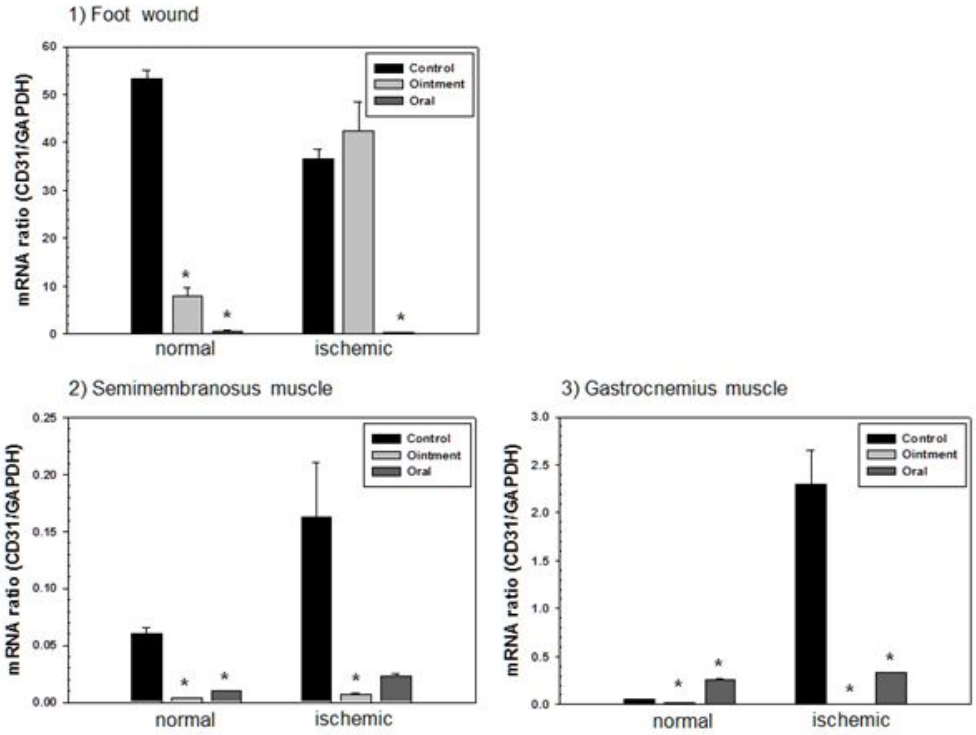


Figure 16. Effect of topical and oral administration of DMHE on CD31 mRNA expression in 1) foot wound, 2) semimembranosus and 3) gastrocnemius muscles. * $p < 0.05$ *versus* the control.

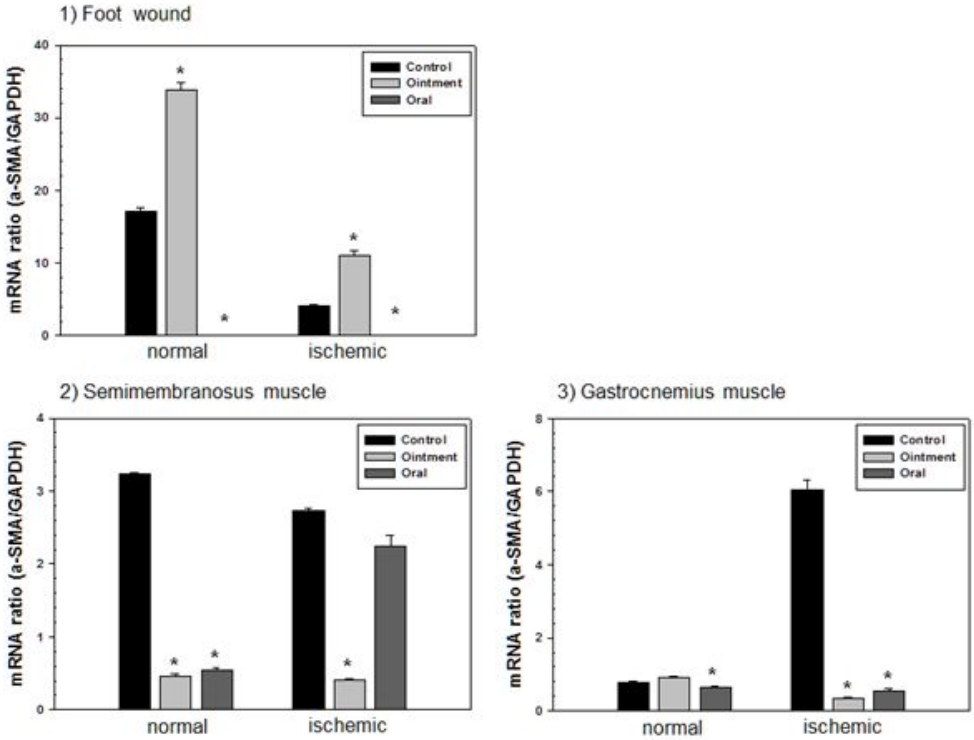


Figure 17. Effect of topical and oral administration of DMHE on α-SMA mRNA expression in 1) foot wound, 2) semimembranosus and 3) gastrocnemius muscles. * $p < 0.05$ *versus* the control.

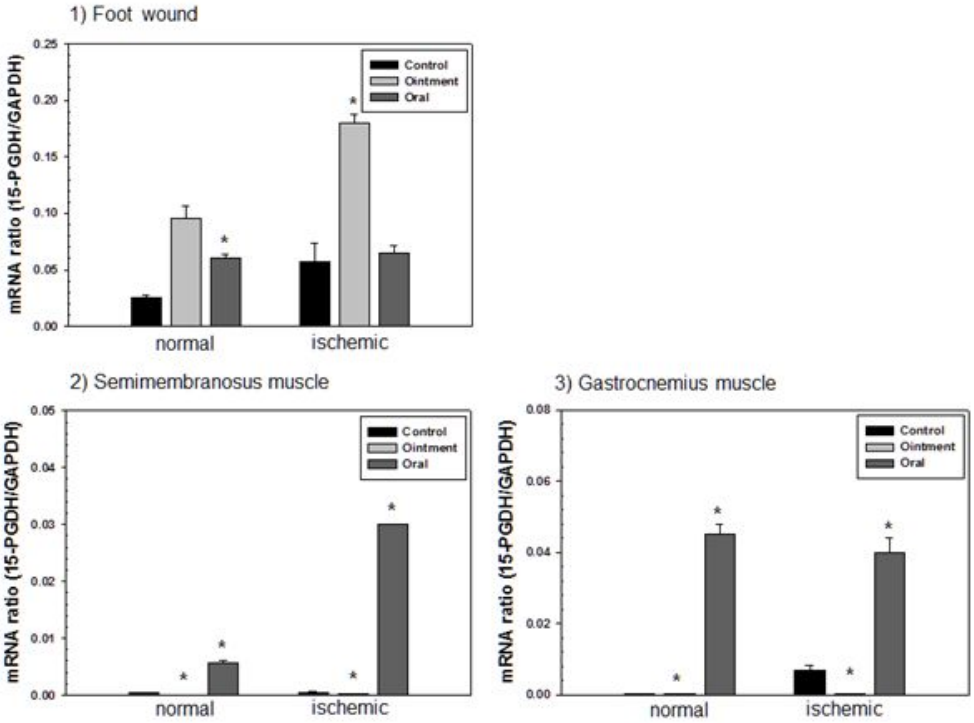


Figure 18. Effect of topical and oral administration of DMHE on 15-PGDH mRNA expression in 1) foot wound, 2) semimembranosus and 3) gastrocnemius muscles. * $p < 0.05$ *versus* the control.

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이런 부족한 저에게 전공의 시절부터 많은 가르침을 주시고 힘든 일이 있을 때마다 항상 마음 가득 힘을 주셨던 지도교수님이신 김권천 교수님께 감사의 인사를 드립니다.

또 학문적으로 여러 가지를 경험하며 익힐 수 있는 기회를 주시고 인생의 선배로서 조언을 아끼지 않으셨던 최철희 교수님.

그리고 전공의 시절부터 묵묵히 지켜봐 주시고 큰 버팀목 같은 역할을 해주신 민영돈 교수님.

연구에 대한 열정과 진지함을 보여 주시고 항상 따뜻한 조언으로 힘을 주셨던 김경종 교수님.

박사과정을 통해 처음으로 인연을 맺었지만 위트와 날카로운 지적으로 논문을 지도해 주신 조훈 교수님.

늦은 논문이지만 마지막까지 논문 지도를 위해 힘써주신 모든 교수님들께 감사드립니다.

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