의과대학 석사 학위논문

The effect of Korean Red Ginseng Extract on flap survival and angiogenesis in rat model

동물 모델에서의 피판 생존과 혈관 생성에 대한 홍삼 추출물의 효과 분석

2016년 8월

서울대학교 대학원
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이 논문을 의학 석사학위 논문으로 제출함.

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논 문 초 록

Abstract

The effect of Korean Red Ginseng Extract on flap survival and angiogenesis in rat model

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Background: Despite its poor predictability of survival, random pattern skin flap has been the most commonly used reconstructive method until recently. To overcome its shortcomings, there have been numerous pharmacologic trials and research to enhance survivability. The authors tried Korean red ginseng extract (KRGE), a well-known herb remedy for its outstanding angiogenecity and vasculogenic properties.

Methods: A total of 36 male SD rats weighing 300 to 350 g were used. Rats were divided into three groups, and 12 rats were

distributed into each group. Group 1 was the control, where the rats were not given KRGE, group 2 contained rats given 100 mg/kg KRGE for two weeks, and group 3 contained rats given 200 mg/kg KRGE over the two-week period. Skin flap survival was measured after 7 days postoperatively.

Also, using micro SPECT-CT, we evaluated vasculogenesis by direct visualization on the third postoperative day. By immunohistochemistry, we confirmed the increase of new vessel formation, and from polymerase chain reaction studies, we investigated the overproduction of numerous growth factors.

Results: Compared to the control group, the experimental groups showed significantly lower necrotic areas of the flap. It was possible to confirm that the oral intake of red ginseng helps flap survival in a random pattern animal model, as demonstrated by the increasing growth factor production and angiogenesis.

Conclusion: The results provide a valuable reference for further preclinical studies of KRGE in flap surgery and various reconstructive methods in the plastic surgical field.

Keywords: Panax ginseng; Korean Red Ginseng extract; reconstructive surgery; angiogenesis; random flap; flap survival; molecular imaging

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Introduction

Random pattern skin flap is one of the main operative methods used in reconstructive surgery. Although the operation is very versatile and easy to perform, the postoperative survivability of the flap is frequently unsatisfying. The flap survivability depends on two major factors, a sufficient blood supply to the flap and the prevention of ischemic-reperfusion injury, although that is not completely comparable to the true ischemia-reperfusion phenomenon in axially perfused flaps[1]. Seeking a method to ensure better flap survival has been a prevalent topic of research in the reconstructive surgical field, as countless numbers of different surgical methods and medications have been studied and clinically tried. Among those, vasodilators, such as prostaglandin E1, anticoagulants, and the administration of topical growth factors, such as epidermal growth factor (EGF) or fibroblast growth factor (FGF) have been adapted by a majority of surgeons and are currently used in flap surgeries [2-4].

Ginseng is a widely beloved herbal remedy in Asia, particularly in northeastern Asia. There are many types of ginseng, but among those, Korean red ginseng, aged more than 6 years, is the most well-known and it has been used in many medical fields and is consumed daily by many individuals [5]. In cardiovascular medicine, the angiogenic and vasculogenic potential of ginseng have been widely studied[6], and in

oncology, its chemopreventive actions have been garnering attention[7]. Various recent Korean red ginseng studies have shown its antithrombotic activities, protective effects in myocardial cells against ischemia-reperfusion injuries, and also the strong angiogenic potential of ginsenosides has been shown by both in vitro and in vivo experiments.

From such results, we hypothesized that consuming Korean red ginseng could have beneficial effects on the survivability of skin flaps. The main objective of the study was to evaluate the effect of red ginseng by using a rat dorsal skin flap model, and to analyze the results with additional growth factor assays, immunohistochemistry, and micro-SPECT-CT scans.

Materials & Methods

Animals

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital. A total of 36 male Sprague-Dawley rats weighing around 300 grams each were used. Animals were randomly assigned to the non-treatment contrast group (n=12), the 100 mg/kg medication group (n=12), or the 200 mg/kg medication group (n=12). Each dosage

and the number of rats per group was set by a review of literature [5, 6, 8], previous pilot studies, and statistical power analysis. With every other condition homogenized, the animals were given standard care and diet with 12 hour night and day cycles. Every rat was caged separately to avoid flap injury by cannibalism.

Materials

Korean red ginseng extract (KRGE) provided by the Korea Ginseng Corporation Central Research Institute (Daejeon, Korea) were used in the experiment. The KRGE was manufactured by the Korea Ginseng Corporation, Seoul, Korea from the roots of a 6-year-old red ginseng, Panax ginseng Meyer, harvested in Korea.

KRGE was made by steaming fresh ginseng at 90–100◦C for 3 hours and then drying it at 50–80◦C. KRGE was prepared from red ginseng water extract, which was extracted at 85–90◦C for 8 hours by circulating hot water three times. The water content of the pooled extract was 36% of the total weight.

KRGE was analyzed by high-performance liquid chromatography. KRGE was found to contain major ginsenosides (Rb1: 8.11 mg/g, Rb2: 3.16 mg/g, Rc: 3.32 mg/g, Rd: 0.91 mg/g, Re: 2.12 mg/g, Rf: 1.04 mg/g, Rg1: 2.01 mg/g, Rg2s: 1.06 mg/g, Rg3s: 1.14 mg/g) and other minor ginsenosides.

KRGE was given to the animals in the treatment groups for 2 weeks (1 week preoperatively and 1 week postoperatively). KRGE dissolved in distilled water was given at the respective dosages, 100 mg/kg and 200 mg/kg, daily.

Operation

Under general anesthesia using isoflurane inhalation and an intraperitoneal injection of zoletil, a 2 x 9 cm flap was uniformly designed on the hemidorsum of the rats after shaving the dorsal hair to constantly develop skin necrosis if kept untreated. The modified Mcfarlane flap design that we used in the study was introduced by Oh et al. [9], as this modified design allows for easy comparison between the operated side and the normal contralateral side, which was left untouched during the surgery. After the musculo-cutaneous flap, including the skin, subcutaneous tissue, and panniculus carnosus, was elevated, the flap was sutured back into its original location.

Assessment of flap necrotic area

We evaluated the flap survivability by measuring the necrotic surface

area on the postoperative seventh day. After euthanasia using a CO2 chamber, the necrotic area and total flap area were marked on transparent paper. Using transparent paper allows for more accuracy in measuring the relatively concave or convex surface areas than photographic measurements [9]. Then, the marked transparent paper was scanned with a conventional digital scanner (Hewlett-Packard, Palo Alto, CA), and quantitative measurements were taken with ImageJ software (NIH, Bethesda, MD). The area was calculated by pixel numbers, and the percentage of necrotic area to total flap area was noted.

Histological analysis

After the necrotic area was measured, a 1 x 2 cm full thickness skin specimen was excised from the middle portion of the flap. The samples were embedded in paraffin, and routine hematoxylin and eosin staining was done. Also, to measure the microvessel density by immunohistochemistry, we stained the slides with rabbit polyclonal antibody (1:100; Abcam, Cambridge, MA) against rat von Willebrand factor and CD31 antibody (PECAM-1). On 10 randomly chosen regions of interest, each under 40x magnification, the microvessel density was measured on CD31 and vWF stained slides by two independent blind observers. The average of two observed vessel numbers in subcutis level

was compared.

Visual analysis of neovascularization

Three days after the operation, we performed micro-SPECT-CT analysis and the results were obvious. A 99mTc-RGD peptide micro SPECT-CT device (Bioscan corp., Seattle, WA) was used for the direct visual assessment of angiogenesis inside the flap, and four rats from each of the three groups were evaluated in this manner. Molecular targeting by RGD, the ligand of integrin αvβ3, was used to visually confirm endothelial neovascularization and has been used mainly in tumor targeting and antiangiogenic drug response evaluation [11, 12].

Reverse Transcription Polymerase Chain Reaction

With excised specimen, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), and platelet derived growth factor (PDGF), expression was analyzed with RT-PCR. Band densitometry was performed with the Quantity One software version 4.6.6 (Bio-Rad Laboratories, Hercules, CA), and the results were expressed as the mean ratio in relation to the GAPDH band intensity.

Statistical Analysis

Statistical comparative analysis for flap survival rate and microvessel density was performed with SPSS version 21 (IBM corporation, Armonk, NY), by one-way ANOVA comparing the three groups independantly with post-hoc test using Scheffe's method. Statistical significance was set at $p < 0.05$.

Results

Flap survival evaluation

The evaluation of flap survival was measured 7 days after the operation (Fig. 1). The results showed significant differences between the 3 groups. The 200mg/kg intake group (mean 14.617), showed lower necrosis rate compared to the 100mg/kg intake group (mean 25.342) and the control group (mean 35.617), and also there was statistically significant difference between 100mg/kg and control groups (Table 1).

Histological analysis

The microvessel count in the 200 mg/kg group (mean 5.25 ± 0.45 / HPF) was increased significantly compared to the 100 mg/kg group (1.92 ± 0.82 / HPF) or the control group (mean 1.42 ± 0.13 / HPF) (Fig. 2; Table 2.), although no significant difference was found between the 100mg/kg and control groups.

RT-PCR analysis

By reverse transcriptase PCR analysis, a marked increase of bFGF and PDGF signal intensity was confirmed in the 100 mg/kg and 200 mg/kg groups, respectively. (Fig. 3,4)

Visual confirmation of angiogenesis

Compared to the control or 100 mg/kg group, the signal intensity of the 200 mg/kg group was stronger on the area around the elevated flap (Fig. 5). An increased uptake of RGD peptide is related to a higher rate of neovascularization in the operated area, especially in the central portion of the flap where angiogenesis were expected to be the most active.

Discussion

The middle portion of the flap, about 1 cm caudal from the necrotic margin, is known to be the area of transition between stasis and blood flow, and is therefore the region that shows most active angiogenesis and vasculogenesis of choke vessels under hypoxic stress[10]. The most important factor in improving flap survival, is without any doubt, a precise surgical technique and adequate surgical planning. In an attempt to aid the surgeons in achieving more satisfactory results, various methods and drugs have been studied, and are currently in use [2-4]. The main actions of the medications include helping flap survival by increasing blood supply to the flap, or by providing a friendly microenviroment by supressing ischemic-reperfusion injury damage.

The reason we turned our attention to KRGE is that the material is well known to be capable of acting in both manners. From various studies, red ginseng has shown antithrombotic activity by inhibiting platelet aggregation[8], which can be beneficial in flap surgery where there are a limited number of supplying vessels. Also, in coronary artery occlusion, ginsenoside Rg1, one of the main compounds of red ginseng, has shown noticeable angiogenic potential as well as a protective role against cardiac ischemic-reperfusion injury[13]. The animals were

treated with KRGE for two weeks, from 1 week before the main operation. We expected to obtain effects of premedication by reviewing references[14, 15] that showed angiogenesis stimulation and prevention of ischemia-reperfusion injury with 5 to 6 days of preconditioning with red ginseng and ginsenosides.

The result of the present flap survivability study were very good, especially in comparison to other previous studies using different compounds. Surprisingly, the results of the present study were even better results than prostaglandin E1 or EGF, both of which are currently being used clinically. There were statistically significant differences between the 200mg/kg, 100mg/kg, and control groups. The dosages were determined from previous studies that used KRGE in rat or mice models, and the results of pilot studies that were performed by the authors. As the study produced successful results indicating possible dose-dependent effects of KRGE on flap survial, additional studies with more variety of KRGE dosage could reveal definite dose-dependent relationships.

Microscopic findings of operated flap tissue showed a stastically significant increase of mean vessel count per field in the 200 mg/kg ginseng uptake group. The main reason for increased number is closely related to new vessel ingrowth, the most obvious sign of the active vasculogenesis of choke vessels. The PCR study results supported this hypothesis by showing higher expressions of angiogenic growth factors, bFGF and PDGF. Basic FGF and PDGF are also known to have a role in

regenerating ischemic tissues and postinjury tissue repair.

To diagnosis and visualize angiogenesis in an animal model, conventional methods such as angiography, computed tomography, or magnetic resonance imaging have disadvantages including invasiveness and a lack of target probes when it comes to in vivo imaging [11, 16-18]. We introduced molecular imaging with the 99mTc-RGD peptide SPECT, which allows high sensitivity and resolution with accurate tracers. RGD- (Arg-Gly-Asp) containing peptides target integrins, which have been identified as favorable target for imaging angiogenesis. Previous studies with micro-SPECT-CT imaging showed effective results in tracking angiogenesis in animal models of myocardial infarction. We used the micro-SPECT-CT device designed for small-sized experimental animals, developed by Bioscan Corp. (Seattle, WA). As seen in figure 4, the overexpression of integrin was observed in both ginseng uptake groups. In particular, the signal intensity was strongest in the middle portion of the flap, which is known to show the most active angiogenesis of the choke vessels. Although the results were satisfying, quantative comparison of integrin expression between groups was not available because of the limited number of animals (n=4), which should be investigated more thoroughly in further studies.

The results from the experiments show a noticable improvement of flap survival in the ginseng intake animal groups compared to the control group, and additional studies showed various bases for the effect of

KRGE. The results will act as a valuable reference for further preclinical studies of KRGE in flap surgery and various reconstructive methods in the plastic surgical field. The limited number of experimental dosages and lack of quantitative analysis in SPECT study should be covered by additional studies in the near future.

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Table 1. Necrotic area assessments.

Group	N	Average	SD	F	Scheffe	p value
Control[a]	12	35.617	7.1059	21.707*	a>b	0.011**
100mg/kg[b]	12	25.342	7.8957		a>c	0.000**
200mg/kg[c]	12	14.617	8.3686		b>c	0.008**

*p<0.001

** Statistically significant (p<0.05)

Table 2. Microvessel density assessments.

Group	N	Average	SD	F	Scheffe	p value
Control[a]	12	1.42	0.9	28.621*	b>a	0.67
100mg/kg[b]	12	1.92	1.240		c>b	0.001**
200mg/kg[c]	12	5.25	1.765		c>a	0.000**

*p<0.001

** Statistically significant (p<0.05)

Table 3. PCR quantification analysis

	0 (control)		100mg/ml		200mg/ml	
	Volume.	(%)	Volume.	(%)	Volume.	(%)
EGF	2298.3	(100)	3252.1	(141.5)	3923.6	(170.7)
VEGF	540.8	(100)	1493.7	(276.2)	1973.5	(364.9)
bFGF	2702.6	(100)	4875.9	(180.4)	6395.1	(236.6)
PDGF	868.1	(100)	2047.5	(235.9)	3846.6	(443.1)
GAPDH	33011.6	(100)	33480.2	(101.4)	33953.0	(102.9)

Fig. 1. Comparison between flap survival results of control group (Left) and 200mg/kg group (Right). Necrotic area can be easily demarcated and measured.

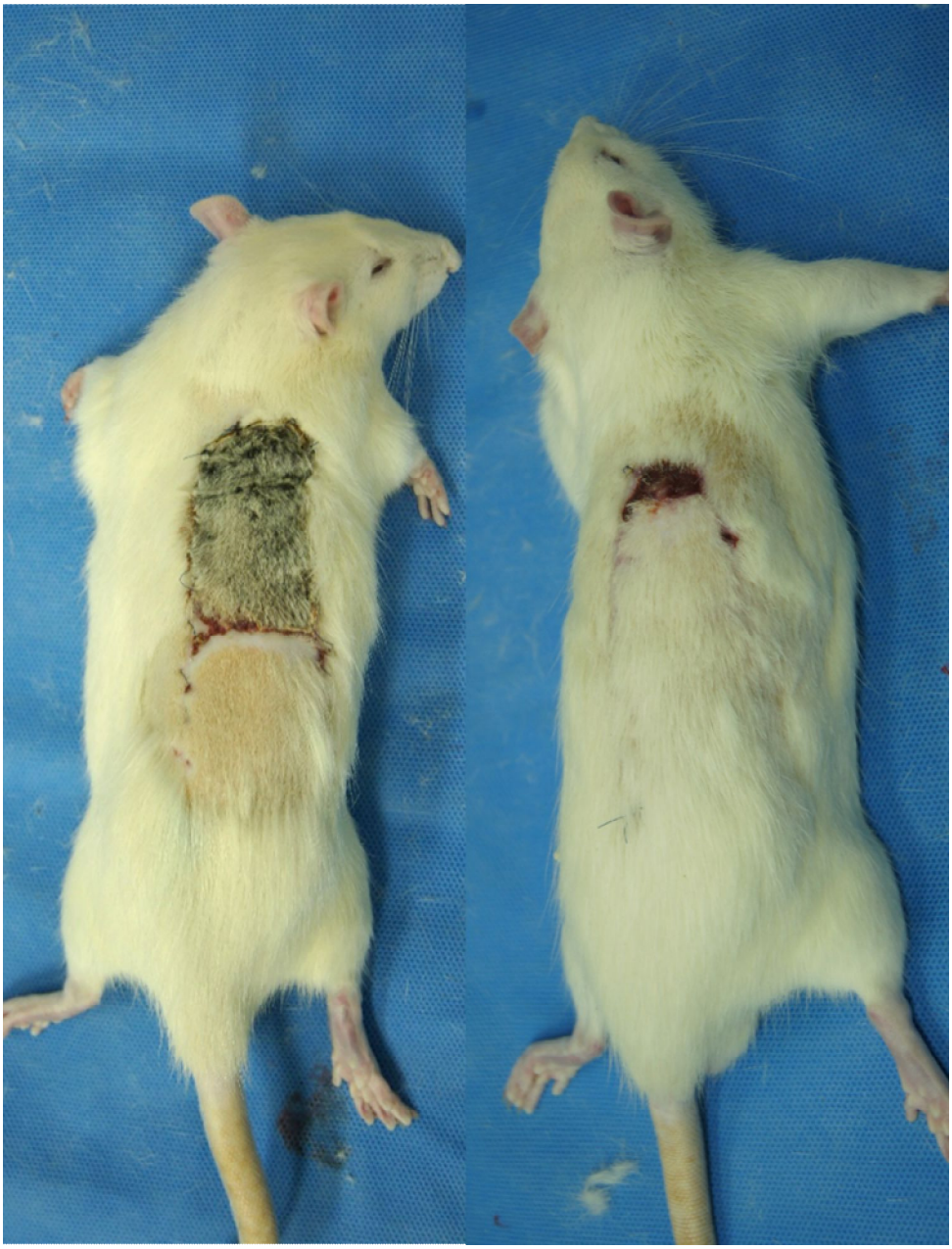


Fig. 2. (Above, left to right) vWF stained microscopic slide of control group, 100mg/kg group, and 200mg/kg group. (Below, left to right) CD31 stained microscopic slide of excised specimens from each group. By observing the slide under 1:40 magnification, a total of 10 fields were examined from each section slide and the number of microvessels were compared between groups. Black arrows indicate microvessels.

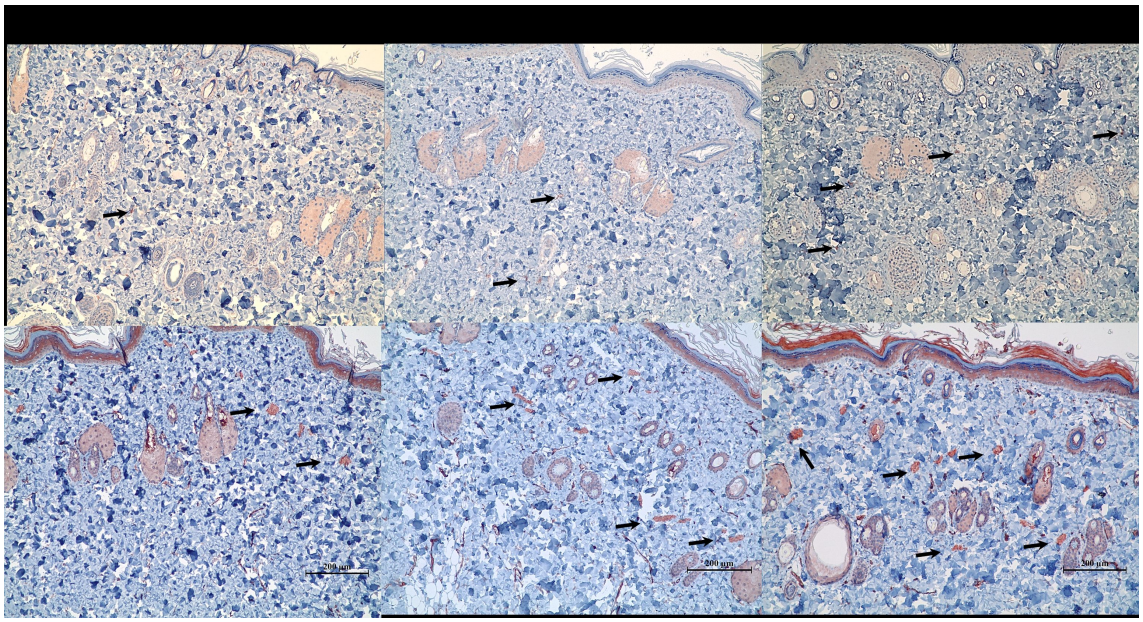


Fig. 3. Result of PCR study. A marked increase in the basic fibroblast growth factor (bFGF) (third row) and platelet derived growth factor (PDGF) (fourth row) in the ginseng intake groups was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). Epidermal growth factor (above row), vascular endothelial growth factor (second row) showed not enough difference between three groups.

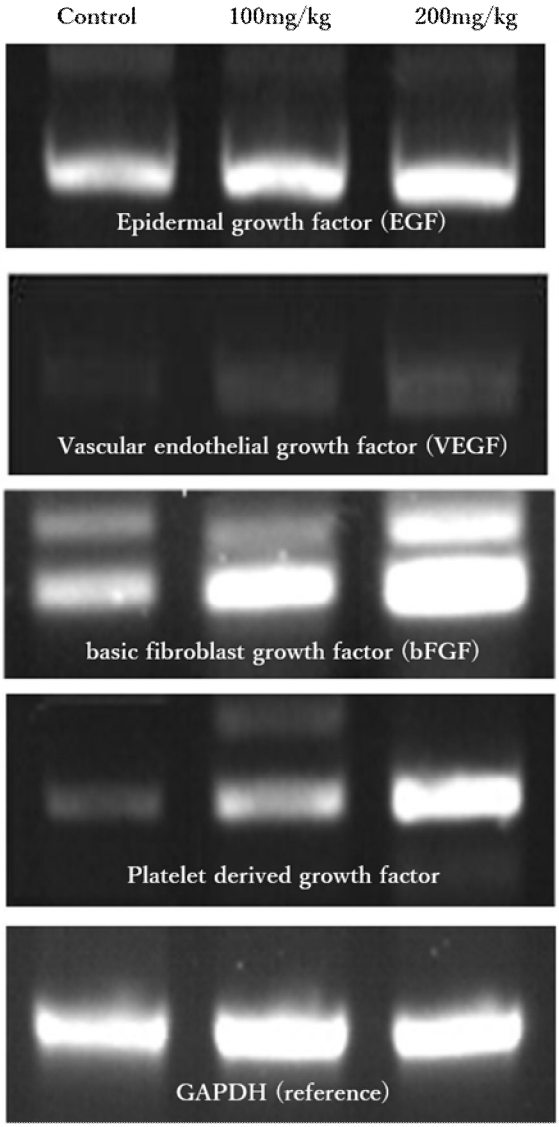


Fig. 4. Quantification analysis of RT-PCR study.

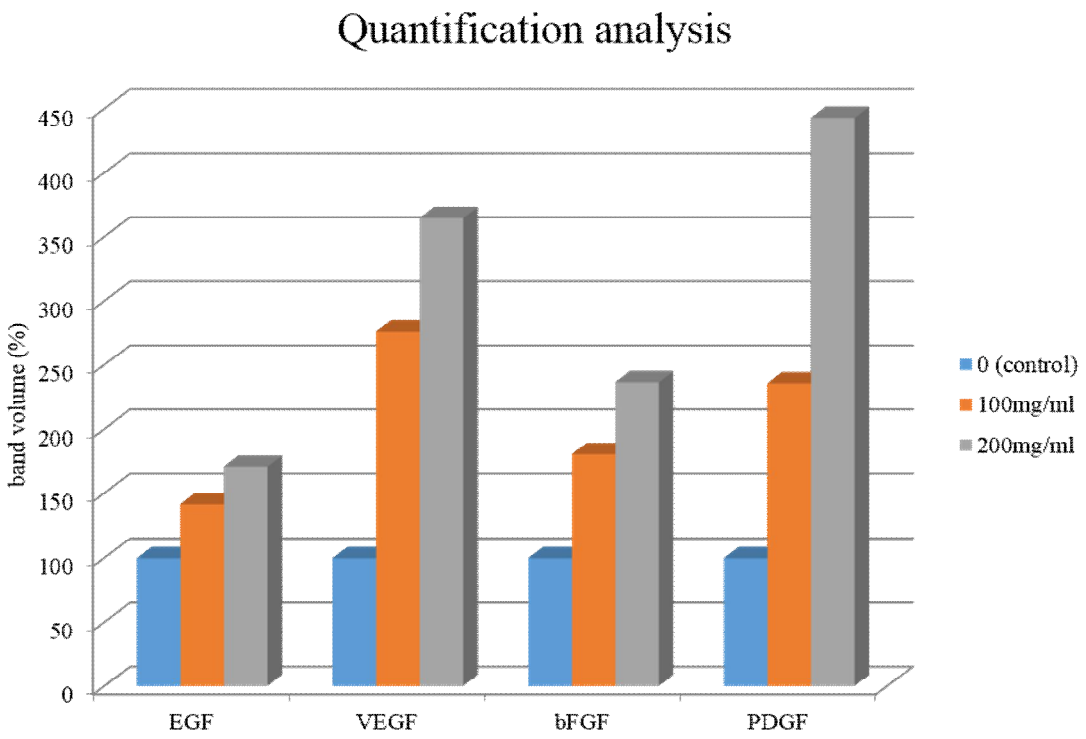
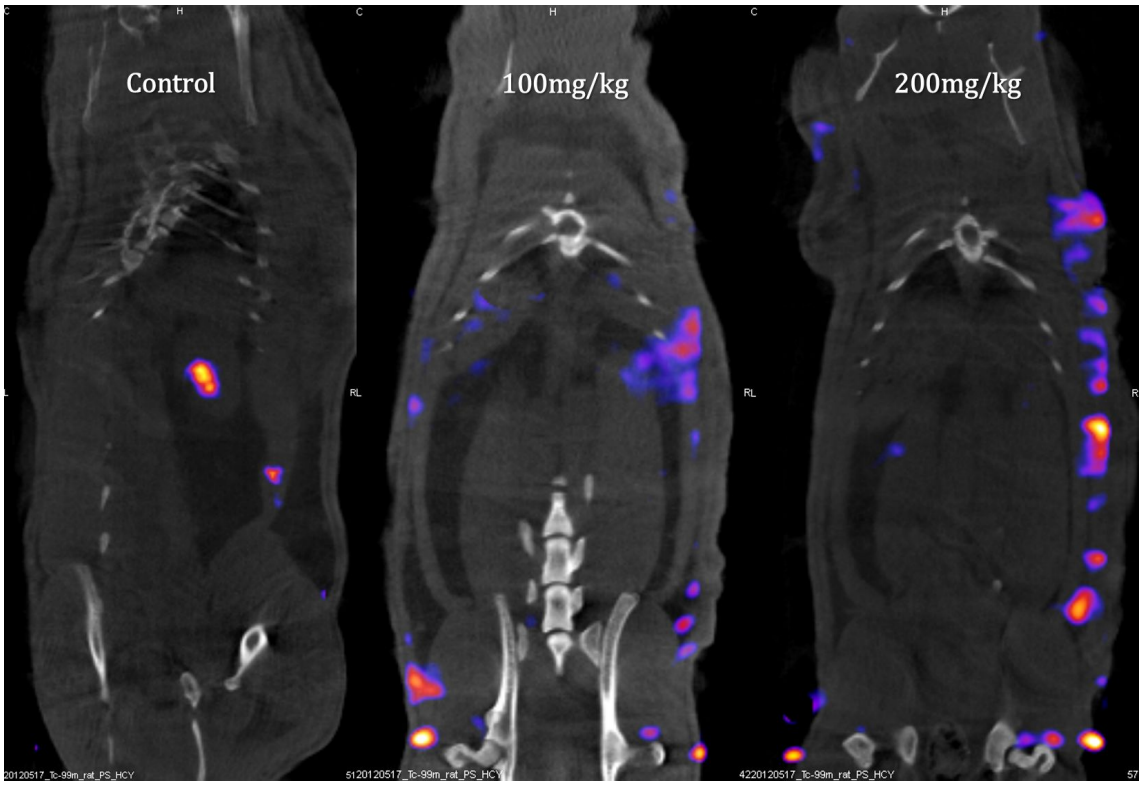


Fig. 5. Increase of neovascularization in 200 mg/kg group by micro SPECT-CT. The bright spots indicate the location of the flap operation, showing different signal intensities in the three groups. The strongest signal intensity can be seen in the mid-portion of the representative 200 mg/kg group animal.



요약 (국문초록)

배경 : 재건 성형의 영역에서 난축 혈관형 피판은 생존에 대한 예측이 어려움에도 불구하고 최근까지도 가장 흔하게 사용되어 지는 수술 방법이다. 피판 생존의 가능성을 높이기 위하여, 지금까지 다수의 약물 실험 및 임상 시험이 수행되어 왔다. 본 저자들은 오랜 시간 동안 동양에서 사용되어 온 홍삼 추출물이 피판의 혈관 신생과 형성을 향상시키는지에 대하여 실험을 수행하였다.

방법 : 몸무게가 300 에서 350 그람인 총 36 마리의 수컷 쥐가 실험에 사용되었다. 쥐들은 총 12 마리씩 세 군으로 나누어 졌으며, 첫 번째 군은 대조군으로써 홍삼을 공급하지 않은 군이었고, 두 번째 군은 kg 당 100mg 의 홍삼 추출물을 2 주간 공급한 군, 마지막으로 세 번째 군은 kg 당 200mg 의 홍삼 추출물을 2 주간 공급한 군이었다. 피부 피판의 생존 정도는 거상된 후 7 일째에 평가되었다.

그리고 마이크로 스펙트-전산화 단층 촬영기를 이용하여 피판 거상 후 3 일 째에 혈관의 재형성을 시각적으로 확인하고자 하였다. 조직화학염색을 통해 신생 혈관의 증가를 확인하고, 중합 효소 연쇄 반응을 통해 성장 인자의 증가를 확인하고자 하였다.

결과 : 대조군에 비하여, 실험군은 피판 생존율이 통계적으로 유의한 정도로 증가하였으며, 방사선학적 검사를 통해 신생혈관의 증가를 확인하였다. 또한 조직학적 검사 상 혈관 신생을 확인하였으며, 성장 인자 수치 또한 증가하는 것을 확인하였다.

결론 : 본 실험에서 홍삼 추출물을 공급한 개체에서 피판의 생존률이 증가하는 결과를 얻게 되었다. 이러한 결과는, 향후 재건 성형외과학 영역에서 피판술을 비롯한 다양한 수술의 상황에서 홍삼 추출물을 통한 전임상 시험 및 약품 사용에 대한 이론적 배경이 될 수 있을 것이다.

주요어 : 홍삼; 홍삼 추출물; 재건 성형; 혈관 신생; 난축 피판; 피판 생존; 분자 영상

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별첨 1. Table 1 (그룹 별 necrotic area measurement) 의 raw data

	Control	100mg/kg	200mg/kg
1	33.2	41.1	12.2
2	50.3	24.2	19.3
3	30.8	20.8	20.4
4	29.9	12.8	11.5
5	34.6	34.4	10.8
6	42.4	32.9	6.7
7	40.2	27.7	29.8
8	39.8	26.9	3.5
9	35.5	21.3	12.9
10	23.6	19.2	10.3
11	29.1	25	29.2
12	38	17.8	8.8

별첨 2. Table 2 (그룹 별 microvessel density count) 의 raw data

Rat number	Group	Microvessel count
1	Control	2/3
2	Control	1/2
3	Control	0/0
4	Control	0/0
5	Control	1/1
6	Control	2/1
7	Control	1/2
8	Control	2/4
9	Control	3/1
10	Control	2/0
11	Control	1/1
12	Control	2/2
13	100mg/kg	1/3
14	100mg/kg	3/2
15	100mg/kg	5/5
16	100mg/kg	2/2
17	100mg/kg	2/1
18	100mg/kg	1/1
19	100mg/kg	1/2
20	100mg/kg	3/3
21	100mg/kg	1/0
22	100mg/kg	1/1
23	100mg/kg	1/2
24	100mg/kg	2/2
25	200mg/kg	5/5
26	200mg/kg	3/5
27	200mg/kg	4/4
28	200mg/kg	5/6
29	200mg/kg	7/5
30	200mg/kg	6/4
31	200mg/kg	8/5
32	200mg/kg	2/4
33	200mg/kg	4/6
34	200mg/kg	6/4
35	200mg/kg	7/4
36	200mg/kg	6/5