의학과석사 학위논문

Effects of Adipose-Derived Stem Cells on the Survival of Allogenic Skin Graft in Mice: A Preliminary Study

지방유래줄기세포는 쥐의 동종 피부이식의 survival에 미치는 영향: 예비 연구

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ABSTRCT

Effects of Adipose-Derived Stem Cells on the Survival of Allogenic Skin Graft in Mice: A Preliminary Study

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Background: Recently, composite tissue allotransplantation (CTA) was introduced that has considerable potential for application in the field of plastic and reconstructive surgery. CTA consists of varied tissues including skin, fat, muscle, nerves, bone, etc. However, many factors such as immunological rejection, blood

circulation disturbance, infection, may inevitably cause grafting failure. Therefore, improving the survival of allotransplantation has important clinical significance. Skin is considered that has high immunogenicity. Thus, the aim of this study was to evaluate the effect of adipose derived stem cells (ADSCs) on improving the survival of allogenic skin graft in mouse model.

Methods: In this study, BALB/c mice as skin graft donors, and C57BL/6 mice as skin graft recipients were selected as the research objects. The experiment was divided into three groups: no injection group (control group, n=12); mice tail vein injection group (IV group, n=12); and fascial layer of the recipient bed injection group (FL group, n=12). After the operation, the adipose derived stem cells (1.5×10^5/ml) obtained from fat tissue of C57BL/6 mice were injected to the mice via tail vein and fascial layer of the recipient bed. The results of skin graft were analyzed by histology and immunohistochemistry.

Results: On 7th day posttransplantation, the skin survival rate was (67.87%±3.97%) in control group, and (84.87±5.84)% in IV group and (84.5±13.41)% in FL group. Compared with the control group, the skin graft survival rate increased in the experiment groups ($P<0.05$), but there was no significant difference in the IV group and FL group ($P>0.05$).On 14th day, the skin survival rate in control group was (17.87±1.88)%, (52.75±3.28)% in IV group, and (55.88±4.61)% in FL group. There was also significant difference between the experiment groups and control group ($P<0.05$). Histologic changes in the skin allografts paralleled the gross appearance of rejection. The inflammatory reaction was more prominent in control group on 7th and 14th day postoperatively. And the expression of vascular endothelial growth

factorthe (VEGF) in the control group was less than in the experiment groups on 7th and 14th day postoperatively.

Conclusions: This preliminary study suggested that the mice adipose derived stem cells could increase the allogenic skin graft survival. However, compared IV group and FL group, there was no significantly difference on effecting the survival of skin allograft.

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Keywords: allogenic skin graft, adipose derived stem cells (ADSCs), skin survival, composite tissue allotransplantation (CTA)

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1. Introduction

Composite tissue allotransplantation (CTA) had considerable potential for application in the field of plastic and reconstructive surgery, such as traumatic injuries, and extensive tissue loss secondary to burns. CTA is composed of a variety of tissues, such as skin, fat, muscle, nerves, lymph nodes, bone, cartilage, ligaments, and bone marrow. In these tissues, skin is one of the most common tissues in transplantations. However, due to high immunogenicity of skin, subcutaneous effusion, skin fixation and other factors, they may cause graft acceptance and tolerance more difficult to achieve [1, 2]. Finally, these reasons may inevitably lead to failed graft operation, and it will bring the patients great suffering and heavy financial burden. Therefore, improving survival of CTA has important clinical significance.

Mesenchymal stem cells (MSCs), derived from fetal and adult organs, have the capacity to self-renew and differentiate into various tissues including muscle, fat, stroma, tendon, cartilage, and bone [3]. The previous experiments in vitro have revealed that Mesenchymal stem cells (MSCs) can suppress the T cell response and directly inhibit T cell and B cells proliferation [4-6].

Furthermore, MSCs can affect immunologic functions of antigen-presenting cells and inhibit monocyte differentiation into mature dendritic cells [7].

Moreover, as one type of the mesenchymal stem cells (MSCs), Adipose-Derived

Stem Cells (ADSCs) from the adipose tissue, have been proved that they also have the capacity as the MSC [8]. Some experiments in vivo have showed that ADSCs have the potentials of immunosuppressive properties, anti-inflammatory [8-10]. In some recent researches, they showed that the ADSCs can also promote vascular endothelial growth factor (VEGF) synthesis, thus, ADSCs could participate the process of neovascularization [11].

Based on these characteristics of the Adipose-Derived Stem Cells (ADSCs), at first step, we were to make primary research to study the effect of adipose derived stem cells on improving the allogenic skin graft survival in allogenic mouse model, and we also studied the effect of different injection ways on the allogenic skin graft survival in mouse model.

2. Materials and methods

2.1 Animals and study design

8-week old male BALB/c mice were used as skin graft donors, and 8-week-old male C57BL/6 mice (Koatech, Korea) were used as skin graft recipients. Because we could harvest two pieces of skin from the donor mice, so in this study we used eighteen BALB/c mice and thirty-six C57BL/6 mice. While another ten C57BL/6 mice would be used to harvest adipose tissue to culture the adipose tissue-derived stem cells. According to the way of injection after skin transplantation, the experiment was divided into three groups: no injection group (control group, n=12); mice tail vein injection group (IV group, n=12); and fascial layer of the recipient bed injection group (FL group, n=12). Specifically, ADSCs at a concentration of 1.5×10^5/ml were given to IV group and FL group mice after skin allograft.

2.2 Preparation of ADSCs

2.2.1 Isolation and Culture of ADSCs from the C57BL/6 Mouse fat tissue

ADSCs were obtained from subcutaneous adipose tissue of the C57BL/6 mice and washed by phosphate-buffered saline (PBS). The fat was finely minced and digested with collagenase type I (Sigma) in incubator for 30 minutes at 37°C. Then, the cell suspension was mixed with the DMEM-low glucose (Invitrogen, EUA)

supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Invitrogen) and then centrifuged 300g/5min. Subsequently, the cell pellet was washed twice by PBS and at last the cell pellet was resuspended in medium. Cells were plated and incubated at 37℃ in 5% CO2. The adherent cells were maintained in culture until 5 passages. At passages 5, when the cells were on reaching 90% confluence, the cells were digested with 0.25% trypsin/EDTA acid at 37℃, washed by PBS and centrifuged, and resuspended in the 0.1ml PBS for injection. Meanwhile, the number of cells were adjusted at 1.5×10^5/ml.

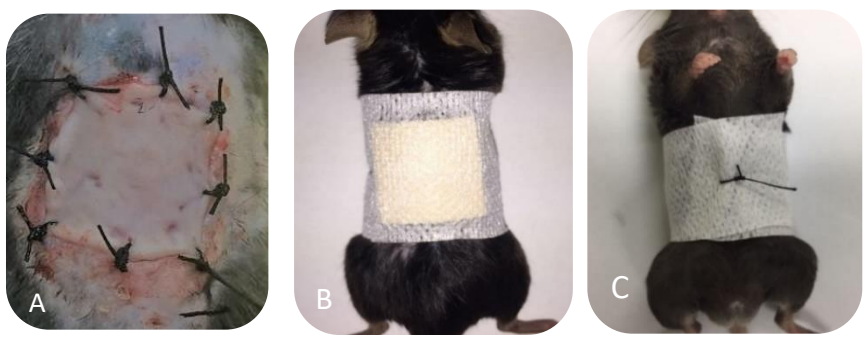
2.2.2 The PKH26 labeling and 4, 6-Diamidino-2-phenylindole labeling

To determine if there would be ADSCs in the transplant specimens, after the passage 5 cells were prepared, we used PKH26 Red fluorescent cell linker kits (Sigma Aldrich) to mark the cells. When the tissues were harvested on 7^{th} day postoperation, tissues were made as sections, and then the tissues were counterstained with DAPI (Sigma-Aldrich).

2.3 Full-thickness skin graft model and injection of ADSCs

After mice were sedated with 2% Isoflurane, 1.5×1.5cm full-thickness skin grafts were harvested from the midline of the back including the panniculus carnosus. During harvesting, the panniculus layer was carefully removed from the bed of the skin graft. The skin graft was kept in saline before being transferred to the recipient site. Then the skin grafts were placed on the midline of the back of the recipient and fixed with simple interrupted sutures (4-0 Silk; AILEE, Busan, Korea) (Figures 1.

A). Then the IV group mice were injected with ADSCs/PBS via tail vein and the ADSCs/PBS were injected in the fascial layer of the recipient bed in FL group. The control group was no injection. Then the mice were dressed with the medifoam (Mundipharma) and Hypafix (BSN medical) (Figures 1. B and C). On day 7 postoperation, we separated the dressing and observed the skin and make sure if there was rejection. The rejection was defined when >50% the graft tissue became necrotic.



Figures 1. A. the skin grafts were placed on the midline of the back of the recipient and fixed with simple interrupted sutures. B and C. after suturing, the surgical site was dressed with medifoam (Mundipharma) and Hypafix (BSN medical).

2.4 VEGF Immunohistochemical Staining and Histologic examination

Immunohistochemistry was used to stain vasoactive endothelial growth factor (VEGF) with a VEGF polyclonal antibody (ab1316, 1:200 dilution; Abcam). Paraffin-embedded tissue from skin grafts was sectioned 4 mm thick. All sections were stained with hematoxylin and eosin. By the optical microscopy, infiltration of

inflammatory cell and neovascularization in skin tissue were observed at day 7 and 14 postoperation.

2.5 Statistical analysis

The data were presented as mean and standard deviation. The data from the different groups were compared using analysis of variance (ANOVA) and Kruskal-Wallis (K-W) test. P values <0.05 were considered statistical significance.

3. Results

At 7 days postoperation, the dressing was removed. The regions of survival and necrosis on skin transplantation were clearly observed. In the control group, partial skin was rigid and black. However, the skin in IV group and FL group appeared pink-white, soft, and normal (Figures 2). We observed the skin again on day 14, most of skin in control group was necrosis, but there were still most of skin surviving in both experiment groups (Figures 3). To evaluate the survival rate of skin transplantation, we used Image J software (National Institutes of Health) to calculate it. In control group, the mean and SD of the viable percentage of the skin graft was (67.87±3.98)%, and (84.88±5.84)% in the IV group, and (84.5±13.41)% in the FL group on day 7 postoperation. The survival percentages of experiment groups were significantly higher than the survival percentage in the control group ($P < 0.05$). The mean percentage of the surviving area between the 3 groups was shown in the Chart 1 and Table 1. However, between the IV group and FL group, there was no significate difference ($P > 0.05$). On day 14 after skin graft, the mean and SD of the viable percentage of the skin graft was (17.87±1.88)% in control group, and (52.75±3.28)% in the IV group, and (55.88±4.61)% in the FL group. Between the control group and experiment groups, there were still significant differences.

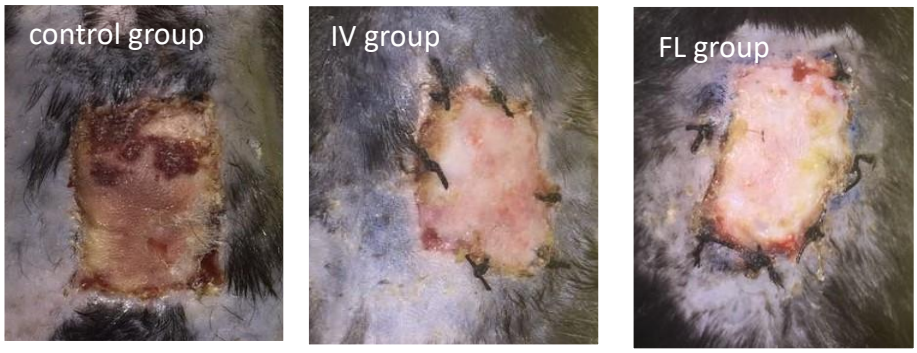


Figure 2. On 7th day there was obvious changing in the control group, partial skin was necrosis, however the skin in both IV group and FL group appeared pink-white, soft, and normal.

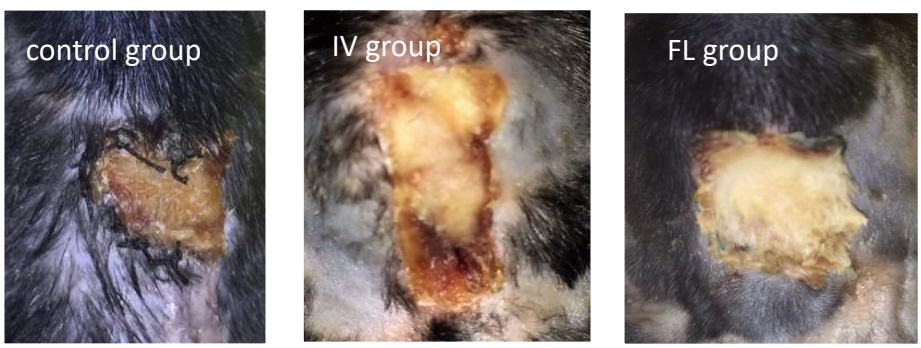
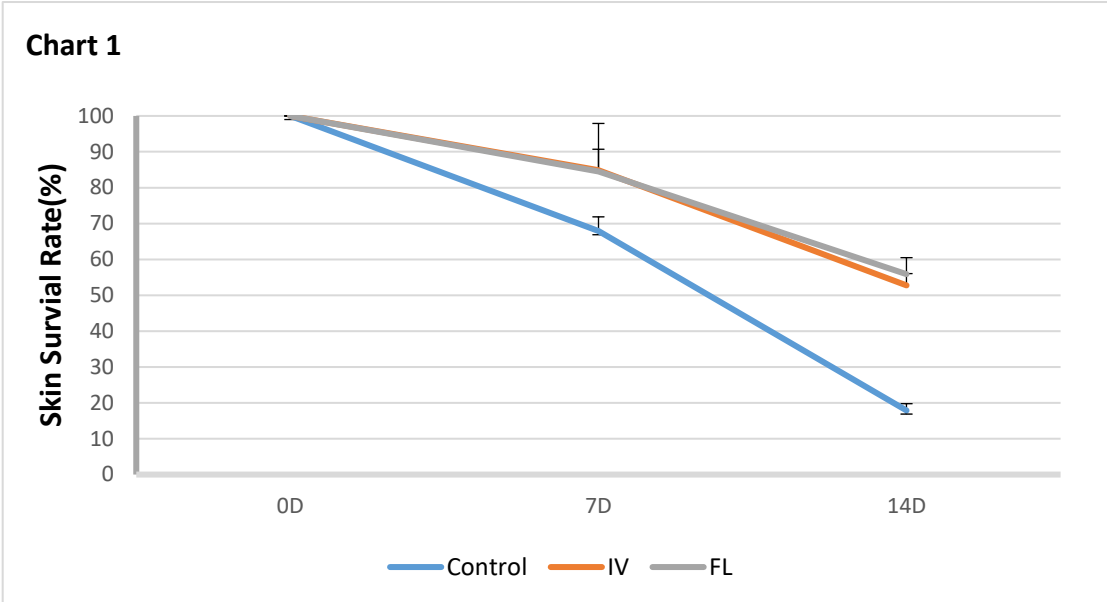


Figure 3. Almost all the skin had been necrosis in the control group on 14th day. However, most of the skin still survived in IV group and FL group.



Observation period	Control group (%)	IV group (%)	FL group (%)
		Mean (SD)	
7 day	67.87 (3.98)	84.88 (5.84)	84.5 (13.41)
14 day	17.87 (1.88)	52.7 (3.28)	55.88 (4.61)

Chart 1 and Table 1. The mean and SD of the viable percentage on 7th and 14th days postoperatively. Compared with the control group, the survival rate in the experimental groups was higher. There was statistical significance between the control group and experimental groups ($P<0.05$). However, there was no statistical significance between the IV group and FL group on 7th and 14th day ($P>0.05$).

After staining the tissue sections with DAPI, the tissue sections from all groups was assessed by fluorescence microscopy on day 7 posttransplantation. The PKH 26-labeled cells could be detected in the graft tissue in both IV group and FL group (Figure 4).

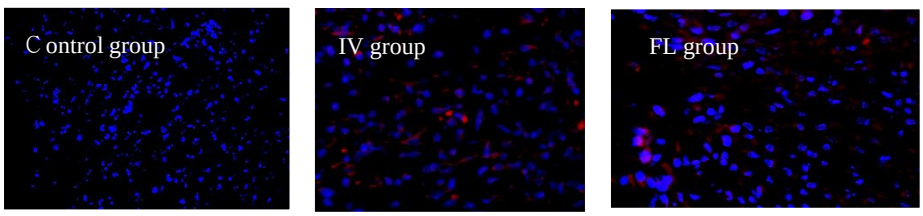


Figure 4. On 7th day posttraslatation, the specimen containing PKH26 labeled ADSCs in both experimental groups were observed by red fluorescence microscope. The red marks showed the PKH26-labeled cells.

Histologically, necrotic sections revealed evidence of acute inflammation with infiltration of monocytes and neutrophils. So we evaluated the histopathologic changes on 7th and 14th days after transplantation, respectively. The histopathology was assessed by the degree of inflammatory cells infiltration on a hematoxylin-eosin stain. At the same time, to evaluate the degree of angiogenesis, the tissue sections were stained by VEGF Immunohistochemistry. The results exhibited that the inflammatory reactions, which were prominent in the control group on 7th day after transplantation, were attenuated by the IV group or FL group (Figure 5. A-C). In VEGF immunohistochemistry, VEGF was less expressed in control group. However, it was prominent in IV group and more expressed in FL group (Figure 5. D-F). On 14th day after transplantation, a considerable number of grafts treated with ADSCs remained undetached with limited inflammatory cell infiltration (Figure 6. A-C). The

expression of the VEGF in control group was still not obvious than IV group and FL group. (Figure 6. D-F)

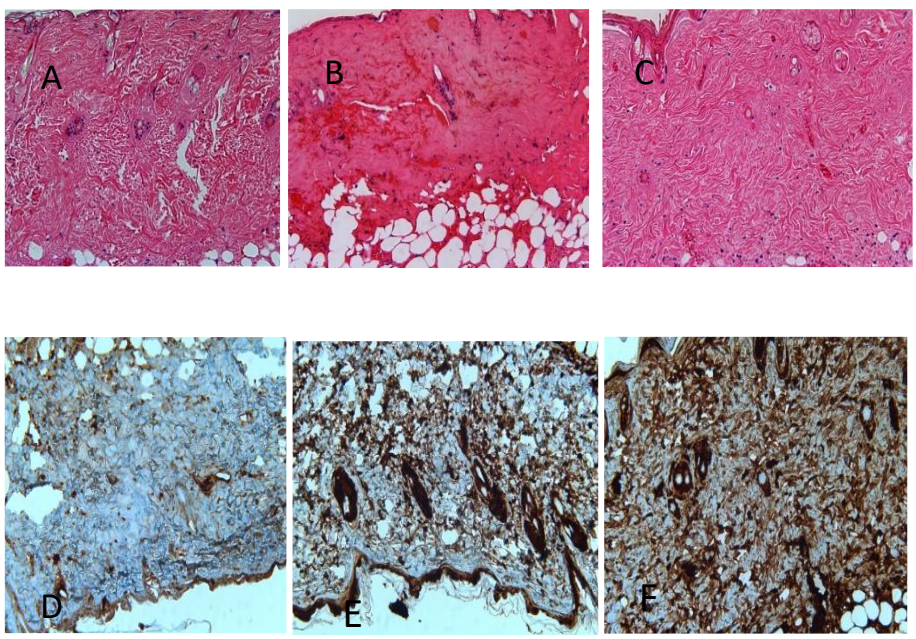


Figure 5. Comparison of histologic changes, the inflammatory reactions had been more prominent in control group on day 7 posttransplantation than the experimentgroups (A-C) (×200). Finally, in control group, VEGF was less expression, but it was evidenced in IV group, and most prominent in FL group (D-F) (×200).

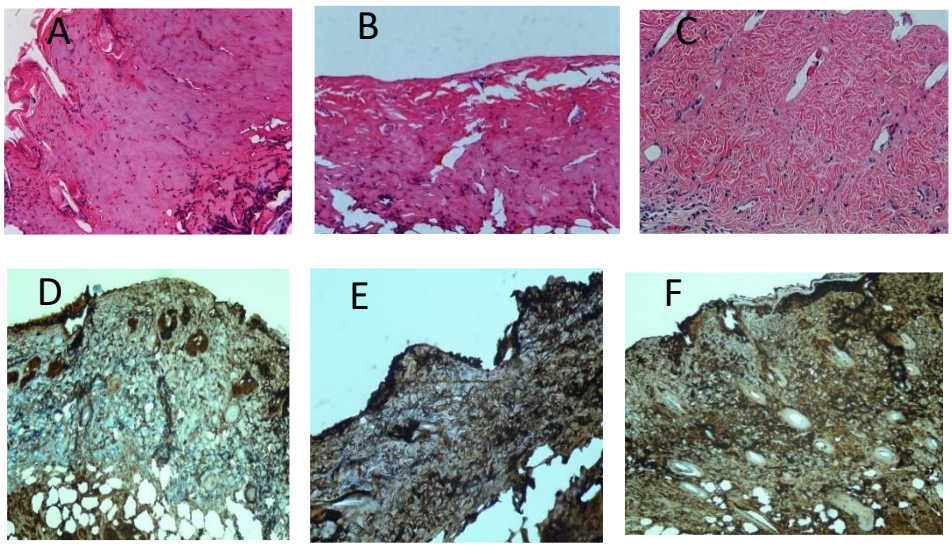


Figure 6. On day 14 posttransplantation, inflammatory reactions were still more prominent in control group than the IV or FL group (A-C) (×200). And the expression of the VEGF was very low than the experiment groups (D-F) (×200).

4. Discussion

Due to a variety of reasons, the survival of autologous skin transplantation is 70%-100% in the clinical surgery [12]. However, because of the factors such as the immunological rejection and the blood supply, the survival of skin allograft will be lower. MSC is another type of tissue stem cell, which is different from hematopoietic stem cells in bone marrow. It has been recognized that MSC has the function of regulating the immune system, and inhibit T cell and B cell proliferation [4-6]. Moreover, MSCs could also differentiate into various tissues including muscle, fat, stroma, tendon, cartilage, and bone, and be involved in angiogenesis [3]. Adipose-Derived Stem Cells (ADSCs), as a type of MSCs, was separated for the first time by Zukpa et al in 2001 [13]. ADSCs have the characteristics of MSCs. And the source of adipose tissues are abundant and ADSCs are easy to be cultured. Due to these points, applying ADSCs will be a new therapeutic tool on skin graft.

In this paper, we have discussed the effects of ADSCs on the survival of allogenic skin graft in mice by different injection methods. We used BALB/c mice as skin graft donors, and C57BL/6 mice as skin graft recipients in this study. The survival of skin grafts were observed and the survival rate was calculated at day 7 and 14 postoperation. The survival rate significantly increased in IV group and FL group than the control group (Table 1). In the skin allograft on mice, the immunological rejection usually occurs in about 7 days [14]. In our study, by observing the skin, it had been verified. On 7th day , the skin was black and rigid in control group, however in both IV group and FL group the skin was still normal, pink-white, and soft (Figure

2). We speculated that ADSCs have played an immunosuppressive role.

PKH26, as a fluorescence dye, can be used to stain the ADSCs in vivo and vitro, and PHK26 do not only affect the survival of ADSCs, and it can also track the cells for a long time [15, 16, 17]. In our study, we used PKH26 and DIAP to stain the cells and sections, and the ADSCs were detected in the IV group and FL group on 7th day. We confirmed that ADSCs remain active and function in the skin.

In addition, by VEGF immunohistochemistry and HE staining, the results also showed that in the two experimental groups, the expression of VEGF was higher than the control group, and inflammatory cells significantly less than the control group. The result indicated that ADSCs could promote the formation of VEGF. In the current study, VEGF have been proved have the ability to promote accelerated neovascularization [18]. These evidences suggested that ADSCs had directly or indirectly involved in the process of angiogenesis. And ADSCs also have anti-inflammatory effect in vivo. All these results, we had obtained, were consistent with the previous researches.

Compared the IV group and FL group, there was no significant difference in survival rate or the results of the immunohistochemistry and histopathology. We considered both ways cloud improve the survival of skin allograft. However, to confirm if there are local effects on bodies by different injection methods, we need further experimental verification. For the future clinical treatment, it will provide a theory evidence.

Finally, due to the limitation of experimental conditions, the mechanism of immune modulation of ADSCs was not studied, however, the fact that adipose derived stem cells could improve skin graft survival had been revealed. In order to study these mechanisms of ADSCs, we are conducting some experiments about the immune modulation of ADSCs. The next step, we will also study the composite tissue allotransplantation such as skin flap graft, combined with ADSCs. In summary, combined with the previous studies, the ADSCs have not only the immunosuppressive effect, but also they play important roles in angiogenesis and anti-inflammatory effects.

5. Conclusions

This preliminary study suggested that the adipose derived stem cells may have the immunosuppressive, anti-inflammatory, and vasculogenic potentials. Due to these characteristics, ADSCs could increase the allogenic skin graft survival. Furthermore, compared the IV group and FL group, there was no significant difference on effecting the survival of skin allograft.

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국문초록

지방유래줄기세포는 쥐의 동종 피부 이식의 survival에 미치는 영향: 예비 연구

서론: 복합조직이식은 피부, 지방, 근육, 신경, 뼈 등 다양한 조직을 한꺼번에 이식을 하는 것으로, 수술 술기 및 면역 억제제의 발달로 인해 최근 재건수술분야에서 미래 의학으로 각광을 받고 있다. 하지만 복합조직이식은 면역성이 강하여 면역 거부반응이 일어나기 쉽고 면역억제제의 용량을 높여야하기 때문에 면역능 저하 및 감염 등의 문제가 발생할 수 있다. 따라서 복합조직이식을 성공적적으로 유지하기위한 핵심은 면역거부반응을 최소화하고 면역관용을 유도하는

것이라 하겠다. 이번 연구 목적은 지방유래줄기세포를 쥐의 동종 피부이식에 이용하여 면역관용을 유도할 수 있는지 알아보는 것이다.

재료 및 방법: Balb/c 마우스의 전층피부를 C57LB/6 마우스에 이식하였다. 실험군 1에서는 마우스의 꼬리동맥을 통하여 지방줄기세포를 주입하였고, 실험군 2에서는 피부이식 주변의 근막조직에 지방줄기세포를 주사하였다. 대조군에서는 세포를 주입하지 않았다. 피부이식의 결과는 수술 후 7, 14일에 육안적인 검정과 조직학적인 분석을 시행하였다.

결과: 이식후 7일 날에 피부 생존률을 계산하였다. 대조군의 피부 생존률은 (67.87%±3.97)% 이었으며, 정맥을 통해 지방줄기세포를 이식한 그룹의 피부 생존률은 (84.87±5.84)% 이었으며, 그리고 주변의 근막 조직에 지방줄기세포를 주사한 그룹의 피부이식 생존률은 (84.5±13.41)% 이었다. 대조군에 비해 실험군에서 피부 생존률이 더 높았으나 ($P<0.05$). 두 실험군 사이에는 통계적인 유의성이 없었다 ($P>0.05$). 이식후 14일 날에 피부 생존률을 계산하였다. 대조군의 피부 생존률은 (17.87±1.88)% 이었으며, 정맥을 통해 지방줄기세포를

이식한 그룹의 피부 생존률은 (52.75±3.28)% 이었으나, 그리고 주변의 근막 조직에 지방줄기세포를 주사한 그룹의 피부이식 생존률은 (55.88±4.61)% 이었다. 대조군에 비해 실험군에서 피부 생존률이 더 높았다 ($P<0.05$). 두 실험군 사이에는 통계적인 유의성이 없었다 ($P>0.05$). 7일 14일에 조직학적인 검사를 통해 실험군에서 염증반응이 적게 일어나고 혈관신생이 더 많이 이루어지는 것을 확인할 수 있었다.

결론: 이번 동물 실험을 통해 지방줄기세포 이식이 항염증반응, 신생혈관형성의 기존을 통해 동종피부이식의 생착률을 높이는데 기여할 수 있음을 증명하였다. 주입경로에 따른 차이는 없었다. 향후 정량적인 분석, 정확한 기전 규명을 시행한다면 나아가 임상 적용을 위한 초석을 만들 수 있을 것이다.

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주요어: 동종피부이식, 지방줄기세포, 피부 생존률, 복합조직이식

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