공학석사 학위논문

Microcurrent Sheet-based Electrical Stimulation Coupled with Electroconductive Cryogel for Wound Regeneration

미세전류 시트의 전기 자극과 전기 전도성을 지닌 크라이오젤을 통한 피부 상처재생

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Electrical stimulation (ES) may provide stimulating signals that can accelerate the proliferation of cells around the wound area. We utilized a microcurrent sheet (M-sheet) for wound regeneration. M-sheet coupled with electrical conductive cryogel was designed to allow electrical flow and the current for an extended period of time in a wet condition. We fabricated electroconductive cryogel with methacrylated gelatin (GelMA). Polypyrrole (PPY), a biocompatible and conductive material that is the polymerized form of pyrrole, was incorporated into GelMA cryogel network to provide electrical conduction. The M-sheet was placed on top of PPy incorporated GelMA for application in the wound regeneration model. The physical and electrical properties of PPy incorporated GelMA (PPy/GelMA) were dependent on pyrrole concentration. Where an increase in PPy concentration enhanced physical and electrical properties. The incorporation of PPy promoted the wound healing process. Moreover, when M-sheet -derived electrical field was coupled with PPy/GelMA, rapid wound closure was observed. It suggests that the combination of both PPy/GelMA cryogel and M-sheet has a synergetic effect on skin wound healing and it provides may provide a novel strategy of ES-based wound therapy.

Keyword : Microcurrent, Electrical stimulation, Cryogel, Wound regeneration

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Chapter 1. The Scientific Background and Research Progress

1.1 Wound healing model and electrical stimulation

Skin is the largest organ in our body. This acts as a barrier to the environment like invasion of various pathogens or harmful substances. [1] However, the skin is frequently injured in various physical and chemical ways, and the skin is structurally and functionally rebuilt by a well-made process. Growth factor and Extracellular matrix(ECM) is mainly involved in this process especially for the guidance of fibroblasts [2] It is known that when healing process occurs, it is important to ensure proper moisture and oxygen levels and to block external microorganism are important. [3] So these principles are followed in Skin treatment.

Representative disease models related to skin include diabetic foot ulcer, burn model and full-thickness wound. Full-thickness wound refers to a wound in which epidermis, dermis and subcutaneous fat are all injured. A wide variety of therapies have been performed and studied to treat these skin wounds.

One common treatment is dressing. In 1958, Odland first discovered blister regeneration faster than the first untreated group. Dressing can prevent external microbial contamination and prevent the spread of infection to other patients. In other words, it serves as a shield between wounds and the outside world. Dressing can also play an important role in modifying the microenvironment of the wound. [5] A commercially representative product is Tegaderm thin film (3M). This keeps the wound environment moist, blocking water from the outside and allowing gas to pass through. [6]

The hydrogel is a polymer of polymers and is primarily used as a scaffold for wound healing. First, Tunable mechanical properties ensure proper elasticity and flexibility to wounds. And hydrogels absorb and retain wound exudate. This promotes fibroblast proliferation and keratinocyte migration. Both play an essential role in the epithelialization of the wound. It is also responsible for the effective delivery of bioactive molecules. [7]

But these methods has some limitation. Current therapies allow structural reconstruction of damaged skin, but functional reconstructions that enable the skin to function intact, such as blood vessels, sweat glands, and nerve tissue, are still difficult.

One way to enable active reconstructions of the skin is to apply an active electrical signal. Skin is a tissue that is sensitive to electrical signals. It has its conductivity from 2.6mS/ cm to 1 x 10^{-4}mS/c [8, 9] Even under normal conditions, the potassium pump runs different potentials inside and outside the tissue. When a wound occurs, the wound becomes negatively charged and electrical potential tends to collect toward the wound. Following this direction, the cells migrate. Research has been carried out to treat wounds using this principle. There are existing studies that allow electrical stimulation to flow through the wound, but these methods also have the disadvantage that patients feel uncomfortable or have to carry large equipment.[10-13]

Chapter 2. Microcurrent Sheet-based Electrical Stimulation Coupled with Electroconductive Cryogel for Wound Regeneration

2.1 Introduction

In order to secure the shortcomings of the existing methods of giving electric stimulation to wound region, research on dressing that can give electric stimulation has been advanced. [21, 22]

We use Microcurrent sheet (M-sheet, biosensor). M-sheet is a AgCl/Zn electric dressing that generates current in the wet state by using galvanic effect. The electric potential was measured for more than 24 hours and 0.9V or more in the condition of being immersed in PBS.

We were able to fabricate the sheet in the desired direction for the current to flow. For example, the electric potential can be directed from outside the wound to the inside of the wound, and the dots can be arranged closely to give compact electrical stimulation. We used a dots-shaped M-sheet that can give compact electrical stimulation to the wound for the experiment. The M-sheet can easily provide electrical stimulation in a desired direction without requiring heavy equipment.

In order to better transmit this electrical stimulation to the wound, we thought of using a electrically conductive cryogel together. We thought this would have a synergistic effect on wound regeneration this is because it fills wound region.

Electrically conductive polymers are known to regulate cell adhesion, differentiation, proliferation, and migration of electrically excitable cells. It will therefore apply to skins that are electrically excitable cells. Therefore, many studies have used conductive polymers as wound dressing materials. Representative conductive polymers include polypyrrole(PPy), polyaniline, polythiophene. [9] We selected polypyrrole with biocompatibility and coated it to gelatin-methacryloyl(GelMA) based cryogel. Cryogel's macroporous properties make itself affinity ligands as adsorbents for cell chromatography. [15] We filed the wound with this PPy/GelMA cryogel and placed an M-sheet on it to create an effective wound regeneration model. This serves to diffuse the microcurrent generated by the M-sheet well into the wound.

2.2 Materials and methods

2.2.1 M-sheet preparation

The M-sheet fabricated by Biosensor Laboratories, Inc (Seoul National University, Seoul 08826, Republic of Korea).

It was made by printing on microfiber. This does not require an external power source, can generate an electric field by the redox reaction of AgCl and Zn .

$$\text{Cathode: } AgCl_{(s)} + e^- \rightarrow Ag_{(s)} + Cl^-_{(aq)} \qquad E^o_{red} = 0.222V$$

$$\text{Anode: } Zn \rightarrow Zn^{2+} + 2e^- \qquad E^o_{ox} = -0.762V$$

$$E^o_{cell} = E^o_{cathode} - E^o_{anode} = E^o_{red} - E^o_{ox} = 0.222+0.762 = 0.984V$$

When M-sheet is wet with electrolytes, it constructs a galvanic cell, Silver chloride is reduced and its standard potential is 0.222V while Zinc is oxidized and its standard potential is − 0.762V. The standard potential of the galvanic cell is 0.984V, it is the potential that this dressing can provide.

The electric potentials of 0.924V were measured in 1xPBS for 30 minutes using Keysignt 34410A digital multimeter, comes out similar to theoretical value 0.984V.

2.2.2 Synthesis of PPy/GelMA Cryogel

In brief, dissolving a porcine skin gelatin into PBS at 10% w/v at 60°C. Then 8% (v/v) methacrylic anhydride was added at 0.5 ml/min at 50°C for 3 hours. Then GelMA solution was dialyzed for 1week to remove residues and salts. It is stored at -20°C. Then GelMA cryogel was performed with ammonium persulfate (APS) as the initiatior agent and N,N,N′,N′ -tetramethylethylenediamine (TEMED) was used as a catalyst. To start the cryopolymerization, 1.5% w/v GelMA solution with 5% (w/v) APS and 0.25% (v/v) TEMED was poured into a cylindrical-shaped polyethylene mold, and placed in a –20˚C freezer [20]

In order to incorporate PPY network on to the GelMA, GelMA cryogel was soaked in pyrrole monomer solution 5mM, 10mM, 20mM, for 6 hours.

Then chemical oxidant ($FeCl_3$) was for 6 hours. It starts PPy polymerization in cryogel networks, forming a PPy/GelMA cryogel.
And it needs to be washing , so PPy/GelMA cryogels are immersed and shaked in 70% etoh for 3 days

2.2.3 Characterization of Cryogel

In order to observing the internal structure of the cryogel, SEM analysis was performed. All samples were lyphophilized and cut horizontally with the blade to expose internal structure. After that, it was mounted on the SEM mount using carbon tape and the sample was coated in platinum, palladium under vacuum for 2 minutes. Images were measured by a JSM-7610F Scanning Electron Microscope (JEOUL USA, Inc.)at 10uA and 10kV.

For measuring the swelling ratio(Q), the cryogels (n=3) were lyphophilzed. And Soaked in DW. Making time point and measuring swelling ratio of the each point. Swelling ratio was calculated through the following equation.

$$Q = \frac{W_{wet}}{W_{dry}}$$

Young's modulus was measured by UTM. All samples were made 8mm in diameter and 4mm in height. The samples were pressed at a speed of 1mm/min. The young's modulus was calculated from using the linear region of the stress-strain curve(5-15% strain).

2.2.4 Conductivity of Cryogel

To analyze the electrical properties, electrochemical impedance spectra (EIS) and electrical conductivity of the sample were measured. For EIS measurement, an alternative sinusoidal potential of 10mV was applied to the hydrogel transferred between parallel gold coated glass electrodes. The impedance was then measured in a frequency from 1 to 10^5 using a computer-assisted electrochemical device (VersaSTAT3, Princeton Applied Research, Princeton, NJ). For conductivity measurement, sheet resistance was measured in a voltage range from -0.5 to 0.5 V at a scan rate of 50 mV/s by linear scanning voltammetry using 4-point probe method.

2.2.5 In vitro cell viability test

Gels go in to washing step, 100% etoh for 2h and soaked in growth media for 3 days. We use NIH3T3 cells. Preparing 96 well, and put $2.5x10^4$ cells in each well. In each well, 200ul of the control media, the media soaked in the gel was added. Viability of the cells was measured by live/dead assays at day 0, 1, 3, and 5. Each well was stained, after that quantified, and imaged by EVOS Cell imaging Systems (Thermo Fisher)

2.2.6 In vivo wound healing test

Animal experiments were performed using protocols approved by the Seoul National University Institutional Animal Care and Use Committees. BALB/C nude mouse (female, age 6 weeks) were purchased from orient Bio(Orient Bio Inc, Seongnam, Korea). All mice were anesthetize with respiratory anesthetics (Isoflurane) during the experiment. First, the anesthetize mice were laid down and punched through the skin using an 6 mm bio-punch. The experimental groups ere PBS treated, PPy 10mM/GelMA, PPy 20mM/GelMA with/without M-sheet. Samples were collected on day 3, 5, 7, 10, 14. Data was obtained by measuring the extent to which the wound size was reduced.

2.3 Results and Discussion

2.3.1 Charaterization of Cryogel

As the concentration of pyrrole monomer and FeCl3 increased, the color of PPy / GelMA cryogel increased from white to black. And this color change means the amount of PPy network in the cryogel. Cryogel of pyrrole 0 mm, 1 mm, 2 mm, and 5 mm that were not used in the experiment were not black or tended to be partially stained, so cryogel of pyrrole 10 mm and 20 mm, which were judged to be uneven throughout the gel, were used.

Cryogel has a microporous structure. This property makes cell easy to migrate. We used cryogel because we thought that the porous structure could be coated with pyrrole to increase the density of the pyrrole network. The internal structure of gel was examined using SEM. SEM images show interconnecting microporous structure. (Figure) Some flakes were found in PPy[10], PPy[20]/GelMA through the pores. It means that the PPy polymerization is well occur within GelMA cryogels. As the concentration of pyrrole monomer and FeCl3 increased, the amount of flakes increased. The porosity of GelMA, Ppy[10]GelMA is 58%, 37%, and Ppy[20]GelMA exhibited 32%.

The mechanical properties of PPy/GelMA cryogel is showed in Figure . As the concentration of pyrrole monomer and FeCl3 increased, Young's modulus is increased. Formation of PPy networks in the cryogels could lead to increased modulus of the cryogels. GelMA, PPy[10]GelMA exhibited Young's Modulus of 25 ± 9 kPA, 63 ± 5 kPA, And PPy[20]GelMA exhibited 170 ± 12 kPA.

A material having a high swelling ratio is suitable for artificial dressing because it can well absorb cell exudate. Swelling ratio is appeared in another aspect. GelMA showed 3271% ± 221, Ppy[10], PPy[20]/GelMA showed 2142% ± 186, 1941% ± 350. As the concentration of pyrrole monomer is increased, swelling ratio is decreased. It might be dense pyrrole network filled in pores. so it cause less swelling ratio

2.3.2 Conductivity of Cryogel

It was essential to measure the conductivity of the conductive gel, which was used to spread the M-sheet current evenly over the wound region. Conductivity and EIS were measured. The conductivity of GelMA is 0.02, Ppy[10]/GelMA, Ppy[20]GelMA is 0.108, 0.121. The higher the pyrrole concentration, the higher conductivit. it is also similar aspect in impedance data. PPy[20]GelMA is appeared the lowest impedance than other groups.
In Sumi Yang's paper using the same material, pyrrole, the conductivities of PPy [5] and PPy [10] / Alginate gel were 0.057 and 0.11. [14]

2.3.3 In vitro cell viability test

The M-sheet just puts it on the wound, but the conductive cryogel goes directly into the wound region. Therefore, it was important to measure the cell viability and toxicity of conductive cryogel.On day 0, 1, 3, and 5, the cell viability did not show a big difference by around 3%. However, cell viability was 95.2% ± 0.7% for Day 5's Ppy20 / GelMA.
Compared to GelMA, which is known to have excellent biocompatibility as a biomaterial, Ppy10/GelMA showed similar cell viability. However, PPy20 / GelMA showed poor cell viability in D5. Therefore, we utilized PPy10 / GelMA cryogel in the next in vivo wound healing test.

2.3.4 In vivo wound healing test

In this experiment, In Day 3, there was no significant difference than other time point in each group. In Day 5, Ppy10 + M-sheet group showed the highest wound reduction rate of 32% ± 8%. In Day 7, Control, M-sheet group showed the similar wound reduction rate of 42% ± 3%, 41% ± 1%. but Pyrrole experimental groups was found to have faster wound regeneration than control group. Ppy10 group, and PPy10 + M-sheet group showed 27% ± 5%, 13% ± 6%. This may be a result of synergy between pyrrole's electrical conductivity and GelMA's biocompatibility as scaffold. In addition the results with M-sheet were also better than without. It can be showed that the electrical stimulation of M-sheet, and the biocompatibility and conductivity of PPy10 / GelMA gel showed positive roles in wound size reduction, respectively. I also confirmed the synergy up to my result in the application used for this.

Chapter 3. Conclusion

We coated pyrrole to impart electrical conductivity to GelMA cryogel with biocompatibility. The wound was filled with PPy / GelMA cryogel. And the electrical stimulation was applied to the wound by placing the M-sheet generating current in the humid environment on the gel. We thought that a conductive gel would deliver the current generated by the M-sheet better, and the experiments actually showed synergistic effects in wound reduction test.

In gel preparation, it was also seen that the color gradually changed from white to black as the amount of pyrrole and FeCl3 increased. This may also be evidence of a well formed polypyrrole network. [14]

Mechanical and electrical properties generally increase with the amount of conductive polymer [14, 16] In results, the properties and condcutivity were measured according to the trend.

GelMA hydrogel is a widely used material in biomedical applications because of its appropriate biological properties and tunable physical characteristics. GelMA hydrogels have key characteristics similar to the native extracellular matrix. For example, cell attaching, matrix metalloproteinase responsive peptide motifs. [17-19] The cell viability of PPy10 / GelMA cryogel is similar to that of GelMA cryogel in the in vitro test. We think this proves the biocompatibility of PPy10 / GelMA cryogel. PPy20 / GelMA cryogel showed cell viability of around 95% and was excluded from in vivo testing.

In the in vivo experiments, M-sheet and Pyrrole groups showed better effects than control group. In addition, PPy10/GelMA + M-sheet group showed the best effect. The electrical conductivity and biocompatibility of PPy10 / GelMA cryogel and the current of M-sheet are thought to have a syergetic effect. And We think this could be a stepping stone to research using both electrical stimulation and hydrogel at the same time.

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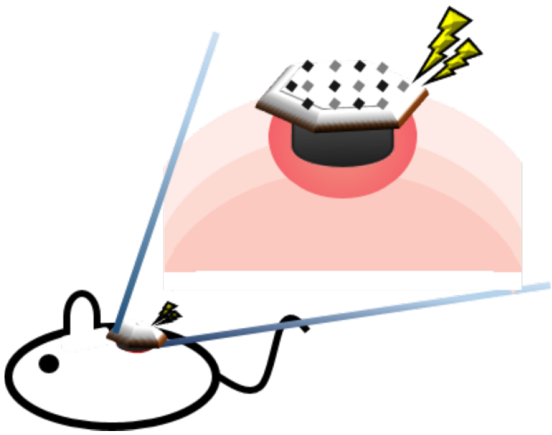

Figure 1　The overall scheme of PPy/GelMA and M-sheet system

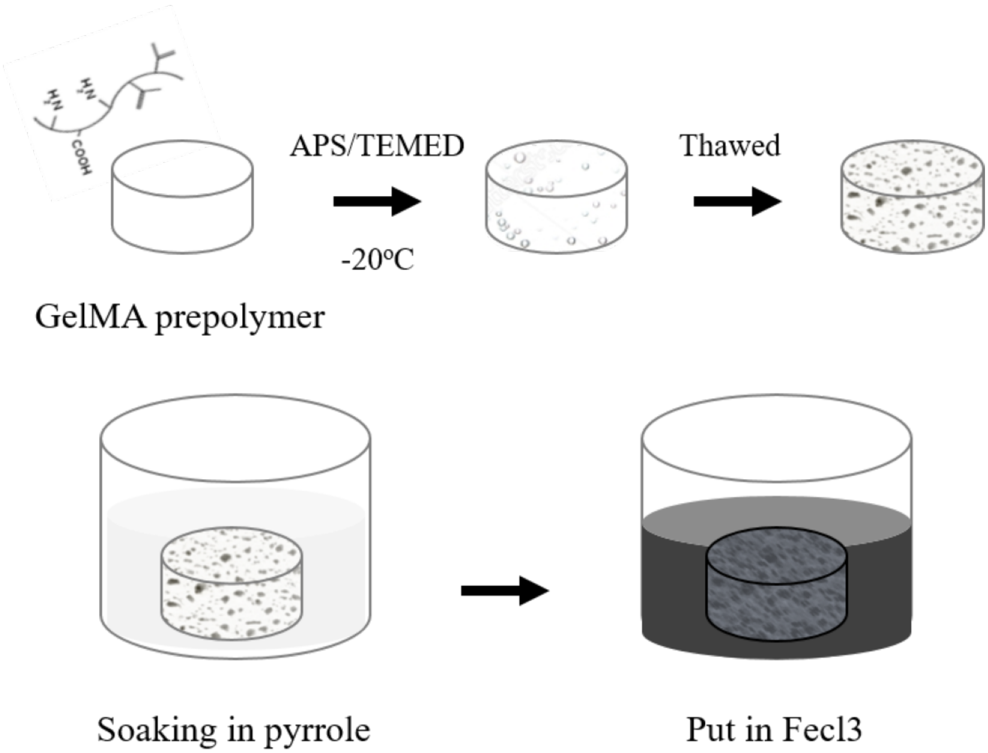

Figure 2 Preparation of PPy/GelMA cryogel

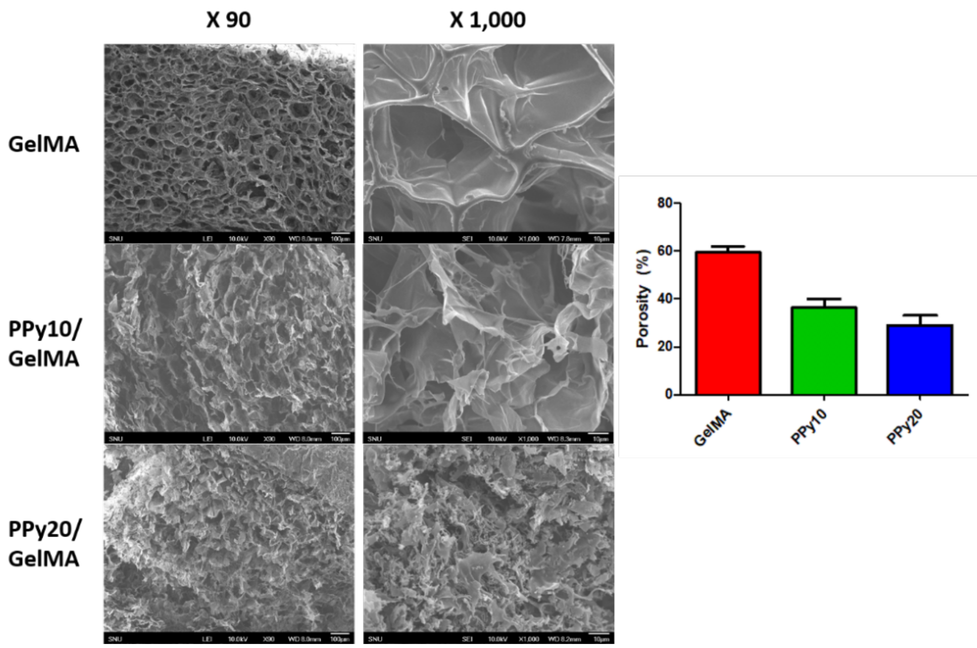

Figure 3 SEM image of PPy/GelMA cryogels

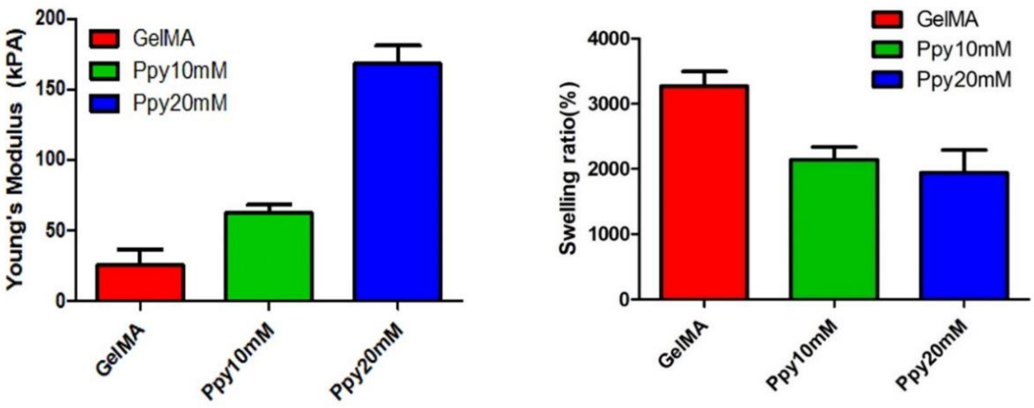

Figure 4　Mechanical property of PPy/GelMA cryogels

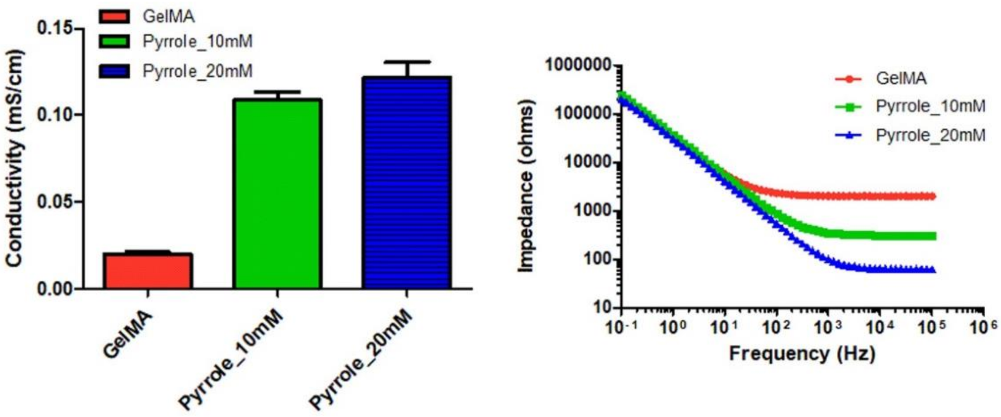

Figure 5 Conductivity of PPy/GelMA cryogel

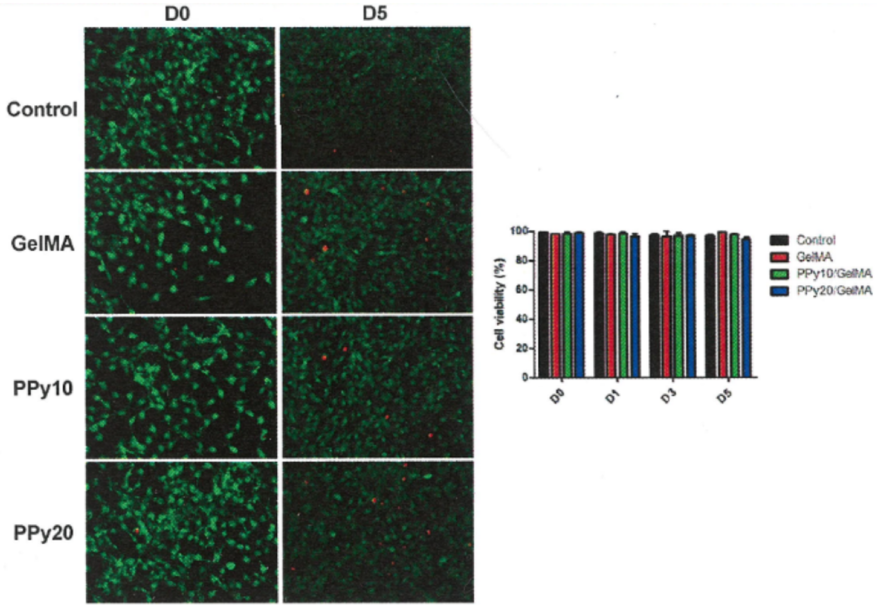

Figure 6 In vitro cell viability test

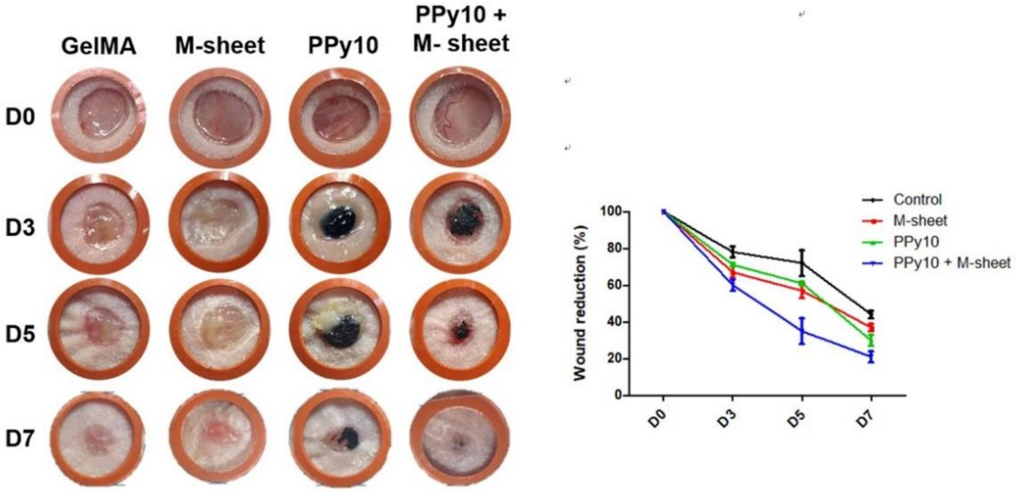

Figure 7　In vivo wound healing test

국문초록

피부 상처 재생을 위한 전기 전도성을 지닌 크라이오젤과 미세전류 시트의 효과

전기적 자극은 상처 부위 주위의 세포의 증식을 가속화 할 수있는 자극 신호를 제공 할 수 있다. 상처 재생을 위해 미세 전류 시트를 사용했습니다. 미세 전류 시트는 젖은 상태에서 장시간 동안 전류를 낼 수 있도록 설계되었다. 전기 전도성을 제공하기 위해 피롤의 중합 된 형태 인 생체 적합성 및 전도성 물질 인 폴리 피롤(PPy)을 메타크릴화 젤라틴(GelMA) 네트워크에 포함시켰다. 미세 전류 시트를 상처 재생 모델에 적용하기 위해 상처 부위에 PPy/GelMA를 넣고 미세 전류 시트를 그 위에 젖은 상태로 올려주었다. PPy/GelMA 의 물리적 및 전기적 특성은 피롤 농도에 의존적이었다. PPy 농도의 증가가 물리적 및 전기적 특성을 향상시키는 경우. PPy의 도입은 상처 치유 과정을 촉진시켰다. 더욱이, 미세 전류 시트에서 나온 전기 자극이 PPy/GelMA와 함께 사용됐을 때 더 빠른 상처 재생이 관찰되었다. PPy/GelMA 크라이오젤과 미세 전류 시트의 조합은 피부 상처 치유에 시너지 효과가 있으며 전기적 자극 기반 상처 치료의 새로운 전략을 제공 할 수 있음을 시사한다.

주요어: 미세전류, 전기자극, 크라이오젤, 상처재생

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