석사 학위논문

# Development of Glycerol-Rose Bengal-Polidocanol (GRP) foam for enhanced sclerosis of a Cyst

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**2020 년 1 월**

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지도교수 이강원

이 논문을 정수현 석사 학위논문으로 제출함

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# Abstract

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Polycystic kidney disease (PKD) is a common genetic disorder that comes with a proliferating and enlarging cyst that ultimately leads to loss of kidney function. Since an enlarged cyst is the primary factor for limited kidney function, the vast cyst is surgically removed by laparoscopic deroofing or by sclerosant, which is a relatively nascent treatment method that entails complications and sometimes failure due to cyst fluid refilling and infection. In this study, we suggest a more stable and effective polidocanol foam with glycerol and Rose Bengal (GRP form) to prevent cyst regeneration and irritation that is caused by required body movement during treatment. GRP form inhibits cellular proliferation and disrupts cellular junction, e-cadherin, and cyst formation. This advanced form also elongates foam retention time and retards foam degeneration in comparison to polidocanol foam only. The GRP foam shows to be a safe and effective treatment as a commercial grade polidocanol foam form from an in vivo study. Thus, this study provides an advanced polidocanol form by adding glycerol and rose-Bengal to help existing sclerotherapy.

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**Keywords : Cyst, Sclerotherapy, foam, Glycerol-Rose Bengal-Polidocanol Foam**

**Student Number: 2018-23054**

# Contents

## Introduction

Polycystic kidney disease (PKD) is a systematic disorder and patients suffer from innumerable cyst proliferation and enlargement from the age of thirty. Multiple enlarged cysts lower kidney function and dramatically undermine patients' quality of life through flanking abdominal pain, infected cyst, or hypertension(1). Therefore, relieving the cystic burden is essential in both improving clinical progression and safeguarding the quality of life of patients.

One method to ablate symptomatic renal cyst is performed by sclerotherapy. Sclerotherapy has been studied for renal cyst ablation for three decades(2). Originally, doctors used ethanol solution to ablate the cyst, which caused too much pain to the patients(2, 3). Presently, active agents such as polidocanol is used for sclerotherapy. Studies reported that polidocanol is safer and more effective than other sclerosants such as sodium tetradecyl sulfate (STS)(3-5). Furthermore, polidocanol product Asclera® is used in clinics, for varicose veins, and 3% polidocanol was used to ablate the problematic cysts(6, 7). The low content of polidocanol was especially developed by foam sclerotherapy, which is a more efficient and safer method than solution sclerotherapy as foam retains the form at the site of injection and is more stable than solution(8). Renal cysts and hepatic cysts are often ablated through foam sclerotherapy(3, 5-7, 9, 10). Since thorough contact to the rostral part of the cyst is vital in preventing the regeneration of cysts, longer foam form retention time and shorter foam degeneration time are also important. To elongate foam retention time, previous studies used

thickening agents such as glycerol, xanthan gum or xylene(11, 12). However, these studies did not demonstrate what affect these additives to foam had on the sclerosing effect on the targeted cells and the possible adverse immunogenicity of the foam materials.

The utmost goal of the research is to investigate readily applicable and enhanced treatment methods for PKD patients. Thus, we selected 3% polidocanol foam as the base material. To induce better retention time of the foam form, glycerol was also added. Glycerol is also a Federal Drug Administration (FDA) approved material that is used in clinics for other treatment methods. Rose Bengal was added, which can induce collagen crosslinking and decrease the possibility of infection during treatment(13-15).

The aim of this study is to investigate the sclerosing effect of these foam stabilizers and to confirm the safe applicability in vivo. In this study, we developed advanced polidocanol form as a sclerosant by adding glycerol and Rose Bengal (RB) as a foam stabilizer(11, 12) and photosensitizer, respectively. Glycerol could augment the sclerosing effect and the elongate foam retention time(11). Rose Bengal could also stabilize the foam and confer an additional effect by emitting ROS (Reactive Oxygen Species) after activating with a laser(16-18). In addition, we examined decreased cyst forming activity of Madin-Darby Canine Kidney (MDCK) cells after the foam treatment and clinical usability of this glycerol-Rose Bengal-polidocanol (GRP) foam from *in vivo* studies.

## Results and Discussion

Various percentages of polidocanol is used for the patients. However, 3% was selected, which is the highest administered dosage possible and exerts the most cytotoxic and membrane destabilizing effect on MDCK cells, thus producing a more feasible experiment. All foam was prepared using the standard Tessari method with the foam solution and air ratio of 1:4.

Glycerol was used as a potent factor for stabilizing sclerosant foam in polidocanol form. Glycerol in 3% polidocanol foam was examined as a function of the concentration of glycerol. 10% glycerol in polidocanol foam exerts the most stable foam form in RT(room temperature) by viscosity (Figure 1A), foam coarsening time (Figure 1B), and initial foam volume (Figure 1C). Figure 1D demonstrates the image of the form whereas Figure 1E shows the ratio of the foam height to width data for how high the initial foam form is retained over time. The prolonged time of the foam's form retention is important because it enables sclerosing of rostral cystic lining cells without the patient moving during the treatment.

**Figure 1. Glycerol enhances the sustainability of polidocanol foam for cyst**

**sclerotherapy** (A) Foam half time increases until glycerol is added more than 10% (v/v) and decreases after additional glycerol input. The viscosity of the foam also decreased likewise. (B) Foam coarsening time. Foam coarsening time refers to the time when initial foam breakage occurred. (C) Initial foam volume. (D) Representative video capture image of foam. (E) Unregenerated foam height/width data for 60 seconds.

RB (Figure 2A) was added in polidocanol form to investigate the multiphoton-activated collagen crosslinking of the organ cyst lining. Half time (Figure2B) by various concentrations of RB showed that 1% (w/v) RB enhances foam stability. Microscopic imaging of the foam also demonstrates this point (Figure 2C). When using 1% RB foam at the same time point (– 10s) after foam injection, large held air bubbles are smaller in size than those of 0% and 10% RB foam. Thus, these results suggested that RB also stabilizes the foam.

**Figure 2. Structure of polidocanol and Rose Bengal and its effect on foam stability.** (A) Structure of polidocanol (up) and Rose Bengal (below). (B) Foam half-time refers to the time until foam degenerates into half of the original volume of the solution. 1% Rose Bengal content increased foam stability about 20%. (C) Microscopic image of foam consisting of various Rose Bengal concentrations. Bubble size and lamina thickness explain the increased and decreased foam half-time for 1% and 10% Rose Bengal foam,

respectively (scale bar = 200 μm).

The effects of glycerol in polidocanol form were investigated *in vitro*. Unfortunately, the experimental means of the testing effect of the foam on the cellular level was scant. Some reported having cells glued on the plate before foam treatment. However, this would fail to mimic the real cystic environment. Many reported having ex vivo experiments when patients' veins are removed from the body and are treated with foam. However, as this study aims to investigate sclerosing foam onto a PKD organ derived cyst, this was not deemed a reasonable tool for an experiment.

Hence, this study devised a quick and easy tool with the transwell system. Transwell is used to test cell migration and permeability. The membrane of the transwell is permeable for foam to be in contact with the cells attached below and allows handy removal of the foam with a pipet to retrieve the cells attached on the membrane. This study tested both 3D culture and 2D culture systems on the transwell (Figure 3A). The 3D culture system was the cyst forming activity of the MDCK cells after foam treatment, whereas the 2D culture system examined lactate dehydrogenase (LDH) activity, or the degree of destabilized cellular membrane caused by the foam. The LDH assay result showed that more stable glycerol-polidocanol foam was more effective in cellular membrane destabilization (Figure 3B) and thus was more cytotoxic. This was also demonstrated by the live and dead imaging of the transwell membrane, where alive cells were still attached to the membrane to be imaged green. As known, the increasing concentration of

polidocanol in foam resulted in a decrease in live cells attached to the membrane (Figure 3C). The dead affected cells are considered to have fallen off. The inverse relationship between foam concentration and either LDH activity or the number of green signals indicate that this study's method of testing foam activity is a valid experiment method (Figures 3B, 3C). These results showed that glycerol-polidocanol foam exerts an enhanced effect in comparison to any other foam tested by this study.

**Figure 3. Glycerol in polidocanol foam better destabilizes cell membrane** (A) Scheme for *in vitro* foam analysis. (B) LDH results. (C) Live and dead image. No red signal is detected from all cells affected by the foam, which are detached from the transwell membrane.

Another concern of glycerol-polidocanol foam is whether it can cause extravesical necrosis when leaked to other parts of the organ. Extravisional necrosis can bring extensive and even fatal organ failure and trauma(9). Although foam sclerotherapy is less likely to cause extravisiocal necrosis than solution sclerotherapy, the cytotoxicity of the GRP form MDCK cells was tested and compared with 3T3-L1 cells (Figure S1). The LDH activity of 3T3-L1 cells from the glycerol-polidocanol foam was significantly lower than that of the MDCK cells. The increase of LDH activity shows that the concentration of polidocanol in the foam increases and bolsters validity of this study's

transwell foam testing method.

After confirming that the glycerol-polidocanol foam is more effective in destabilizing the MDCK cell membrane, the foam was investigated for altered cell proliferation and the expression of cellular junction protein by cell replating. Cells treated with the foam are washed, displaced from the transwell membrane, counted, and cultured either in a 2D without collagen matrix for proliferation and fluorescence imaging or in a 3D collagen matrix for cyst formation (Figure 4A). The results showed that the glycerol-polidocanol foam retarded cell proliferation more than the polidocanol foam alone. As shown in Figure 4B, proliferation of MDCK cells after polidocanol foam treatment increases, but glycerol-polidocanol foam shows a lag phase from 48 to 72 hours. Also, the glycerol-polidocanol foam induced less e-cadherin protein expression, which is confirmed by immunofluorescence imaging (Figure 4C). The non-treated group was used as a control group. While both the control group and the polidocanol foam-treated group show the expression of E-cadherin, the glycerol-polidocanol foam-treated group did not show the explicit E-cadherin expression as much as the other two groups. A decreased or a delayed expression of e-cadherin noticeable by the imaging suggest that the glycerol-polidocanol foam can better treat a PKD cyst or a cyst from various cystic diseases as e-cadherin relates to the metastasis, cyst and lumen generation, which are related to cyst refilling or regeneration(19-21). Accordingly, fewer MDCK cells successfully generated a cyst after 7 days when MDCK cells are treated with foam and replated into a 3D collagen matrix.

In comparison with the control group, the polidocanol foam-treated group and the glycerol-polidocanol foam-treated group both showed a significantly less number of visible cysts (Figure 4D). However, the difference between the two different foam-treated groups was not significant, though the number of cysts from the glycerol-polidocanol foam-treated group were less than the polidocanol foam-treated group.

**Figure 4. Glycerol in polidocanol foam attenuates proliferation and E-cadherin expression** (A) Scheme for in vitro foam analysis. After foam treatment, viable cells are collected and replated for proliferation and protein expression analysis. (B) The cell proliferation graph showed augmented foam-treated cells proliferates slower than polidocanol foam-treated cells. (C) Protein expression 3 days after cell replating. Augmented foam-treated cells fail to form a visible e-cadherin junction like polidocanol-treated cells. (D) MDCK cells after 3D replating show less cyst generation and a smaller cyst.

Herein, RB was not added in the foam since it could interfere with the imaging and the assay. However, we believe that added RB would not significantly alter the result of this investigation as RB does not confer an additional cytotoxic effect (Figure S2). Added RB with various concentrations in 3% polidocanol foam did not significantly alter the exerted LDH activity and cytotoxicity of the treated cells (data not shown).

RB was used to enhance foam stability and elongate time until foam degeneration for advanced sclerotherapy of the cyst (Figures 2B, 2C). In addition, added RB as a component of the foam could enhance the effect. Hence, RB was used to ablate the cyst lining cells when activated by the laser. As shown in Figure S3, this study conducts a test to confirm that RB can be delivered into the area by foam injection and a subsequent washing step as a standard sclerotherapy of a cyst. Collagen was placed on the 96 well plate and foam was injected with various concentrations of RB in glycerol-polidocanol foam and then washed out. The well plate treated with foam, were red from RB in the foam. When lighted with a 532 nm laser for 1 minute, RB-treated collagen was more cross-linked in terms of rate (Figure S3, A) and degree (Figure S3, B). Also, this study showed that ROS was generated when treated with the RB-added foam and irradiated with a 532 nm laser for 1minute.

The safety and efficacy of the GRP form were examined by in vivo system to compare with a conventional sclerosant. The ethanol sclerotherapy is used conventionally to ablate a cyst of PKD patients. The GRP foam and ethanol were injected in the subcutaneous area of the mouse and irradiated multiphoton laser for RB activation (Figure 5A). After treatment, the mice were sacrificed to investigate the acute immune response. H/E staining showed that the GRP foam was slightly more immunogenic than ethanol, but not statistically significant (Figures 5B, 5C).

**Figure 5. Multiphoton laser irradiation after rose-bengal-foam treatment suggests**

**deep tissue treatment methods for cyst ablation** (A) schematic diagram of animal experiments. (B) Cell counts per unit area demonstrate that immune cells on a given area of the image do not significantly differ from the ethanol and foam-multiphoton laser-treated group. (C-D) H/E representative image from the ethanol-treated group. (E-F) H/E representative image from the RB foam and the multiphoton laser-treated group (scale bar = 200 μm).

The overall scheme of the GRP foam is shown in Scheme 1. MDCK cells are known for their cyst forming activity inside the 2 mg/ml collagen matrix (Scheme 1F). However, ROS emission by RB and the effect of GRP foam induces MDCK cells to be less potent in cyst formation (Scheme 1D) and proliferation than the untreated cells (Scheme 1E) by treating the glycerol-RB-polidocanol foam (Scheme 1B) and laser (Scheme 1C) for RB activation within the 3D collagen matrix.

**Scheme 1. Schematic diagram of glycerol-Rose Bengal-polidocanol foam's mechanism of action.** (A, F) MDCK cell proliferates and form a cyst (F) when cultured in a collagen matrix (A). (B) When treated with the Rose Bengal foam, polidocanol affects the cell membrane by ultimately killing the cells while Rose Bengal dyes the collagen matrix. (C) Green light activates Rose Bengal and reactive oxygen when Rose Bengal is emitted. (D, E) MDCK cells fail to proliferate and form a cyst (D) and fails to

proliferate as the untreated cells (E). The dark blue line represents collagen crosslinking.

## Conclusions

In this study, effective polidocanol foam was developed that not only retains its initial volume and height but also decreases proliferation of the affected cells. MDCK, cyst forming cells that are used in PKD studies, were used to investigate the foam's ability to debilitate MDCK cyst generation by direct treatment and replating experiments. The induced GRP form destabilizes the cell membrane of MDCK and inhibited expression of E-cadherin, a cellular junction protein that also plays a vital role in cyst formation in PKD. In addition, the number of MDCK cells and the size of cysts were decreased by the GRP form in the 3D cell replating culture. Additionally, this study suggests a quick and handy way of testing sclerosing foam *in vitro* using a transwell system. Thus, this study suggests an advanced polidocanol form including glycerol and rose-bengal that facilitates PKD treatment research in sclerotherapy as well as a readily applicable means to better treat patients who suffer from endless pain and hardships.

## Acknowledgment

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## Material and Methods

Foam preparation and analysis

The physical property of the foam was analyzed as previously described(11, 12). Foam viscosity is measured with the Brookfield DV2T Viscometer. An image of the foam was captured at each time point (0, 15, 30, 45, 60 seconds) up to 1 minute in RT. All foam was prepared by Tessari method with a solution to air ratio of 1:4.

Cell culture and analysis

Madin-Darby Canine Kidney (MDCK) cells were purchased from ATCC and cultured as provider's instruction. The MDCK cells were cultured in a 3D collagen matrix as previously described(22, 23).

For foam-treated cellular LDH assays, cells were incubated at the bottom of the transwell membrane and were insert by placing the insert upside down on the 6 well plate. 28 ul of the culture medium was placed onto the insert to protect the cell from drying out. Then, the cells on the transwell were placed in the 24 well and were cultured for a day before

the assay. When treating foam to the transwell, the medium was removed from the inner insert. Then, 100 ul foam was injected for 1 minute in each well. The media was collected immediately for the LDH assay. LDH assays were conducted as described by the provider's instruction. Live and dead (L/D) assays and cellular junction protein expression analysis was conducted by Image J.

*In vitro* Rose Bengal (RB) analysis

Collagen (2mg/ml) were prepared by mixing either PBS or PBS with different RB concentrations. We measured absorbance at 548 nm or 315 nm (fibrillogenesis) with a microplate reader.

Animal Studies

All animal experiments conformed to the National Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Seoul National University (IACUC2018-2-15).

# Figures and Supplementary Materials

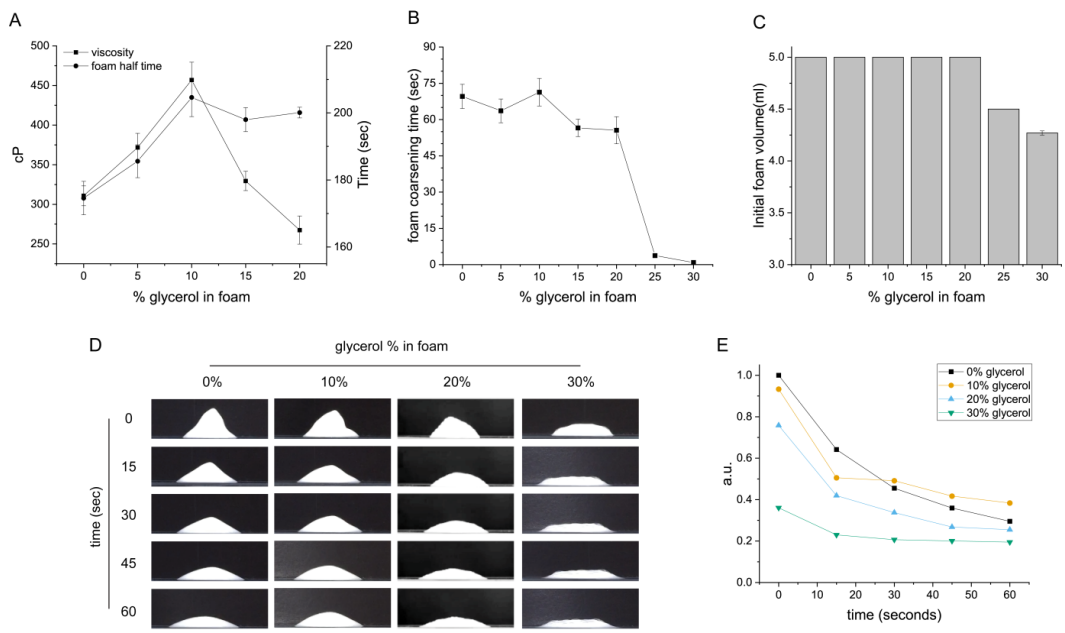


**Figure 1. Glycerol enhances the sustainability of polidocanol foam for cyst sclerotherapy** (A) foam half time increases until glycerol is added more than 10%(v/v) and decreases after additional glycerol input. The viscosity of the foam also decreased

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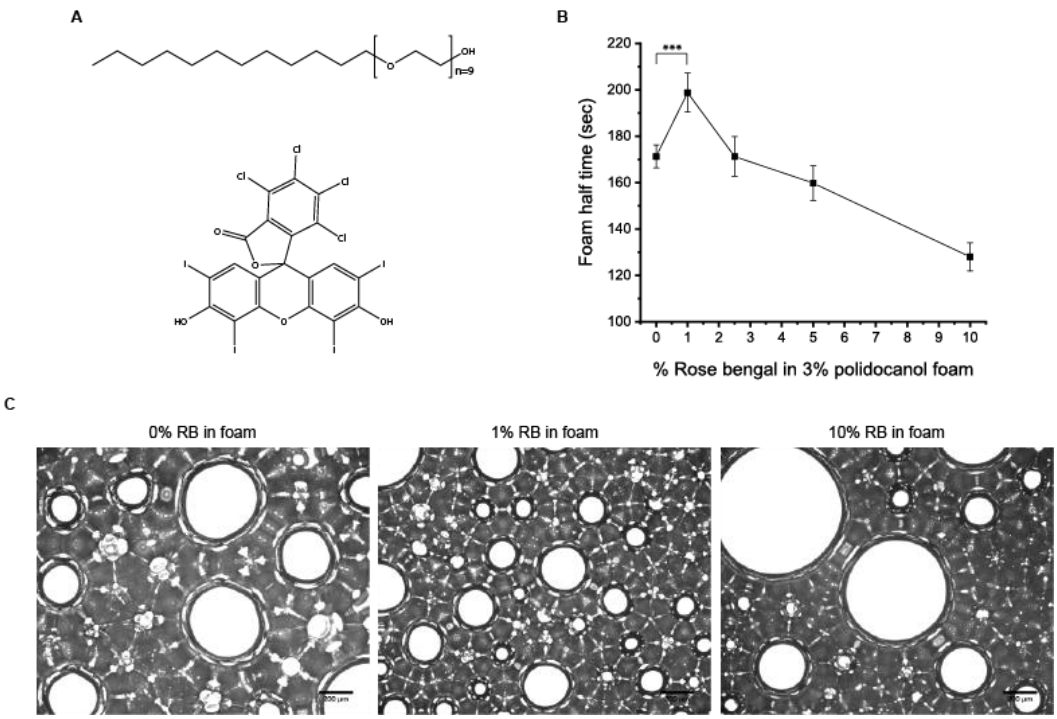


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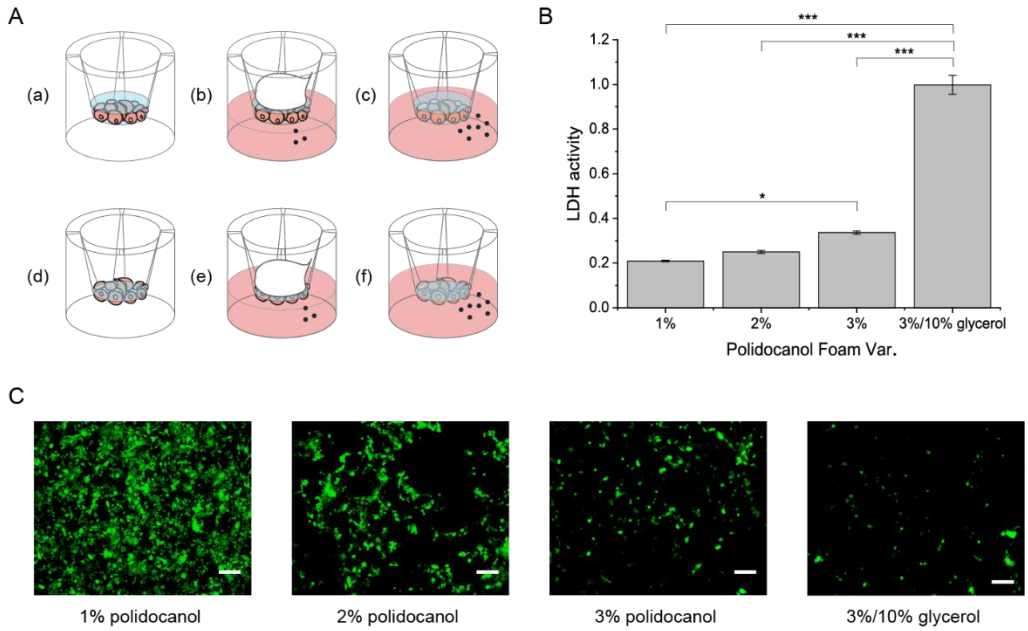


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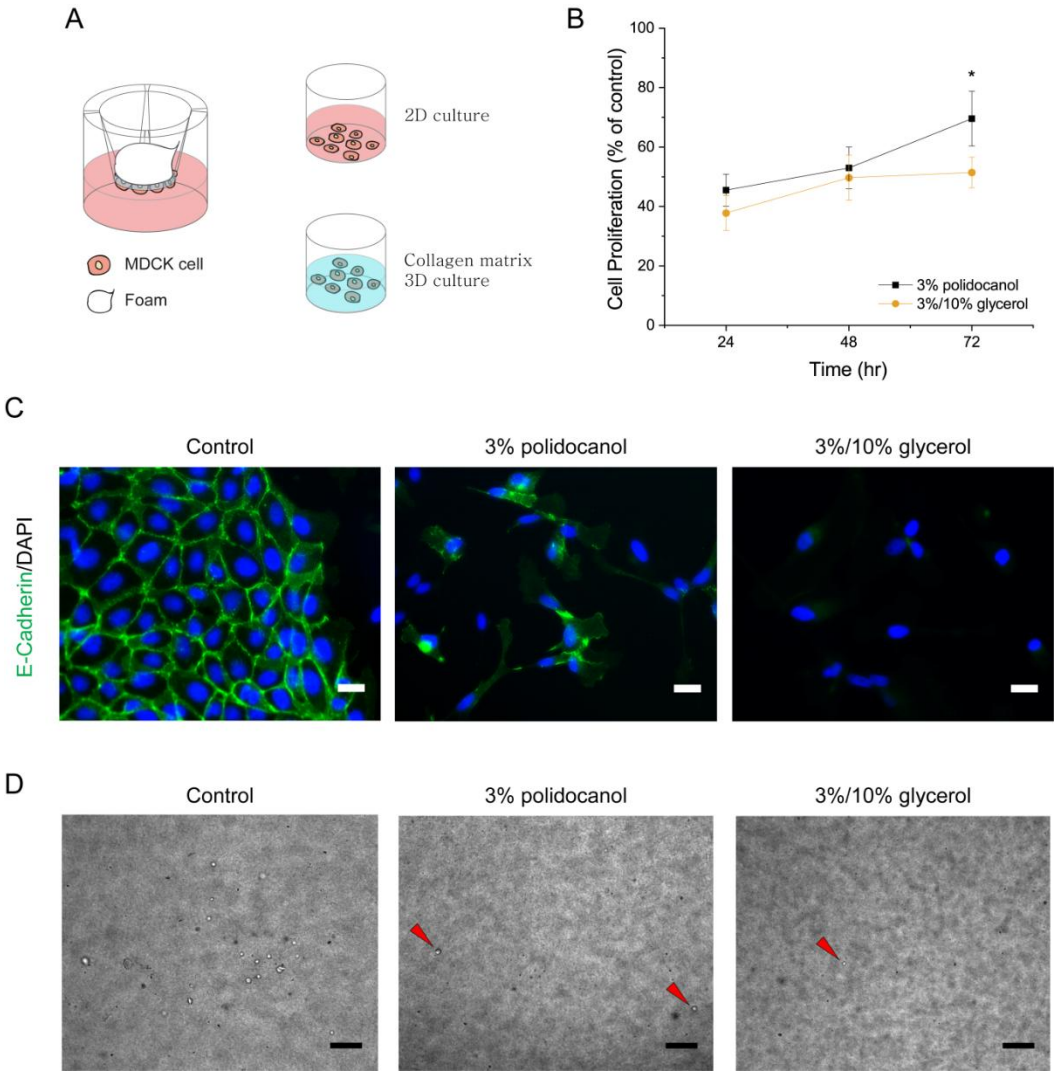


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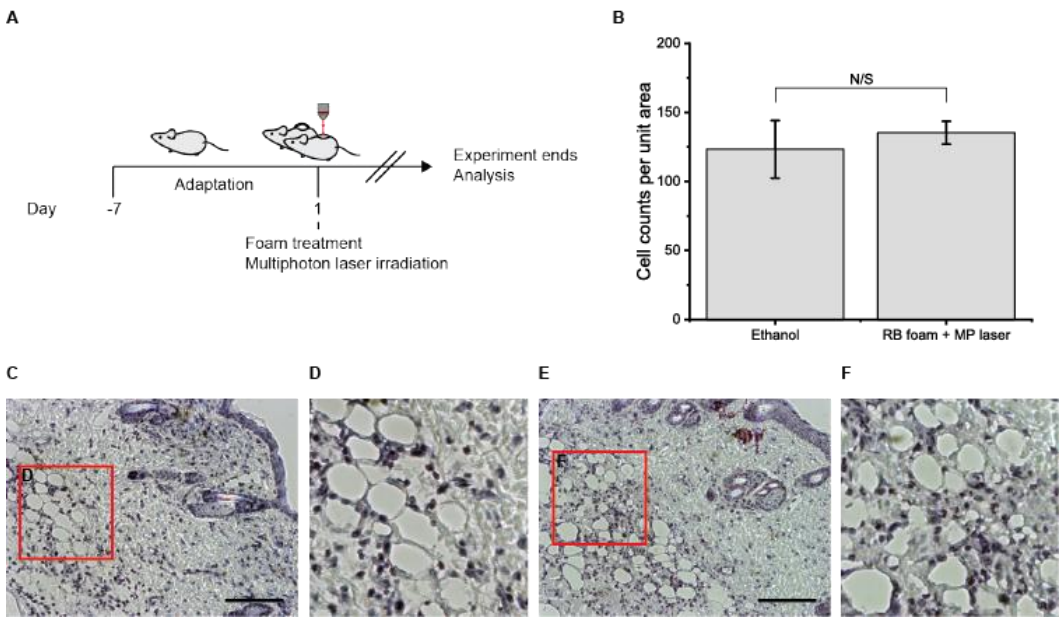
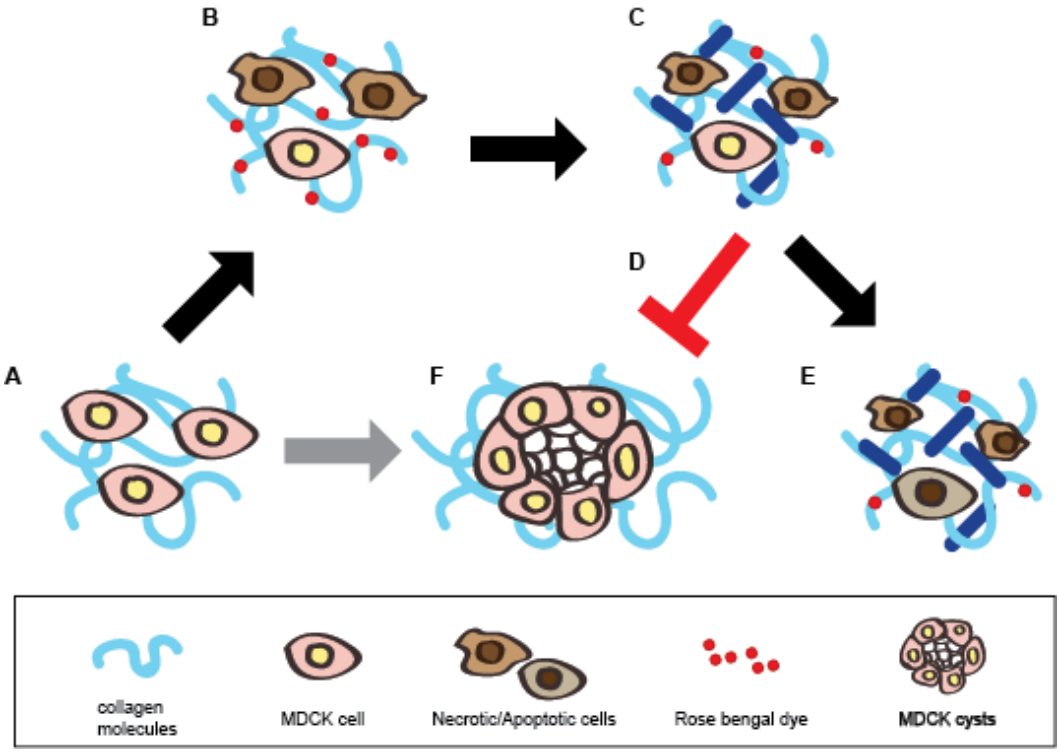
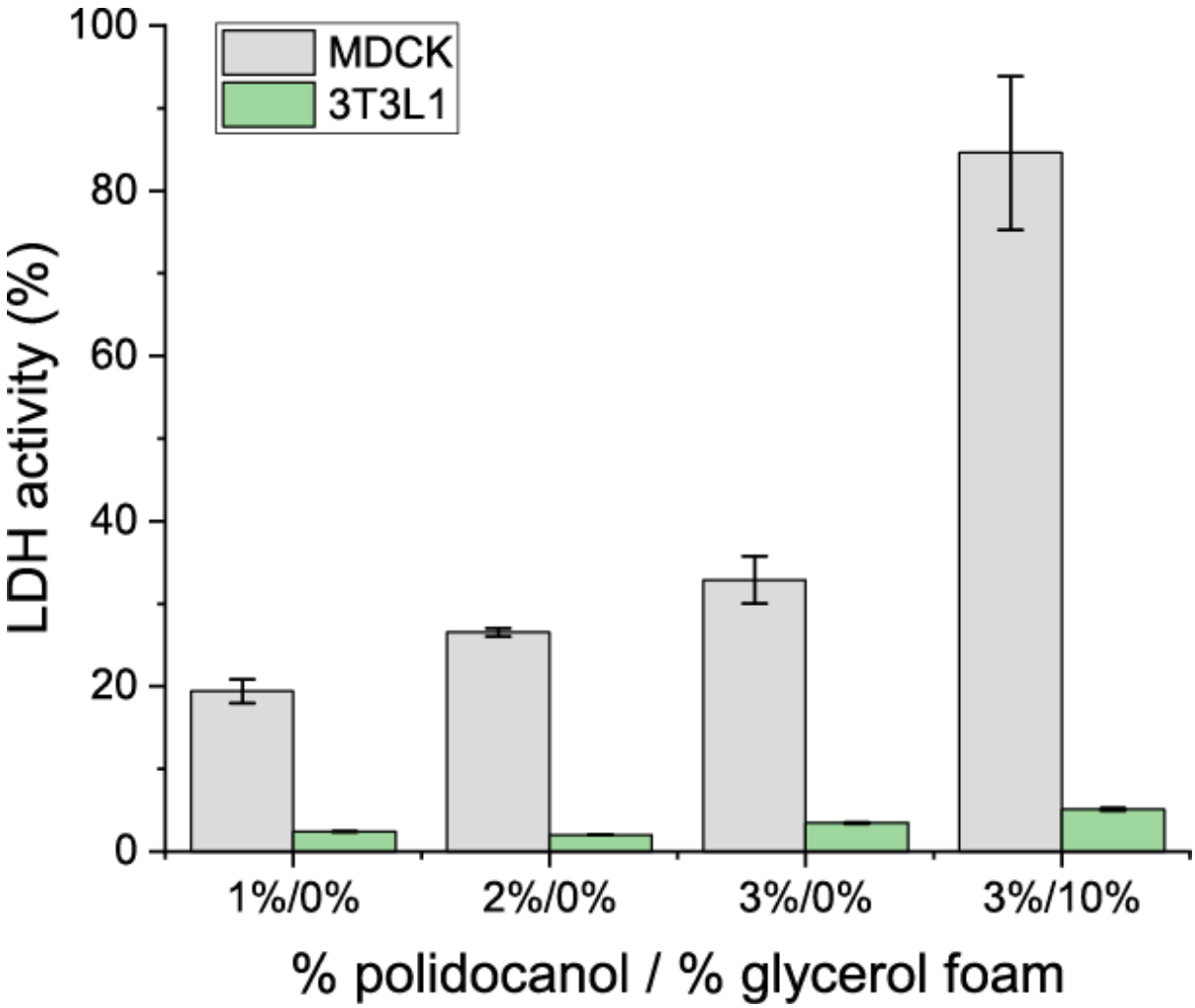


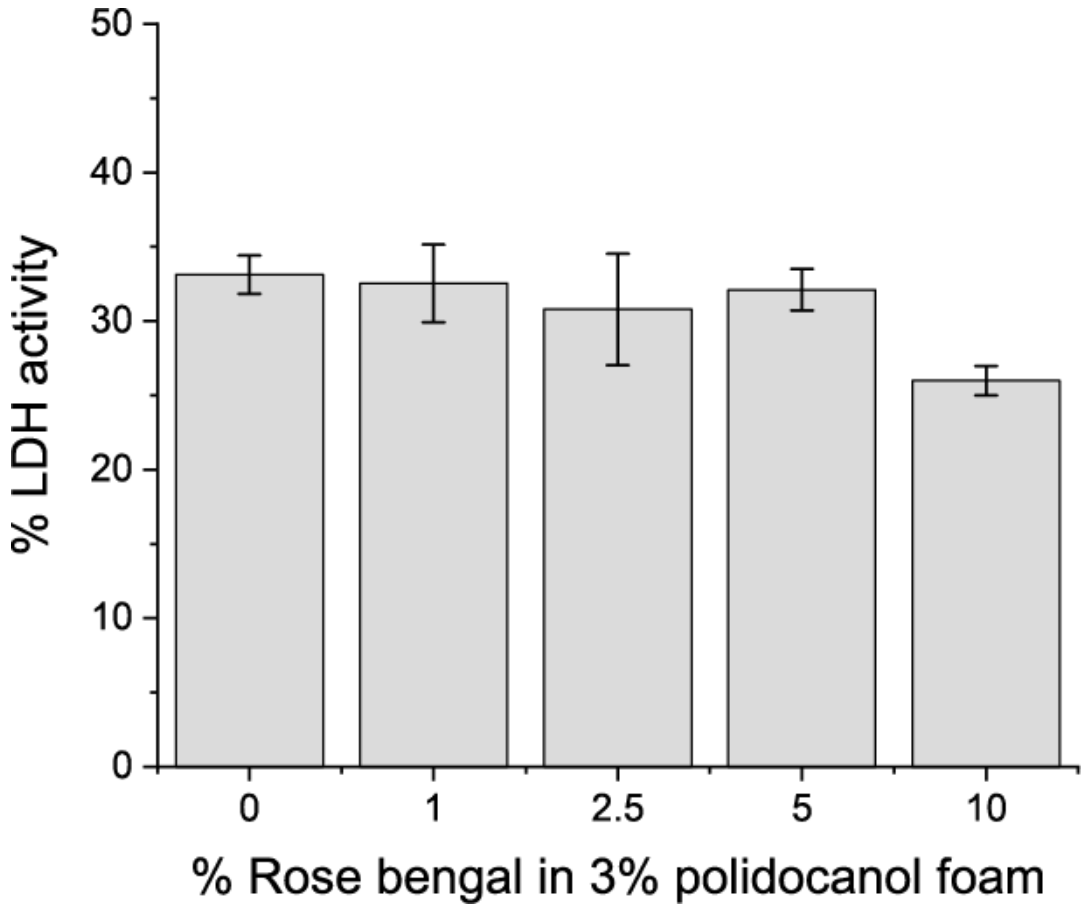

**Fig5. Multiphoton laser irradiation after Rose-bengal-foam treatment suggests deep tissue treatment methods for cyst ablation** (A) schematic diagram of animal experiments. (B) Cell counts per unit area demonstrating immune cells on given area of the image do not significantly differ from ethanol and foam-multiphoton laser treatment group. (C-D) H/E representative image from ethanol-treated group. (E-F) H/E representative image from RB foam and multiphoton laser-treated group. (scale bar = 200μm)



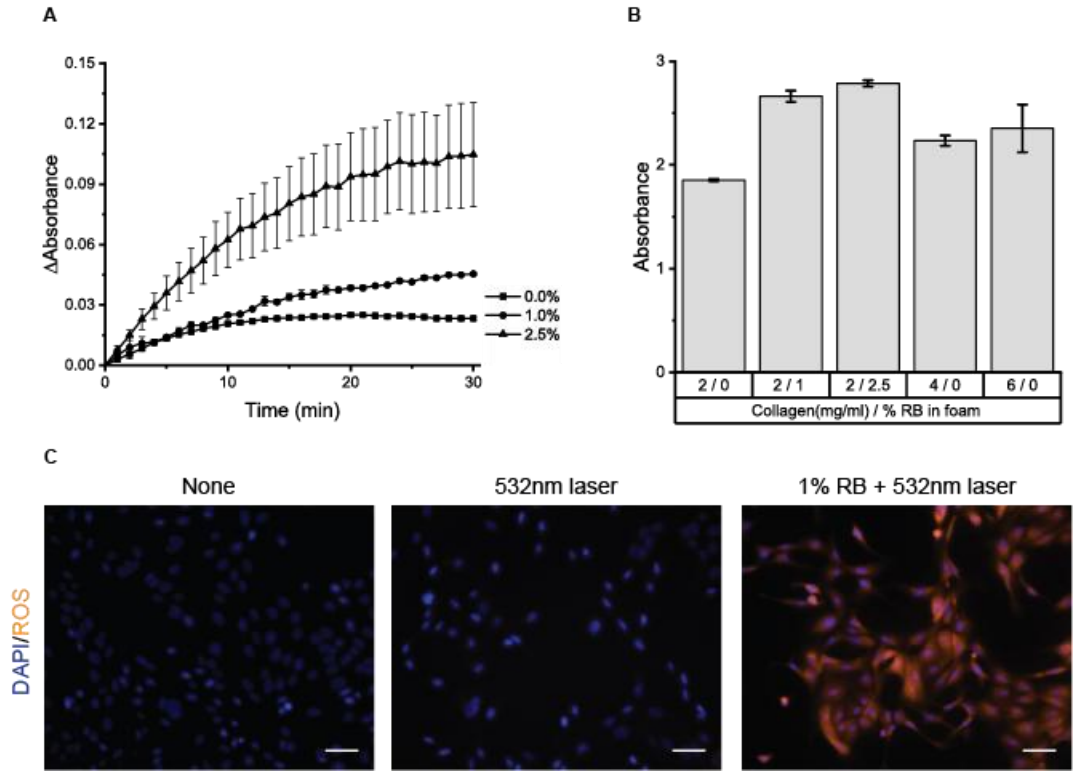

**Scheme 1. Schematic diagram of glycerol-rose Bengal-polidocanol foam's mechanism of action.** (A, F) MDCK cell proliferates and form cyst (F) when cultured in collagen matrix (A). (B) When treated Rose Bengal foam, polidocanol affects cell membrane ultimately killing the cells while rose Bengal dyes collagen matrix. (C) Greenlight activates rose Bengal and reactive oxygen from rose Bengal is emitted (D,E) MDCK cells fail to proliferate and form cyst (D) and fails to proliferate as the untreated cells (E). The dark blue line represents collagen crosslinking.




**Supplementary Figure1. Policodanol-glycerol-foam is not as cytotoxic to T3TL1 cells as to MDCK cells**




**Supplementary Figure 2. Rose Bengal does not alter cytotoxicity of the polidcoanol-glycerol-rb-foam**




**Supplementary Figure 3. Rose Bengal delivered by foam is activated by 532nm laser irradiation and cause ROS generation.**

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# 요약(국문초록)

다낭성 신장 질환 (PKD) 은 궁극적으로 신장 기능 상실을 초래하는 가장 흔하게 발생하는 유전적 장애로, 낭종이 증식하고 확대한다. 확대 및 증식한 낭종은 신장의 기능을 저해하는 주요 요인이기 때문에 경화제로 광대한 낭종을 외과적으로 제거한다. 이 연구에서 글리세롤과 로즈벵갈을 폴리도카놀 폼에 첨가하여 안정적이고 효과적인, 글리세롤-로즈벵갈-폴리도카놀 폼을 갭라하였다(GRP v폼). GRP 폼은 세포 증식을 억제하고 세포 접합에 쓰이는 E-cadherin의 발현을 억제한다. 이 GRP폼은 폼의 유지 시간을 연장하고, 기존의 폼보다 세포독성이 뛰어남을 증명했다. GRP폼은 또한 기존의 상업적 등급의 폴리도카놀 폼보다 효과적이고 이와 동등하게 안전함을 실험적으로 밝혀냈다.

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**주요어 : 낭종, 경화제, 폼, 글리세롤-로즈 뱅갈-폴리도카놀 폼**

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