



ORIGINAL ARTICLE

Evaluation of pleurodesis by poly- ϵ -caprolactone (PCL) gel in an animal model using New Zealand white rabbits



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Fibronectin

Summary *Background/Purpose:* Pleurodesis with biomaterial implant is an emerging treatment method for pleural diseases. However, the ideal biomaterial or the optimal form for the common diseases is still under investigation. In our previous study, Poly- ϵ -caprolactone (PCL) membrane produces significant pleurodesis in New Zealand White rabbit animal models. *Methods:* We investigate the Poly- ϵ -caprolactone (PCL) gel pleurodesis by animal models using New Zealand White rabbits, which were sacrificed for examination after one month. Thirty-Six New Zealand White rabbits were randomized into three groups equally to undergo procedures. Gross pleurodesis scoring was evaluated. Additionally, inflammation and fibrosis scoring were done under microscopic evaluation, as well as Western blot analysis.

Results: Gross evaluation of pleurodesis score revealed that lower concentrated PCL gel (10%) produced moderate pleural adhesion, while higher concentrated PCL gel (25%) showed significantly higher pleurodesis scores. ($P < 0.05$) Control group with thoracostomy alone produced almost no pleurodesis ($P < 0.05$). Western blot showed fibronectin expression was more evident in the 25% PCL gel than 10% one.

Conclusion: PCL gel induced significant degree of pleurodesis in the rabbits. The 25% PCL gel produces more intensive adhesion than 10% one. Fibronectin plays an important role in the process of pleurodesis. Further study is required for the clinical application of the promising biomaterial with gel form.

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1. Introduction

More and more need for pleurodesis using some kind of biomaterial is noted in recent years when treating pleural diseases.^{1–3} Different biomaterials will result in pleurodesis with various degree and extent to meet the needs of different pleural diseases. For example, pleurodesis of the upper pleural cavity can prevent recurrence while preserves maximal lung function in primary spontaneous pneumothorax. Nonetheless, mechanical pleurodesis or apical pleurectomy carry some risks of complications, such as post-pleurodesis pain or bleeding.^{4–10} Traditional methods of creating pleurodesis include mechanical abrasion and instillation of chemical irritants. The recurrence rates after these methods are widely varied from different reports.^{8,9} However, the recurrence rates of pneumothorax after thoracoscopic surgery ranged between 5 and 12%, which are higher than the rates with open thoracotomy. It is postulated that a less intense pleural inflammatory reaction is induced by thoracoscopic procedure than by thoracotomy.^{11,12} Thoracoscopy gains its worldwide popularity in thoracic surgery recently, whether uniportal or multiportal.^{13,14} Therefore, we need a potential alternative adjuvant to increase the intensity of pleural inflammation and thereby to prevent pneumothorax recurrence. The biomaterial membrane pleurodesis is one of the best choices. Therefore, it was reasonable to design an appropriate biomaterial membrane to cover lung bullae with inducing adhesion, without mechanical stimulation and trauma, to treat pneumothorax. Our previous study using PCL membrane to induce pleurodesis proved that this biomaterial is feasible in animal study.³ Poly- ϵ -caprolactone (PCL) is a widely-used FDA-approved biomaterial. The degradation time is approximately 24 months when degraded by hydrolysis only.^{15–19} However, PCL membrane cannot be delivered without surgery. We want to transform the PCL membrane to PCL gel, hoping to gain the same effect. In the current research, lapin models were again used to see if PCL gel can also produce similar effect of pleurodesis as PCL membrane.

2. Materials and methods

2.1. PCL gel preparation

Briefly, 4% PCL (Sigma–Aldrich, USA) in dichloromethane were drop-mixed with 1% PVA (volume ratio of 1:4) and then sonicated at 80 W for 30 min. After that, PCL particles were centrifuged at 7500 g for 10 min and supernatant discharged. Nanoparticles were washed with MilliQ-water, lyophilized and stored in a moisture free environment. For PCL gel preparation, the lyophilized PCL particles were

diluted in 1% CMC. 10% and 25% PCL gel were prepared for animal study usage.

2.2. Cell culture

Human fibroblast Hs68 cells were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan, R.O.C). Cells were expanded in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biological Industries) and antibiotic/antimycotic (penicillin G sodium 100 U/mL, streptomycin 100 g/mL, amphotericin B 0.25 g/mL; Gibco-BRL Life Technologies, UK) at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. Once 90% confluent, cells were trypsinized using 0.2% trypsin with 0.1% EDTA (Sigma Aldrich) and resuspended in the same medium until ready for experiments.

2.3. Immunocytochemistry

After 4 days of incubation, cells were washed with PBS, fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% triton X-100 for 10 min. The cells were blocked in 2% bovine serum albumin overnight and stained by incubation with anti-fibronectin antibody (MAB1937, EMD Millipore, USA). Fibronectin was then visualized using fluorescence-conjugated secondary anti-mouse IgG (Santa Cruz, USA). DAPI (Invitrogen) was used as the nuclear marker. Images were taken with a fluorescent microscope (Leica DMI 6000).

2.4. Western blot analysis

Cells or tissues were lysed with a lysis buffer containing Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Germany) for 30 min on ice and then proteins were collected followed by centrifugation. The protein concentration in the supernatants was measured by a Bicinchoninic acid (BCA) protein quantification kit (Pierce Biotechnology, USA). Proteins were separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. The membranes were immersed in the primary antibodies, anti-fibronectin & anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), overnight at 4 °C. After washing several times, the membranes were immersed in the horseradish peroxidase-conjugated secondary antibodies for 2 h and visualized with an enhanced chemiluminescence detection system (Millipore, USA).

2.5. Animal subjects and general design

We assigned 36 New Zealand White rabbits (2.5–4.0 kg) randomly and equally into three groups: (a) 10% PCL gel

pleurodesis [10% PCL group ($n = 12$); (b) 25% PCL gel pleurodesis [25% PCL group ($n = 12$); (c) control [control group ($n = 12$)]. The protocol was approved by the Institution's Committee on Investigations Involving Animal Subjects at National Taiwan University. (20170552) All animals were housed in the facilities of National Taiwan University. The methods and procedures were carried out in accordance with the relevant guidelines, including any relevant details.

2.6. Anesthetic and surgical techniques

We induced general anesthesia with intramuscular ketamine hydrochloride (Ketalar, 35 mg/kg; Pfizer, Shinchu, Taiwan) plus xylazine hydrochloride (Rompun®, 5 mg/kg, Bayer, Leverkusen, Germany) to the animal subjects. We continuously monitored arterial blood pressure and transcutaneous oxygen saturation via percutaneous ear arterial catheter and pulse oximeter. Besides, ECG monitoring using standard limb leads was continuous, too. We gave Penicillin G benzathine (40,000 U/kg IM) prophylactically. Antimicrobial skin preparation with povidone-iodine was performed before skin incision. All procedures were done with aseptic techniques. One 5-mm thoracostomy wound were made in the hemithorax over the sixth intercostal space. 2 ml PCL gel instillation was performed in study groups while rabbits in control group received nothing but thoracostomy. Rabbits in the PCL gel groups underwent 2 ml PCL gel spray in the parietal pleura. The upper half of the parietal pleura, above the 5th rib anteriorly and 7th rib posteriorly, was sprayed by PCL gel. After the procedure, the incision was then sutured in layers. At the end of the procedure, no chest tube was inserted into the pleural space. After resumption of spontaneous breathing, the rabbits were then sent to the animal center for post-operative care.

2.7. Macroscopic and microscopic evaluation

One month after the surgery, injection of pentobarbital sodium (Nembutal; 120 mg/kg) via ear vein was given to the New Zealand white rabbits and they were euthanatized by. The affected thorax was soon open after thoracotomy. We submerged entire thorax in 10% formalin solution for 3 days. The macroscopic evaluation of pleurodesis degree were performed by following criteria: 0 indicates normal pleural space; 1 indicates no adhesions but pleural space inflamed as evidenced by redness and fibrin deposition; 2 indicates a few scattered adhesions (25%); 3 indicates generalized scattered adhesions (25–75%); and 4 indicates complete obliteration of the pleural space by adhesions (75–100%). At the same time, samples of the parietal pleura, contiguous visceral pleura, and lung from each hemithorax were obtained from the areas of greatest adhesion and placed in neutral-buffered 10% formalin. The routine histological examination and stained with hematoxylin-eosin were performed for the tissue. The microscopic slices were evaluated for the presence of inflammation and fibrosis on a five-point scale (0: absent, 1: equivocal, 2: mild, 3: moderate, or 4: marked).²⁰

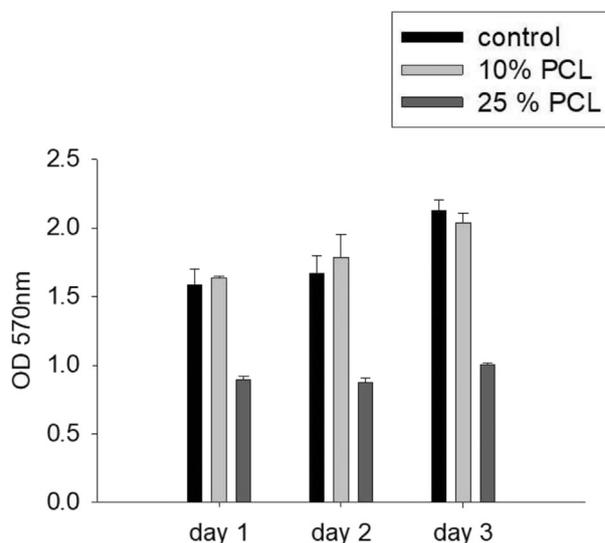


Figure 1 Cell Density: 104/cm². Cells proliferate inconsistently on PCL gel with culture time. Compared to 10% PCL gel, fibroblasts exhibited significant lower activity index on 25% PCL gel at each day.

2.8. Statistical analyses

The categorical variables were compared using χ^2 and Fisher's exact tests, while the continuous variables with Student's t test. P values of less than 0.05 were regarded as significant. Statistical analyses were performed using SPSS release 18 (SPSS Inc., Chicago, Ill). All statistical tests were two-sided.

3. Results

3.1. Fibroblast proliferation

Hs68 fibroblast proliferation on PCL gel was evaluated by the application of alamar blue. Fig. 1 shows cells proliferated inconsistently with culture time. Compared to 10% PCL gel, fibroblasts exhibited a significant lower activity index on 25% PCL gel.

3.2. PCL gel-treated pleurodesis in a lapin thoracic model

Representative images of the hemithorax after gel pleurodesis are presented in Fig. 2. Macroscopic evaluation of pleurodesis score (Table 1) showed that 10% PCL gel produced moderate pleurodesis, while 25% PCL gel exhibited significantly higher pleurodesis scores. ($P < 0.05$) Control group without PCL gel instillation produced almost no pleurodesis ($P < 0.05$). Microscopic evaluation of the parietal and visceral pleura are demonstrated in Fig. 2 and Table 2. PCL gel with two different concentrations induced moderate to severe inflammation and fibrosis in the parietal and visceral pleura when compared with the control group ($P < 0.05$). Similarly, the highest scores of inflammation and fibrosis of the parietal and visceral pleura in this study was observed in the 25% PCL gel group.

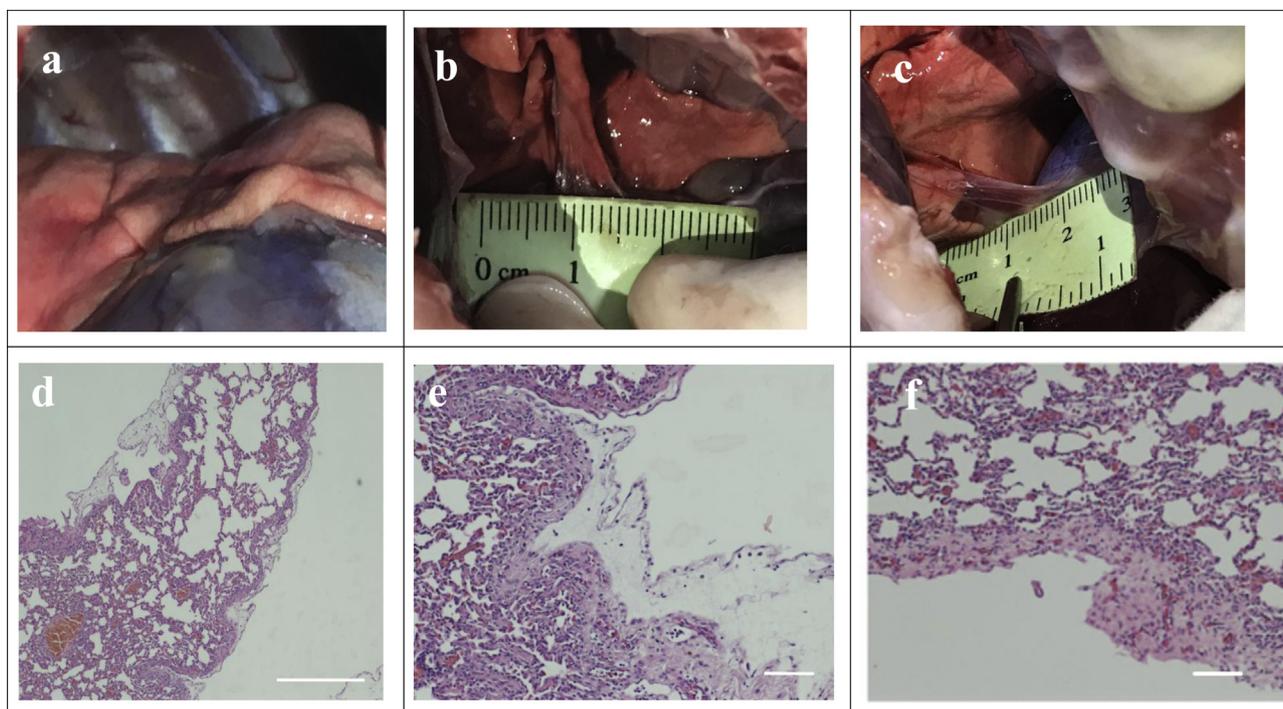


Figure 2 The gross view of hemithorax 30 days after various procedures. (a: control group, b: 10% PCL gel, c: 25% PCL gel). (d, e, f) The corresponding microscopic view of the pleura 30 days after procedures (d: control group, e: 10% PCL gel, f: 25% PCL gel). Scale bar: 250 μm .

Table 1 The pleurodesis scoring during macroscopic evaluation of pleural cavities after PCL gel pleurodesis.

Method	Score
10% PCL gel (n = 12)	2.5 \pm 0.3
25% PCL gel (n = 12)	3.9 \pm 0.5#
Control (n = 12)	0.3 \pm 0.2*

#P < 0.05 when comparing 25% PCL gel and 10% PCL gel.

*P < 0.05 when comparing Control with 10% PCL gel or 25% PCL gel.

3.3. Fibronectin expression

Fig. 3 shows the immunochemical staining of fibronectin was enhanced on both groups, more on 25% than 10% PCL gel. It indicated that more fibronectin was secreted by Hs68 fibroblasts when stimulated with PCL gel with higher concentration.

4. Discussion

More and more need for better pleurodesis material is noted for pleural diseases. Take pneumothorax for example, biomaterial pleurodesis gradually become one of the treatment choices.³ Generally speaking, choosing one specific pleurodesis method should be relied on the specific pleural disease entity. In other words, the degree and extent of pleurodesis required are different for various pleura diseases. The most intensive pleurodesis may not always be the best, especially for young men.⁴ The conventional pleurodesis methods, mechanical pleurodesis or apical pleurectomy, may cause some complications, such as severe pain or bleeding.^{4–10} Therefore, our previous work was reasonable to design an appropriate membrane to cover lung bullae with inducing adhesion, without mechanical stimulation and trauma, to create pleurodesis. We proved PCL membrane-induced pleurodesis can be achieved with satisfactory results.³ In current study, we

Table 2 Microscopic examination of pleural cavities after PCL gel pleurodesis.

Method	Parietal pleura inflammation score	Visceral pleura inflammation score	Parietal pleura fibrosis score	Visceral pleura fibrosis score
10% PCL gel (n = 12)	2.0 \pm 0.4	2.1 \pm 0.7	2.2 \pm 0.4	2.3 \pm 0.6
25% PCL gel (n = 12)	3.0 \pm 0.4*	3.1 \pm 0.5*	2.9 \pm 0.3*	3.1 \pm 0.4*
Control (n = 12)	0.2 \pm 0.1**	0.2 \pm 0.1**	0.3 \pm 0.2**	0.2 \pm 0.1**

*P < 0.05 when compared 10% PCL gel with 25% PCL gel.

**P < 0.05 when compared control with others.

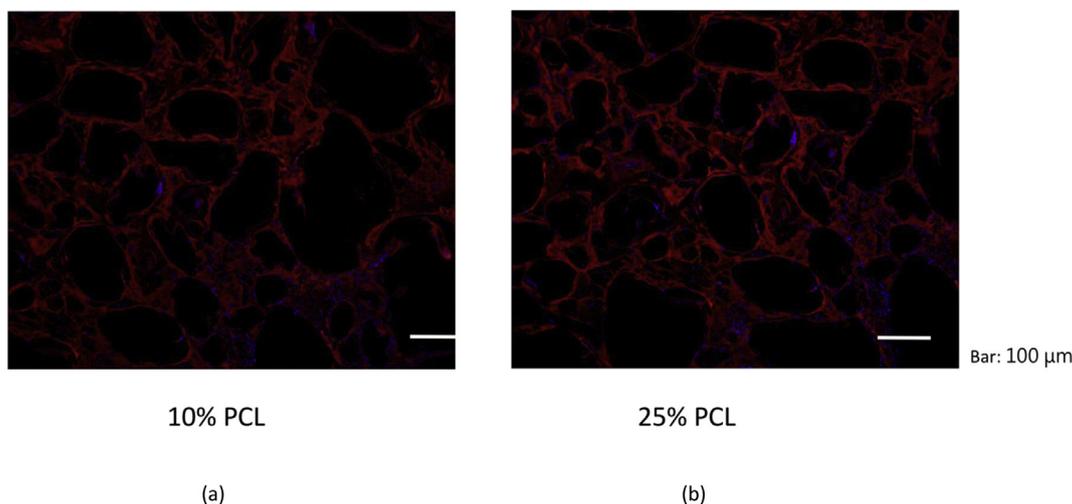


Figure 3 The immunohistochemical staining of fibronectin was enhanced more on 25% PCL gel(b) than 10% PCL gel(a), indicating that more fibronectin was secreted by Hs68 fibroblasts when stimulated by PCL gel with higher concentration.

supposed that the same kind of biomaterial, i.e. PCL, could induce pleurodesis in gel form, as well. The gel form of PCL has the advantage of more fluidity over the membrane form. Moreover, gel form can be delivered without surgery. If it can be clinically used, the delivery of gel form PCL requires only catheter insertion instead of operation. Therefore, it can be used not only in the operative room but also ward or emergent room.

New Zealand White Rabbits were used to test whether PCL gel-induced pleurodesis also could be achieved. We found that gel pleurodesis could be safely performed and was able to induce adhesion in rabbits with the results comparable to PCL membrane. Further, we confirmed that PCL gel caused significant effect of adhesion and the 25% PCL could cause greater adhesion than 10% one. As previous study showed, the seal integrity was achieved in two ways. First, PCL gel induced inflammation and thickening of the visceral pleura, causing adhesion between the visceral and parietal pleura. It seals leaking holes in the visceral pleura. The highest pleurodesis scores, as well as pleural inflammation and fibrosis scores, were observed in the 25% PCL gel group. Second, we thought fibronectin is still the key for the adhesion effect. Fibronectin is synthesized by numerous cells, primarily fibroblasts.²¹ It has been known to be important in wound healing and tissue repair, as well as pleurodesis. It is chemotactic for fibroblasts and provides a scaffold for matrix deposition and fibroblast binding.^{21–25} In our studies, we found that fibronectin plays an important role in the adhesion formation, both in the rat and lapin study. The activity of fibroblast was suppressed in 25% PCL more than 10%, which is the same phenomenon in the literature.²⁶ Hence, the PCL gel concentration does influence the degree of adhesion. This appeared to suggest that the PCL gel concentration might be a key factor in controlling the successful rate of treating recurrent pneumothorax. Moreover, the result of this study also shed light on the disseminated pleural seeding with malignant pleural effusion by lung cancer.^{27–29} Since pleural diseases have common therapeutic method with pleurodesis, the PCL gel can be used combining with other biomaterial or drugs to treat the severe condition in the future.

In conclusion, our study showed that PCL gel induced significant degree of pleurodesis in the pleural space. The 25% PCL gel produces more intensive adhesion than 10% one. Fibronectin plays an important role in the process of pleurodesis. Further study is required for the clinical application of the promising biomaterial with gel form.

Competing financial interests statements

There is no conflicts of interests for all authors.

Acknowledgments

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