

# Evaluation on the biocompatibility, bone cell activity and bone regenerative capacity of chitosan-PLLA bilayer porous membrane

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## I. Introduction

Chitosan is copolymer of(1→4) glucosamine(2-amino-2-deoxy-D-glucose) and N-acetyl-D-glucosamine(2-acetamido-2-deoxy-D-glucose) which has been reported as a wound healing accelerator<sup>1)</sup>. Chitosan is biodegradable cationic polysaccharide that is non-toxic and non-immunogenic<sup>2)</sup>. It is also used as an osteoconductive material which induces or stimulates bone formation<sup>3)</sup>. It stimulates proliferation and differentiation of mesenchymally derived cells including fibroblasts, smooth muscle cells, ligament cells and osteoblasts<sup>4)</sup>. The lactic acid is a chiral molecule and it exists in two stereo-

isomeric forms: L-poly(lactic acid) and D-poly(lactic acid). Generally poly(L-lactic acid)(PL-LA) is more frequently employed, since the hydrolysis of PLLA yields L(+)-lactic acid, which is the naturally occurring stereoisomer of lactic acid. The PLLA shows high mechanical strength and toughness and most widely used as synthetic degradable polymers in medicine.

In this study the chitosan mesh was intended for its biocompatible surface of non-immunogenic properties and PLLA membrane was used for enhancing the mechanical support and excluding the connective tissue ingrowth. The purpose of this study was to investigate the biocompatibility of

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the PLLA-chitosan membrane and the biologic effect of this novel membrane as a barrier membrane in bone regeneration.

## **II. Materials and Methods**

### **1. Materials**

Chitosan was purchased from Tae-Hun Bio(Kyungbook, Korea). PLLA(Mw 370,000) was obtained from Purac Bio-chem BV(Gorinchem, Holland). PLLA dissolved in chloroform gave an intrinsic viscosity of 6.30dl/g at 25°C. Span 80 was purchased from Showa chemicals(Osaka, Japan). Collagenase,  $\beta$ -glycerol phosphate, L-ascorbic acid were obtained from Sigma-Aldrich(St. Louis, MO, USA). Trypsin-EDTA, fetal bovine serum,  $\alpha$ -minimumessential medium were purchased from Gibco(Grand Island, NY, USA). All solvents used were of analytical grade.

### **2. Fabrication of chitosan-PLLA membranes**

Chitosan fibers were prepared by extruding 4% chitosan solution in 4% acetic acid into pH 13 aqueous solution. Chitosan fibrous meshes were fabricated by pressing chitosan fibers. PLLA-methylolacrylate solution was cast on between the two chitosan fibrous meshes using a doctoring blade, and solvents were evaporated in air at 25°C for 24 hours and further dried under vacuum for 24 hours to remove residual solvents.

### **3. Preparing the cells**

Osteogenic cell line MC3T3-E1 was used. Cell pellets were resuspended and plated in flasks in 30ml of  $\alpha$ -minimum essential medium( $\alpha$ -MEM) containing 10% fetal bovine serum(FBS) and 1% antibiotic-antimycotic solution. The flasks were maintained in a humidified atmosphere consisting of 95% air and 5% CO<sub>2</sub> at 37°C. After 3 days, hematopoietic cells and other unattached cells were removed from the flasks by repeated washing with  $\alpha$ -MEM. When confluent, the cells were enzymatically lifted from the flask using 0.25% trypsin in 4mM EDTA. The cells were concentrated by centrifugation at  $4 \times 10^3$  rpm for 7 minutes at 4°C.

### **4. Cell seeding into matrices and in vitro culture**

After centrifuging, the supernatant was suctioned away and resuspended in a known amount of media. Cells were counted with a hemacytometer and diluted to  $1 \times 10^5$  cells/ml in complete  $\alpha$ -MEM media containing 15% FBS, 1% antibiotic-antimycotic solution, 10 mM Na  $\beta$ -glycerol phosphate, 50  $\mu$ /ml L-ascorbic acid, and  $10^{-7}$ M dexamethasone. Pre-wetted membranes with complete media were placed in 24-well plates. Aliquots of 100  $\mu$ l of cell suspension were seeded onto the top of the membranes resulting in a seeding density of  $10^4$  cells/membrane. The seeded membranes were left undisturbed in an incubator for 3 hours to allow the cells to attach. Subsequently, an additional 1ml of complete media was added to each well.

Medium was changed every 2 to 3 days.

## 5. Cell proliferation

Cell proliferation was measured at 1, 7 and 14 days. At each time point, media were removed from the wells. The membranes were washed gently with Hank's balanced salt solution (HBSS) to remove any unattached cells and remaining media. The adherent cells were released from the substrate by incubation in 300  $\mu$ l of 0.25% trypsin in 4mM EDTA for 10 minutes. After digestion, the membranes were washed with 300  $\mu$ l of HBSS for collecting retained cells. Cells in trypsin/HBSS solution were counted by the hemacytometer. After counting, the cells in the media were centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was suctioned away and the cell pellet was prepared for alkaline phosphatase (ALPase) activity test.

## 6. SEM view of cell-membrane complexes

Cultured cell-membrane complexes were prepared for scanning electron microscope (SEM) studies at 1, 7 and 14 days. They were incubated at low temperature (in ice) in a fixative of 2.5% of glutaraldehyde for 20 minutes and then washed in PBS for 10 minutes twice. The complexes were then incubated for 20 minutes in a postfixative of 1% aqueous  $\text{OsO}_4$  and subsequently washed with PBS for 10 minutes. Samples were dehydrated through ascending ethanol (70, 80, 90, 95 and twice in 100%), allowed to air dry overnight and shadowed with 20 nm Au/Pd.

## 7. Measurement of alkaline phosphatase (ALPase) activity

Production of ALPase was measured spectroscopically at 1, 7 and 14 days. Removed cells from the membranes were homogenized with 200  $\mu$ l of double distilled water (DDW) and sonicated for 1 minute in ice. 50  $\mu$ l of cell lysate were mixed with 100  $\mu$ l of 0.1M glycine-NaOH buffer, 50  $\mu$ l of 15mM para-nitrophenol phosphate (PNPP), 50  $\mu$ l of 0.1% Triton X-100/saline, and 50  $\mu$ l of DDW. Aliquots were incubated for 30 minutes at 37°C. After incubation, each tube was added 1.25 ml of 0.1N NaOH and placed in ice. The production of paranitrophenol (PNP) in the presence of ALPase activity was measured by monitoring light absorbance at 405 nm. The slope of absorbance versus time plot was used to calculate the ALPase activity.

## 8. Detection of mRNA by RT-PCR

Total RNA was isolated from the samples. 0.7  $\mu$ l of 14.2M  $\beta$ -mercaptoethanol ( $\beta$ -ME) was added to 100  $\mu$ l of Lysis Buffer for each sample. 100  $\mu$ l of Lysis Buffer- $\beta$ -ME mixture was added to each cell sample and was vortexed until homogenized. An equal volume of 70% ethanol to the cell lysate was added and then mixed thoroughly by vortexing for 5 seconds. This mixture was transferred to a seated RNA-Binding Spin Cup. It was spun in a microcentrifuge at maximum speed for 1 minute and the filtrate was discarded. DNAase treatment was followed as the manufacturer's instructions. 500  $\mu$ l of 1x High-Salt Buffer was added and

spinned and the filtrate was discarded. Then 600  $\mu\text{l}$  of 1x Low-Salt Wash Buffer was added and spinned and the filtrate was discarded. 300  $\mu\text{l}$  of 1x Low-Salt Wash Buffer was added and spinned for 2 minutes to dry the fiber matrix. Spin cup was transferred to a 1.5 ml collection tube. After adding 30  $\mu\text{l}$  of Elution Buffer directly onto the fiber matrix, it was incubated for 2 minutes at room temperature and was spinned again at maximum speed for 1 minute. The purified RNA was obtained in the Elution Buffer in the microcentrifuge tube.

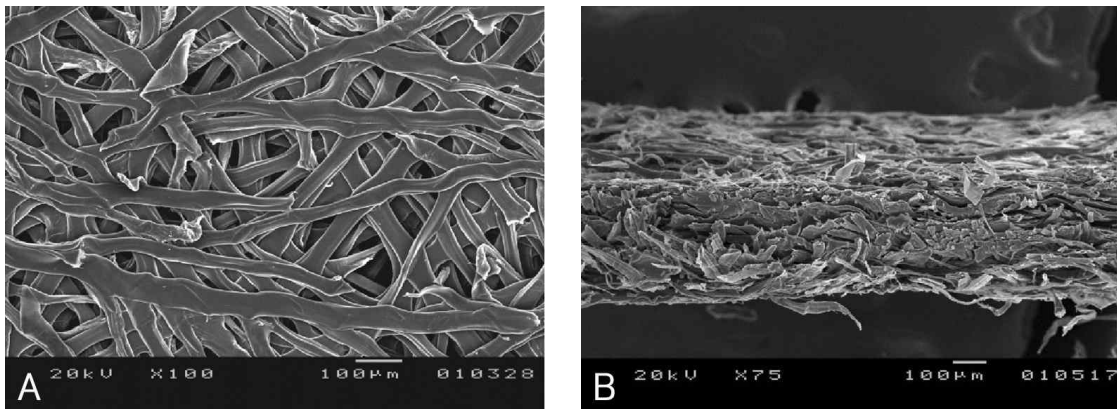
RT-PCR was used to assess transcript expression. cDNA was constructed by using kit. Master Mix containing 2  $\mu\text{l}$  of 10x reaction buffer, 4  $\mu\text{l}$  of 2 mM  $\text{MgCl}_2$ , 2  $\mu\text{l}$  of deoxynucleotide mix, 2  $\mu\text{l}$  of Oligo-p(dT)<sub>15</sub> primer, 1  $\mu\text{l}$  of Rnase inhibitor, and 0.8  $\mu\text{l}$  of AMV reverse transcriptase was mixed in sterile microcentrifuge tube. RNA and sterile water were mixed and was incubated for 15 minutes at 65°C. Following incubation it was kept in ice for 5 minutes. The Master Mix made before was put in the tube and incubated for 10 minutes at 25°C and kept for 60 minutes at 42°C. Reverse transcriptase was denatured by incubating at 100°C for 5 minutes and then cooled down to 5°C.

PCR of the cDNA products was carried out in a final volume of 20  $\mu\text{l}$  containing of 2  $\mu\text{l}$  of cDNA, 1  $\mu\text{l}$  of 20 pmol/ $\mu\text{l}$  of each of the forward and reverse primers, and 16  $\mu\text{l}$  of autoclaved DDW. PCR protocol was 30 cycles of denaturing(94°C, 1 minute), annealing(52°C, 2 minutes) and primer extension(72°C, 1 minute), which was controlled by the DNA thermal cycler(Mastercycler Gradient, Eppendorf,

Germany). Primers for glyceraldehyde-3-phosphate dehydrogenase(GAPDH), a house keeping gene used for an internal control, collagen type I(CO), osteopontin(OP), and osteocalcin(OC) were designed from the published sequences. GAPDH: forward, 5'-CACCATGGAG AAGCCCGGG G-3' reverse, 5'-GACGGACACA TTGG GGGTAG-3'. CO: forward, 5'-TCTC CAC TCTTCTAGTTCTT -3' reverse, 5'-TTGG GTCATTTCCACATGC-3'. OP: forward, 5'-AC ACTTTCACTCCAATCGTCC -3' reverse, 5'-TG CCCTTTCCGTTGTTGTC C-3'. OC: forward, 5'-TCTGACAAACCT TCATGTCC -5' reverse, 5'-AAATAGTG ATACCGTAGATG CG-3<sup>5)</sup>.

## 9. Animal experiment

8 male Sprague-Dawley rats were used in animal experiments. The animals with chitosan-PLLA membranes were served as test group and the one without membranes were served as control. Rats were anesthetized by intraperitoneal injection of ketamine (30 mg/kg body weight). The surgical site was made in the sagittal plane across the cranium. A full-thickness flap including periosteum was reflected, exposing the parietal bone. An 8mm diameter critical size calvarial defect was created with saline cooled trephine drill. Extreme care was taken to avoid injury to the brain. After removal of the trephined calvarial bone, chitosan-PLLA membranes were implanted into the defects in test group and nothing was applied on the control group. The periosteum and skin were closed using 5-0 chromic gut and 4-0 silk sutures, respectively. Bone regenerative efficacy of the membranes was examined at



**Figure 1.** Morphologic appearance of outer surface of the membrane(A) The cross-sectional view of membrane shows the inner PLLA layer(asterik) and outer chitosan layer with distinct interface(arrows).

4 weeks. The histomorphometric analysis was done at 2 and 4 weeks. The perimeter of newly formed bone was traced and the enclosed area was measured using image analysis software(Image Access).

## 10. Statistical analysis

All measurements were collected from at least in triplicate and expressed as mean standard deviation. The results obtained from the samples at the same time point were analyzed by Mann-Whitney U Test. Values of  $p < 0.05$  were considered statistically significant.

# III. Results

## 1. The morphology of chitosan–PLLA membranes

The surface of the membrane revealed a characteristic thin multifibers of chitosan mesh with a slight roughened surface(Figure

1A). The membrane composed of inner PLLA layer and outer chitosan layer observed distinctly in cross-sectioned view(Figure 1B). The pore size of PLLA layer between two chitosan mesh was fallen between 2 to 10  $\mu\text{m}$ .

## 2. Cell proliferation

To calculate the increasing rate, the cell numbers of 7 and 14 days were divided by the cell number of day 1. The cell proliferation rate of day 14 was significantly increased compare to that of day 7( $p < 0.05$ ) in test group, however, there was no significant difference between two groups at each period(Table 1 and Figure 2).

## 3. SEM view of cell–membrane constructs

At day 1, cells were well attached to the membrane and the cell morphology was round to spindle with elongated cytoplasmic extension(Figure 3A). At day 7, more cells were attached over the membrane sur-

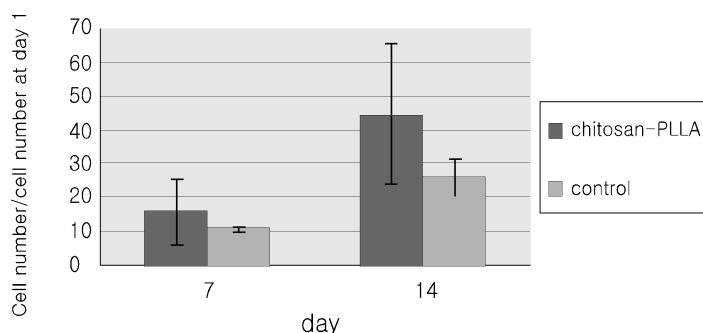


Figure 2. Cell proliferation(cell number/cell number at 1 day)

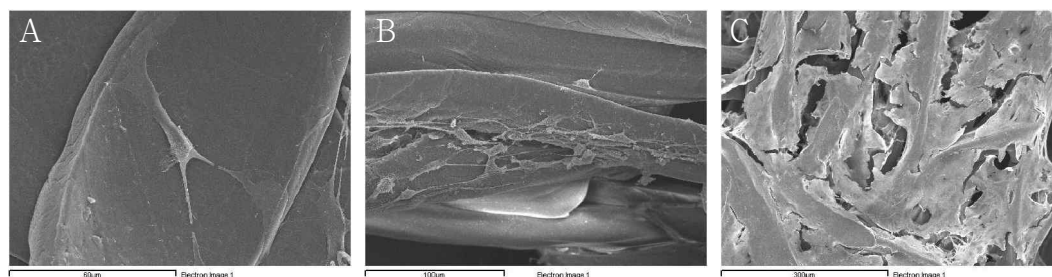


Figure 3. Scanning electron microscopy of chitosan-PLLA membrane.(A) At day 1, cells were well attached to the membrane with elongated cytoplasmic extension(x 1000);(B) At day 7, more cells were attached over the membrane surface(x 500);(C) At 14 days, membrane was filled with proliferated cells(x 200).

face(Figure 3B). At day 14, the number of cells was highly increased and the membrane was filled with the proliferated cells(Figure 3C).

#### 4. Measurement of alkaline phosphatase(ALPase) activity

The ALPase activity had an increasing tendency throughout the periods. There was no significant increase after day 7 and 14 compared to day 1 in test group and there was significant difference between the two groups at 7 days( $p < 0.05$ )(Table 2 and Figure 4).

Table 1. Cell proliferation(cell number/cell number at 1 day)

day	cell number/cell number at day 1	
	Chitosan-PLLA	Control
7	15.99 ± 9.56	11.11 ± 0.56
14	44.62 ± 20.61*	25.81 ± 6.21

\* $P < 0.05$ , as compared with day 7 in each group

Table 2. Alkaline phosphatase activity levels

	Chitosan-PLLA	Control
1	0.031 ± 0.0100	0.033 ± 0.009
7	0.027 ± 0.007	0.033 ± 0.009#
14	0.043 ± 0.014	0.049 ± 0.009*

\* P<0.05, as compared with day 1 in each group

# P<0.05, as compared with chitosan-PLLA

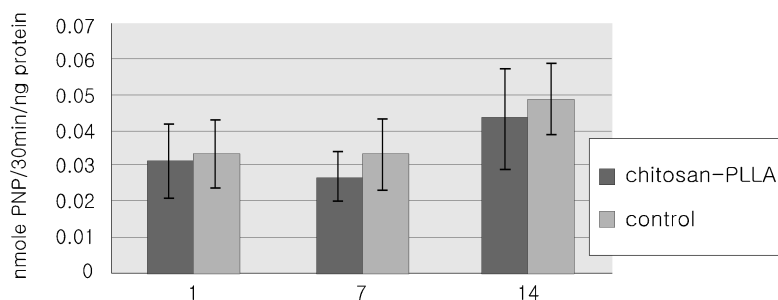


Figure 4. Alkaline phosphatase activity levels

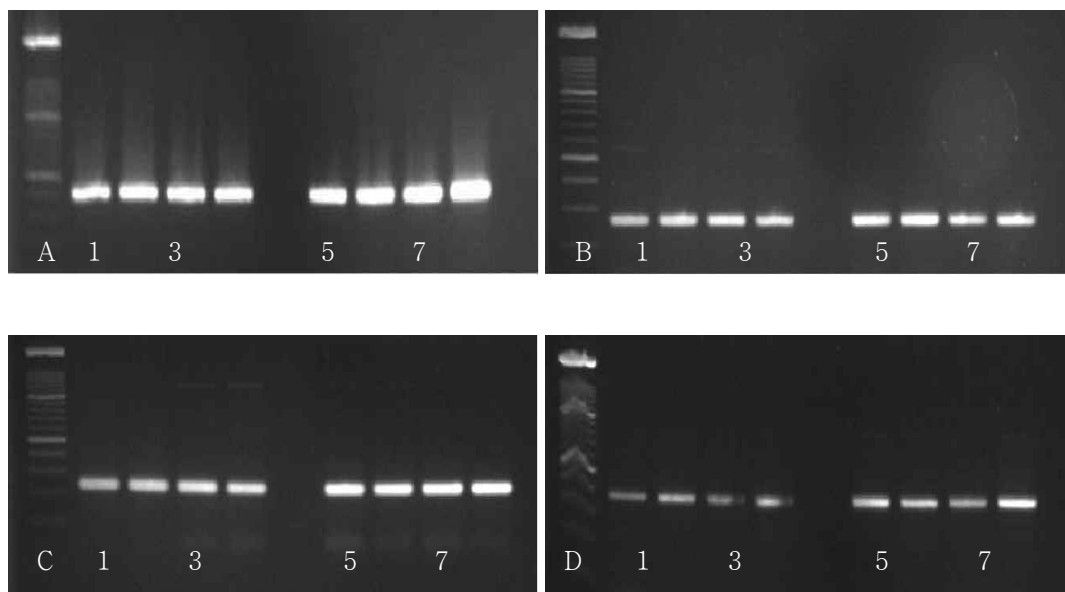


Figure 5. Expression of mRNA in osteoblastic cells.(A)GAPDH(housekeeping gene, 418 bp) was expressed in all osteoblastic cells.(B,C,D)mRNA for COL(250 bp), OP(239 bp), and OC(198 bp) was expressed in all samples.(1,2,3,4: chitosan-PLLA membrane 1,4,17 and 14 days 5,6,7,8: control 1,4,7 and 14 days)

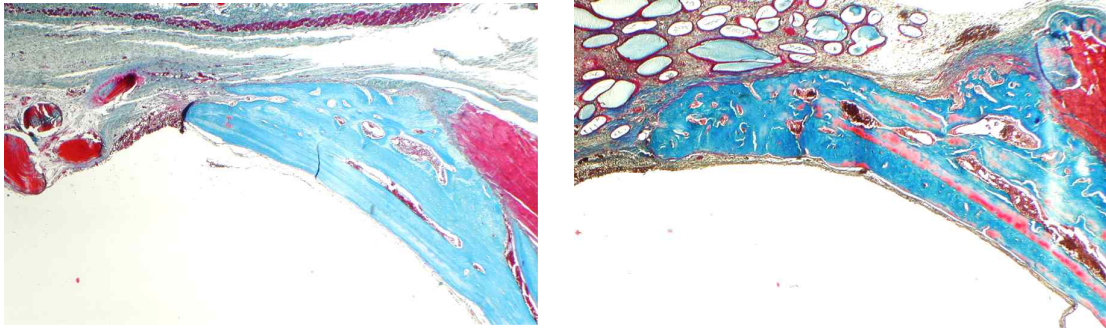


Figure 6. Histologic view in vivo at 4 weeks(A)non-membrane-treated defects were invaded by the thin, loosely organized connective tissue and limited amount of bone repair was observed(arrow).(B)bone regeneration was observed from the periphery toward the center in membrane-treated membrane-treated defects(asterik).

Table 3. Area of new bone in rat calvarial defect

Day	area of new bone(mm <sup>2</sup> )	
	Chitosan-PLLA	Control
7	0.127 ± 0.005	0.044 ± 0.015
14	0.325 ± 0.009	0.117 ± 0.037

\* P<0.05, as compared with day 1 in each group

# P<0.05, as compared with control

## 5. Detection of mRNA by RT-PCR

mRNA for glyceraldehyde-3-phosphate dehydrogenase, collagen type I, osteopontin, and osteocalcin were expressed at 1, 4, 7 and 14 days(Figure 5). The detection of GAPDH mRNA in cell extracts served as a positive control for mRNA recovery and reverse transcription. Seeded osteoblasts retained their biochemical phenotype-specific characteristics for bone formation throughout the entire culture period.

## 6. Bone regeneration experiment in vivo

No specimens revealed any evidence of inflammatory reaction and all wounds showed a good healing response. At 4 weeks after surgery, non-membrane-treated wounds were invaded by the thin, loosely organized connective tissue and limited amount of bone repair was observed(Figure 6A). In membranetreated wounds showed bone regeneration in centripetal fashion(Figure 6B). The results of histomorphometric analysis showed that the amount of regenerated bone in the chitosan-PLLA group was significantly( $p < 0.05$ ) greater than in the control group in both 7 and 14 days(Table 3 and Figure 7).



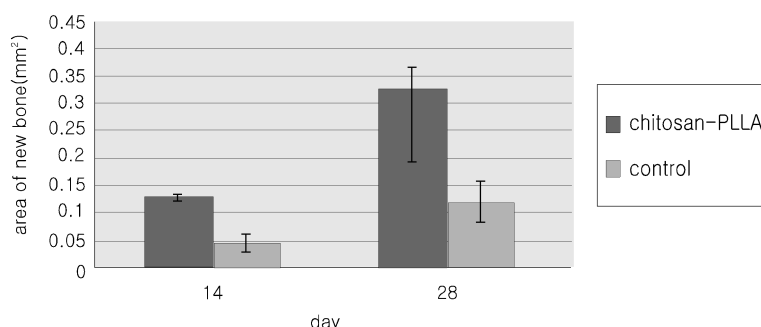


Figure 7. Area of new bone in rat calvarial defect

## IV. Discussion

Chitosan has been reported as a wound healing accelerator<sup>1)</sup>. The effect of chitosan on bone wound healing has been examined. Muzzarelli *et al.* reported improved osseous healing in the defect area<sup>6)</sup>.

The components of culture media were also important in phenotypic expression and retention of osteoblast and matrix mineralization. In this study, the culture media was supplemented with ascorbic acid,  $\beta$ -glycerophosphate and dexamethasone. Ascorbic acid probably stimulates the formation and hydroxylation of collagen, permitting sufficient amount of collagenous matrix to bone deposited<sup>7)</sup>. The organic phosphates appear to be necessary for mineralization. In the study by Bellows *et al.*, nodules failed to be mineralized in absence of  $\beta$ -glycerophosphate while nonmineralized nodules formed in the absence of  $\beta$ -glycerophosphate did mineralized when  $\beta$ -glycerophosphate was added<sup>8)</sup>. Glucocorticoids such as dexamethasone, have been shown to cause an initial increase in the activity of a number of osteoblast-like cell markers<sup>9)</sup>.

The cell number of membranes is lower than that of the control. The membrane had porous structure and all the inoculated cells may not have seeded on the membrane. It might have passed through or overflowed the membrane. To make the day 1 cell number of the membrane similar to that of the control, we could increase the number of seeded cells in the membrane. The surface of polystyrene dish has been known to have good cellular attachment and show rapid cellular confluency in incubation period<sup>10)</sup> and higher increasing rate of the membrane implies good cellular activity.

The initial attachment of osteoblastic cells to the membrane was observed by SEM 1day after incubation. The morphology of the cell was round to spindle with elongated cytoplasmic extension. As time goes on, the cells started to aggregate and the membrane is partially covered with the proliferated cells.

All the RNA samples used in the study showed the single band of GAPDH gene sequence, indicating that the extracted RNA samples were intact and not degraded<sup>11)</sup>. Osteoblasts express various phenotypes such as elevated levels of ALPase activity and par-

athyroid responsiveness.

Osteopontin, osteocalcin, and bone sialoprotein may play important roles in cementogenesis, osteoblast differentiation and bone mineralization<sup>12)</sup>. The expression of ALPase activities suggests that seeded bone marrow cells were differentiated into the osteoblasts. During osteoblast differentiation, the increase in ALPase activity occurs earlier than does osteocalcin production. Osteocalcin production occurs preferentially in mature osteoblasts<sup>13)</sup>. In present study, chitosan-PLLA membrane retained phenotype-specific characteristics as osteoblast, which implies good cellular adaptability.

## V. Conclusion

To investigate the biocompatibility of the PLLA-chitosan membrane and to evaluate the biological effect of this novel membrane, osteoblast-like cells were applied to the test membrane. We performed a histologic and tested biochemical approach in vitro. We also evaluated the bone regenerating activity of this membrane using rat calvarial defect in vivo.

1. Seeded cells were well attached to the membrane used. Cell proliferation was significantly increased after 7 and 14 days compared to 1 day ( $p < 0.05$ ).
2. SEM view of day 1 showed well attached cell to the membrane. And that of day 7 and day 14 showed increased cell population over times.
3. Expression of collagen type I, osteopontin and osteocalcin mRNA was detected in all samples.

4. At cultured cells, alkaline phosphatase activity was maintained during whole periods.

5. Chitosan-PLLA membrane promoted osseous healing as compared to controls.

These results suggest that the novel chitosan-PLLA porous bilayer membrane has a favorable biologic effect on osteoblast cells. Within the limit of our study, it is concluded that the chitosan-PLLA membrane has a good cellular adaptability. Further studies are needed for long-term evaluation of the membrane as a barrier membrane in vivo before applying in clinic.

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## Chitosan-PLLA 다층 다공성 차폐막의 생체적합성, 골세포활성도 및 골재생 능력에 관한 연구

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이 연구의 목적은 새로이 제작된 chitosan-poly(L-lactic acid)(PLLA) 다층 다공성 차폐막의 생체적합성 및 골세포활성도 및 골재생능을 평가하는 것이다.

제작된 차폐막을 24 well에 넣고 clonal osteoblast-like cell line(MC3T3-E1)을 접종한 군을 실험군으로, 차폐막을 사용하지 않은 대조군으로 하였다. 배양 1일, 7일 및 14일째에 각 well에서 세포수를 측정하였다. 주사전자현미경을 이용하여 차폐막에 부착된 세포의 형태관찰을 시행하였다. RNA 추출 및 RT-PCR을 실시한 후, agarose gel상에서 전기영동하여 조골세포 표식자인 collagen type I(COL), osteopontin(OP) 및 osteocalcin(OC) mRNA의 발현을 관찰하였다. 제작된 매트릭스의 생체적합성 및 골재생능을 관찰하기 위하여 백서의 두개골에 직경 8mm의 원형 결손부를 형성한 후 차폐막을 이식한 군을 실험군으로, 아무 것도 넣지 않은 군을 대조군으로 하여 4주 경과 후 실험동물을 희생시킨 후 조직학적관찰을 시행하였다.

시간경과에 따른 부착세포수 관찰결과, 배양 14일까지 조골세포의 수가 지속적으로 증가하였고, 주사전자현미경으로 세포의 형태 관찰결과, 배양된 세포들은 중층의 형태로 성장하면서 시간경과에 따라 세포가 응집되는 양상을 나타내었다. 관찰 기간동안 COL, OP, 및 OC mRNA의 발현이 관찰되어 배양 전 기간동안 조골세포의 형질이 잘 유지되고 있음을 알 수 있었다. 백서 두개골 결손부에 이식된 차폐막은 염증반응 없이 주위 조직과 우수한 생체적합성을 나타내었으며, 차폐막을 이식하지 하지 않은 대조군에 비해 높은 신생골 형성을 나타내었다.

이상의 관찰결과로 새로이 제작된 chitosan-PLLA 차폐막은 우수한 생체적합성 및 골재생능을 나타냄을 알 수 있었으며, 향후 이를 골조직 재생 및 치주조직유도재생 분야에 응용될 수 있을 것으로 생각된다.

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주요어 : chitosan-PLLA 다공성 차폐막, 생체적합성, 재생

