Polymer(Korea), Vol. 40, No. 2, pp. 181-187 (2016)
http://dx.doi.org/10.7317/pk.2016.40.2.181

ISSN 0379-153X(Print)
ISSN 2234-8077(Online)

조직공학적 인공각막내피를 위한 실크필름의 정제시간이 각막내피세포의 성장에 미치는 영향

이선의 · 차세롬 · 장나금 · 김수영 · 김은영 · 송정은 · 박찬흠* · 강길선†

전북대학교, BIN융합공학과, 고분자나노공학과, 고분자융합소재연구소
*한림대학교 의과대학 춘천성심병원 이비인후과

(2015년 8월 18일 접수, 2015년 9월 30일 수정, 2015년 9월 30일 채택)

Effect of Degumming Time of Silk Films on Growth of Corneal Endothelial Cells for Tissue Engineered Endothelialized Neo-Corneas

Seon Eui Lee, Se Rom Cha, Na Keum Jang, Su Young Kim, Eun Young Kim, Jeong Eun Song, Chan Hum Park*, and Gilson Khang†

Dept. of BIN Fusion Tech & Dept of PolymerNanoSci Tech, Chonbuk National University, 567 Baekje-daero, Jeonju 54898, Korea
**Dept. of Otorhinolaryngology-Head and Neck Surgery, Chuncheon Sacred Heart Hospital, College of Medicine, Hallym University, 1-1 Okcheon, Chuncheon, Gangwon 24253, Korea*

(Received August 18, 2015; Revised September 30, 2015; Accepted September 30, 2015)

초록: 실크는 생분해성 천연고분자로써 이식 후 낮은 면역 거부반응을 일으킨다. 뿐만 아니라, 실크는 다양한 형태로 제조할 수 있으며 제조 방법에 따라 수 마이크로 미터 두께로 조정될 수 있다. 필름 형태의 실크 박막은 매우 투명하며, 높은 수분 및 산소 투과도를 나타낸다. 그러나 실크 단백질인 세리신에 의해 염증반응이 일어난다는 보고가 있으며, 이식 후 분해에 장시간을 필요로 한다. 이 연구에서는 생체 적합성 및 염증반응 등에 세리신 정제시간의 영향을 알기 위해 다양한 정제시간(0, 20, 40, 60분)을 가진 실크로 필름을 제조한 후 접촉각과 세포 생존율 및 RT-PCR을 이용한 염증인자발현 실험을 시행하였다. 정제시간에 따라 세리신 함량을 달리한 실크 필름에 각막내피세포 파종 후 필름에서의 세포 부착도와 증식률, 특정 mRNA의 발현을 평가하였다. 그 결과 실크의 정제시간은 각막내피세포의 성장에 영향을 주었으며 실크필름의 생명공학 신 각막으로써의 가능성을 보여주었다.

Abstract: Silk is a biodegradable natural polymer with low immunological rejection after transplantation. As well, silk can be manufactured in various forms and adjusted to the thickness of several micrometers depending on the manufacturing method. Silk shows high transparency in the form of film, high water and oxygen permeability. However, studies have cited some drawbacks of silk, such as inflammatory response due to presence of silk protein (sericin) and its another disadvantage of silk requires a long period of time to decomposition after transplantation. In this study, silk films with various degumming time (0, 20, 40 and 60 min) were fabricated in order to evaluate the effect of sericin in learns of biocompatibility, inflammatory responses, etc. The experiments such as the contact angle, cell viability and inflammatory factor expression using RT-PCR were performed. Corneal endothelial cells were seeded on silk films and examined to evaluate the degree of adhesion in films, cell's proliferation and specific mRNA expression. This study showed degumming time of silk film fabrication which is a factor for growth of corneal endothelial cells.

Keywords: silk, sericine, film, degumming time, corneal endothelial cells.

Introduction

Silk is a natural protein composed of fibroin and sericin. Silk is a biodegradable natural polymer with the characteristics of bioresorbable, supporting cell growth and good mechanical properties.[1-4] The fibroin is a structured protein and a fibrous protein. The fibroin with β-sheet structure is useful for application to many biomedical and biotechnology as biomaterials because of the ability of biodegradable, good mechanical strength with little immune response.[5,6] The sericin is a sticky protein that surrounds the fibroin fibers. The sericin protects damage, microbial decomposition and digestion of fibroin.[7]

†To whom correspondence should be addressed.
E-mail: gskang@jbnu.ac.kr

Degumming is the pre-treatment process of obtaining only fibroin fiber structure by removing the sericin.[8] According to the related study of degumming, there was a change in the characteristics of silk fibers such as molecular weight, viscosity of the fibroin, packing density and tensile strength.[9] The cause of these changes is molecular changes, bonding breakage and structural changes due to the removal of sericin. In addition, hydrophilic sericin reduces the efficiency of stress transfer at the fiber-matrix composite formation and results in the effect to prevent binding of polymer surface to reduce mechanical properties of composite substantially.[10,11]

Corneal transplantation is the most common transplant surgery to replace a healthy donated cornea to improve vision problems caused by a damaged cornea.[12-14] However, corneal transplantation has limitations due to the shortage of healthy cornea that can be implanted.[15] Therefore the development of suitable scaffolds for regeneration of corneal endothelial cells could be an alternative that overcomes the limitations of insufficient supply healthy donated cornea.[16-18]

In this study, we prepared films by using silk with a unique mechanical properties, and biocompatibility for regeneration of corneal endothelium.[19,20] On the basis of reproduction of damaged corneal endothelium to *in vitro* studies, we conducted to evaluate the effect of different sericin contents to inflammation and growth of corneal endothelial cells.[21]

Experimental

Materials. Four types of silk fibroin from silkworm cocoons (Kyebong Farm, Korea) were used: Different sericin contents on degumming process, boiling time was varied with 20, 40 and 60 min. The cocoons were cut into small pieces and boiled in 2 L of distilled water containing 0.02 M of Na_2CO_3 (Showa Chemical, Japan) to degumming treatments. 7 g of cocoons were heated in a hot water bath for 20, 40 and 60 min, respectively. Raw silk fibers were referred to as 0 min. Afterward hot water treated fibers were washed with cold distilled water and dried at 60 ºC for 24 h. Dried silk fibroin was dissolved in 9.3 M LiBr (Kanto Chem., Japan) at 60 ºC for 3 h. To remove residual LiBr, the solution was dialyzed using dialysis tube (SnakeSkin® Dialysis Tubing 3500 MWCO, Thermo Science, USA) during 62 h. After dialyzation, the solution was lyophilized by freeze drying at -60 ºC, 5 mTorr for 5 days. After degumming, silk films were prepared using solvent evaporation method. The 100 mg of silk was dissolved in 3 mL of hexafluoroisopropanol (HFIP, Sigma, USA) at 60 ºC for 3 h. After all of the solution was poured into a glass dish (50 mm in diameter), dried films were immersed in 99.5% methanol (Samchun chemical, Korea) to make a semi-crystallized water-insoluble film at room temperature for 1 h.[22]

Cell Culture. This study used NIH/3T3 (mouse embryo fibroblast cell line, KCLB21658) (Korean Cell Line Bank, Korea). The NIH/3T3 cells were cultured in RPMI 1640 (Rosewell Park Memorial Institute Medium) containing 10% fetal bovine serum (FBS, Gibco, USA), and 1% antibiotics PS (100 units/mL Penicillin and 100 μg/mL streptomycin).[23] The RAW 264.7 cells (Mouse leukaemic monocyte macrophage cell line, KCLB40071) (Korean Cell Line Bank, Korea) were cultured in DMEM (Dulbecco's Modified Eagle Medium, High Glucose, Gibco) containing 10% fetal bovine serum (FBS, Gibco, USA), and 1% antibiotics (100 units/mL Penicillin and 100 μg/mL streptomycin).[24] Corneal endothelial cells (rCEnCs) were collected from a New Zealand white rabbit (4 weeks old female, Hanil LabAnimal Co., Seoul, Korea). Rabbit eyeballs were extracted, then washed with PBS 1X containing 1.5% PS. After gaining cut cornea from eyeballs, Descemet's membrane (DM) with corneal endothelium was digested with 0.3% collagenase type A (Roche) for 1 day. Then, cells were cultured in EGM-2 (Endothelial Cell Growth Medium, Lonza, USA) with 10% FBS containing 1% PS.[25] The medium was changed every 3 days and all cells were cultured in an incubator at 37 ºC with 5% CO_2 conditions.

Scanning Electron Microscopy (SEM). In order to observe the morphology of silk fibers and surface of films with various contents of sericin, scanning electron microscopy measurement (SEM; Hitachi Co. Model S-2250N, Japan) was carried out. Films were gold coated using plasma sputter (Emscope, SC500L, UK) under argon gas.

Optical Transparency Test. Transparency of silk fibroin films was evaluated by visual observation and spectrum analysis using a SYNERGY Mx spectrophotometer (BioTek®, USA) at a wavelength range from 380 to 780 nm. Samples of transparency were prepared without/with cells on films and measured.[26]

Fourier Transformed Infrared Spectroscopy (FTIR). The physicochemical characterization of silk films was evaluated by Fourier transformed infrared spectroscopy (Perkin Elmer, USA). The samples were analyzed in the spectra range of wave numbers from 500 to 4000 cm^{-1}.

Contact Angle Measurement. To evaluate wettability of fabricated silk films, contact angle expressed from 2 μL water droplet volume on random area of specimens was measured by

contact angle goniometer (CAM-PLUS, ChemInstrument, USA) at 0, 1, 2, 3, 4 and 5 min. Average of measured value was used.

Measurement of Cell Viability. Viability were observed by MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide, Sigma) assay. The cells were seeded on 24-well plate at a density of 1×10^4 cells/plate. 1, 3, and 5 days after the medium of all samples were removed and 100 μL of MTT solution was added to samples for 4 h at 37 °C, 5% of CO_2. The samples were dissolved in 1 mL of dimethylsulfoxide (DMSO, Sigma) until the violet-color crystal was totally dissolved in solution. The solution was pipetted into a 96-well plate and absorbance was measured using an ELISA reader (Emax, Molecular Device, USA) in the wavelength at 570 nm to yield absorbance as a function of viable cell number.[27]

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. After seeding of RAW 264.7 cells in 24-well plates with a density of 1×10^4 cells/plate, the mRNA such as GAPDH, TNF-α, COX-2, IL-1β and IL-6 were confirmed in order to determine the inflammatory cytokines.[28] Then the culture medium was removed and total RNA was extracted from the cells using 500 μL of RNAiso Plus (Takara, Japan) and chloroform (1/5 of RNAiso Plus). After centrifugation at 4 °C, 12000 rpm for 15 min, the supernatants were precipitated with iso-propanol (Sigma) and 5 μL of Polyacryl Carrier™ (Molecular Res Center, USA). Each primer was extended using TOPscript™ One-step RT PCR DryMIX (Enzynomics, Korea). All PCR products were separated via electrophoresis on 1% (w/v) agarose gel containing EtBr (Ethidium Bromide, Sigma) and visualized under UV light (Vilber Lourmat ETX-20.M, France) at 360 nm.[29] And to confirm the phenotype maintenance of rCEnCs from a genetic perspective, RT-PCR conducted based on previous steps. The primers related rCEnCs (β-actin, Aquaporin-1, Na^+/K^+ ATPase and VDAC 3) were purchased from Genotec (Korea).

Proliferation Assay of Corneal Endothelial Cells. To study how well cells grow on films, cultured rabbit CEnCs were seeded on fabricated each silk films with a density of 2×10^4 cells/plate. To evaluate the initial attachment, the samples were fixed with 10% neutral formalin for 20 min after 30 min of seeding. And cells stained with DAPI (Santa Cruz Biotechnology, USA). Images were obtained with a fluorescence microscope and nuclear number was counted (cells/mm^2) using Image J program. At 1, 3, and 5 days after seeding, cell proliferation was tested by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma, USA).[30]

Statistical Analysis. Statistical analysis was carried out using the student's t-test. Differences were considered significant when $p^*<0.05$, $p^{**}<0.005$ and $p^{***}<0.001$.

Results and Discussion

SEM Observation. We observe a change in the material was given a difference in degumming time, using such a fiber to manufacture the film. Different surface morphologies of the raw silk fibers and defummed silk fibers according to different degumming time was observed by SEM. The silk is comprised of fibroin and sericin. The formation is that the fibroin strands were surrounded by many particles of sericin irregularly.[31] The SEM image in Figure 1 is architecture of the silk fibers after degumming, and surface of silk films. The rough surface of raw silk fiber (0 min) appears as large amount of sericin and impurity. The surface of fibers degummed for 20 min shows no sign of destruction, impurity and damage on the fiber surfaces. The surface of fibers degummed for 40 min are almost removed the sericin, and the fibroins are split away from the main fiber axis. The most serious damages were observed at degummed for 60 min, which of silk fibroin strands are more split off. Most films have a smooth surface but rough surface and non-uniform porosity were also observed in each 0 min and 60 min.

Optical Transparency Test. We prepared transparent silk

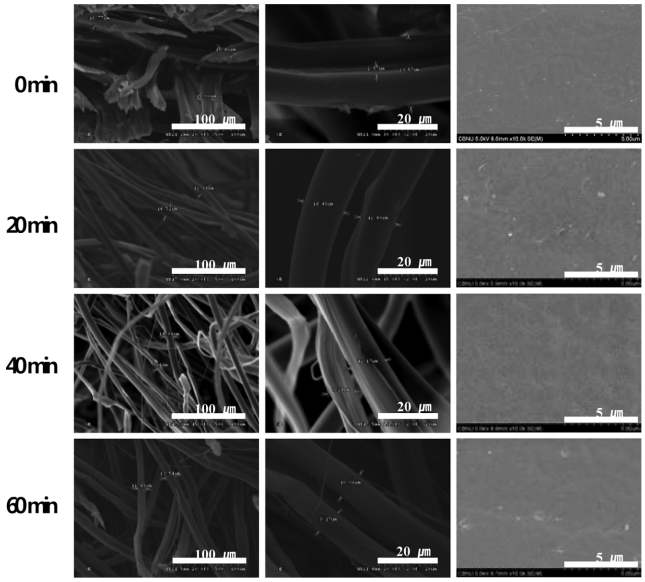

Figure 1. SEM images of silk fibers and surfaces of silk films degummed for 0 (Raw silk fiber), 20, 40 and 60 min (magnification is X500, X1000 and X10000).

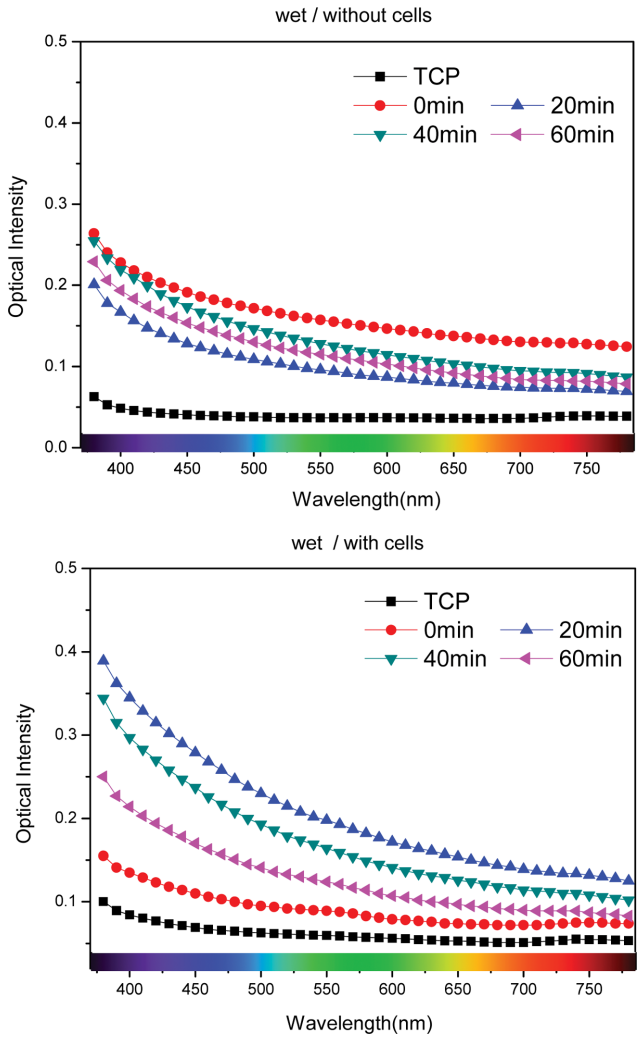

Figure 2. Optical transparency graphs of silk films in the visible range.

films as seen letters through the film. To evaluate the transparency of the film to be used in implants, we compared the turbidity between films with/without cells (Figure 2). The transparency of films without cells degummed for 0 min was reduced due to diffraction and scattering of light by impurities. Generally transparency of films cultured rCEnCs was lower than films without rCEnCs. Unpurified silk film (0 min) was higher than other experimental groups in the graph of transparency with cells. Transparency reduction of all experimental group except the unpurified film was caused by scattering of light when passing through the cells.[32]

Fourier Transformed Infrared Spectroscopy (FTIR). Using the FTIR for analysis of structural changes by different sericin contents was observed (Figure 3). The conformation of silk protein was analyzed using the infrared spectral absorption

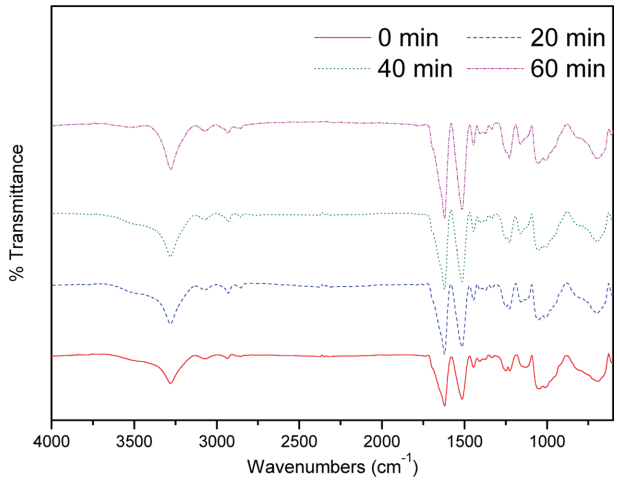

Figure 3. Effect of degumming time on the macromolecular conformation of the silk films.

by the peptide backbones of amide I (1630~1650 cm^{-1}), amide II (1520~1540 cm^{-1}) and amide III (1230~1270 cm^{-1}). Silk films were used in the experiment were not soluble in water, because of the transition to the β-sheet structure induced by methanol treatment.

Therefore the bands of characteristic amide I and amide II peaks in unpurified silk film (0 min) may be attributed to the characteristic β-sheet structure. The spectra of degummed silk films showed the characteristic C=O stretching for amide I, secondary N-H bending and C-N stretching for amide III. The IR spectra indicated that degummed silk films predominantly exists in silk I conformational state.[33]

Contact Angle Measurement. To evaluate the difference in hydrophilicity, contact angle was measured. The hydrophilicity of the films will affect to the rate of the initial adhesion of cells, and cell growth will be increased resulting in the increase of initial rate. In general, the greater the angle between the sample and the droplet indicates hydrophobic, and the smaller the angle indicates hydrophilicity.[34] The contact angle measured for unpurified silk film (0 min) and degummed films (20, 40 and 60 min) was indicated that the random coiled conformation of degummed silk films made it more hydrophilic as compared to unpurified silk film (Figure 4).

Measurement of Cell Viability. Cell viability of silk films containing different content of sericin was evaluated by the MTT assay in Figure 5.

After NIH/3T3 cells seeding on the film, the cell viability in all groups showed a little difference, and statistically significant survival with TCP of positive control on day 3. The cell

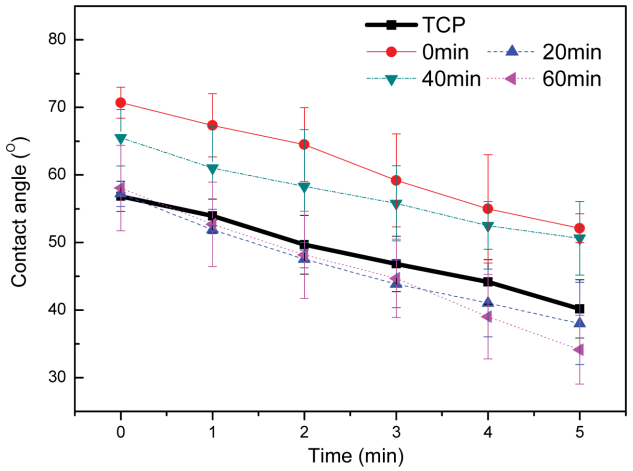

Figure 4. Contact angle of water deposited on the surface of silk films.

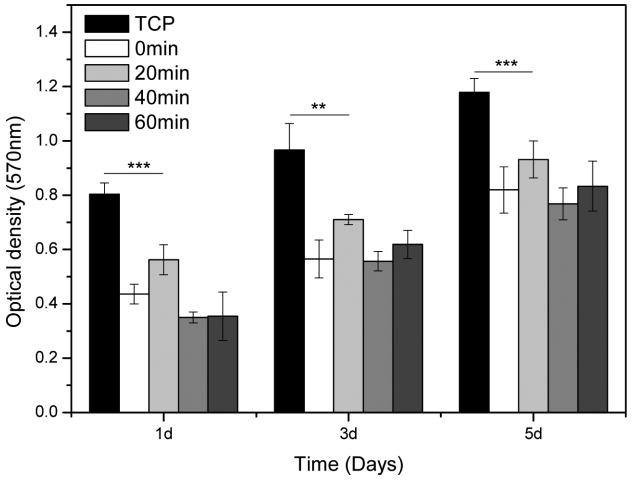

Figure 5. Cell viability of NIH/3T3 fibroblast cells in silk films (*p <0.05, **p<0.005 and ***p<0.001).

proliferation of the film degummed for 20 min was higher than other films in experimental groups. Because the possession of a large amount of sericin may lead to inflammatory responses, also impurity on the film surface will interfere proliferation of NIH/3T3 cells. In the case of all sericin and impurity have been removed hampered cell growth due to degradation products of the rapid degradation.

Expression of mRNA Related Inflammatory Responses. RT-PCR was used to confirm the inflammatory cytokines of silk films containing different content of sericin.

Figure 6 illustrates that the level of gene expression related inflammatory responses after seeding of RAW 264.7 cells. We evaluated the level of TNF-α, COX-2, IL-1β and IL-6 mRNA expression and standardized with GAPDH as the standard of

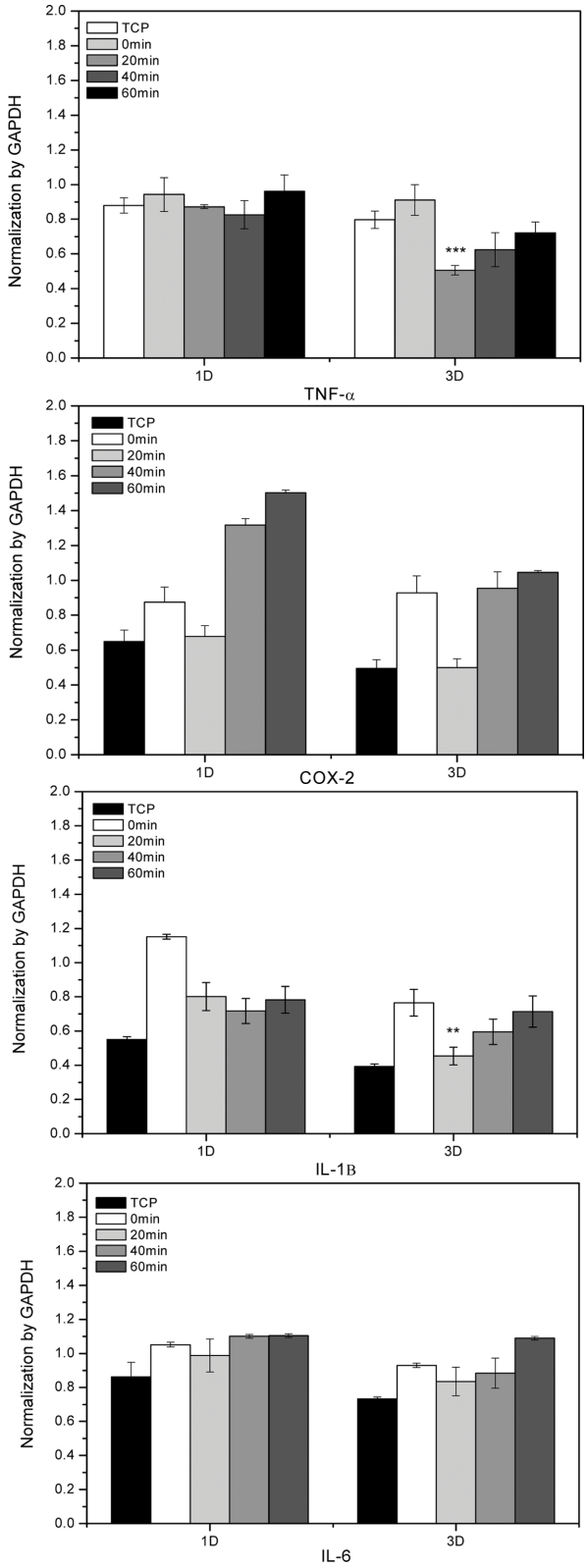

Figure 6. Expression of specific mRNA related inflammatory responses in RAW 264.7 cells (*p<0.05, **p<0.005 and ***p <0.001).

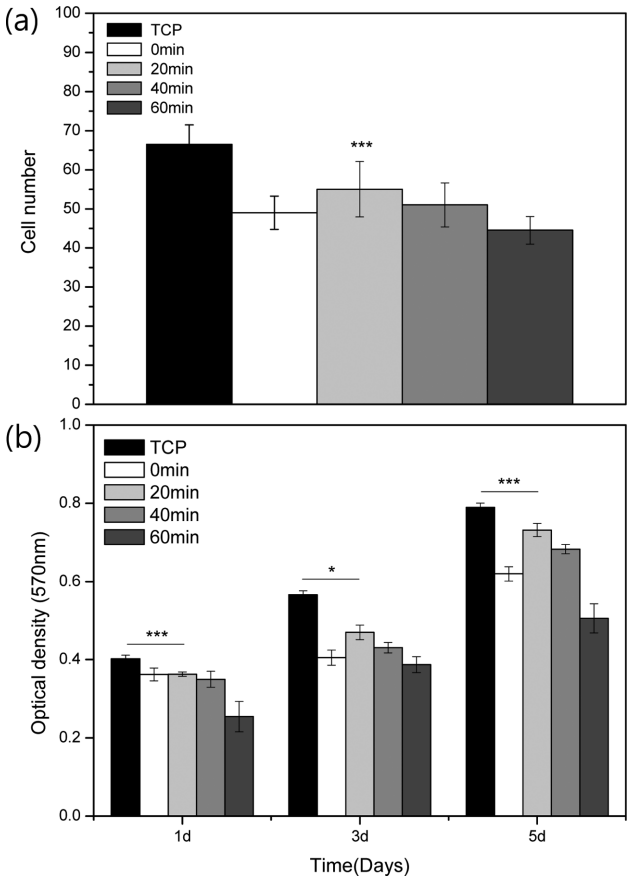

Figure 7. (a) Initial attachment of rCEnCs on silk films; (b) proliferation measurement of rCEnCs using the MTT assay (*p<0.05, **p<0.005 and ***p<0.001).

the inflammatory cytokines. The results shown that the mRNA expression level decreased markedly in degummed films (20 and 40 min) on day 3 compared to 1 day.

Proliferation Assay of Corneal Endothelial Cells. The interaction between cells and surface of the scaffold was important for the initial adhesion of cells on substrate.

30 min after seeding, initial attached cells on films are counted through DAPI staining. The results showed in Figure 7(a). The number of cells was similar in all groups, and higher in films (20 min) following the TCP as a positive control group. We seeded the cells in each group, and were evaluated the proliferation of rCEnCs by MTT assay on day 1, 3 and 5 in Figure 7(b). Over time, cells are cultured in the TCP showed the highest growth. Proliferation of rCEnCs in cultured films degummed for 20 min has to be similar to that seen in cultured TCP. As a result, the film degummed for 20 min has a good condition in the cell growth. The impurities of unpurified film (0 min) and decomposition products of the film (60 min) act as a factor to hinder cell growth.[35,36]

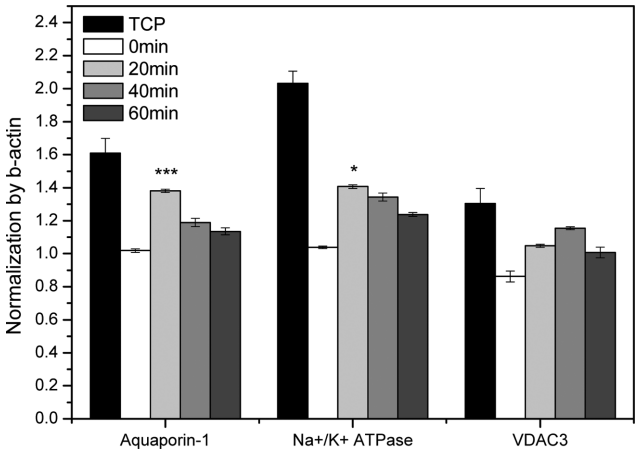

Figure 8. Expression of specific mRNA related the cellular metabolism of rCEnCs (*p<0.05, **p<0.005 and ***p<0.001).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. We have carried out the RT-PCR to determine the effect on the cells of the features and functions according to the content of sericin. Figure 8 shows the normalized expression level using the rCEnCs related primer (Aquaporin-1, Na^+/K^+-ATPase and VDAC 3).

Aquaporin-1 plays an important role in fluid transport which water can flow more rapidly inside the cell.[37] Na+/K+-ATPase has an effect on permeability and transparency through pump function, and VDAC 3 (Voltage-dependent anion-selective channel protein 3) plays a role in apoptotic signaling, and controls mitochondrial permeability.[38] Statistically, mRNA expression on degummed silk films was higher than that of unpurified silk film (0 min).

Conclusions

We hypothesized that the biomaterial structure, and the biochemical composition of silk films with various contents of sericin might be responsible for the attachment and growth of cells. Silk degumming process removes the sericin and some impurities from silk fiber. All the impurities are removed, and damages on the surfaces cannot be found in 20 min degumming from the measurement of surface morphology by SEM analysis. According to the results of contact angle and the initial attachment of cells, impurities and sericin of unpurified silk film (0 min) did not help in the attachment and growth of cells on films. The results of *in vitro* study show that degummed silk films except for 60 min obtain the high cell viability and cel-

lular metabolism. Growth of rCEnCs on degummed (20 and 40 min) silk films was not interfering by the impurities seen in the unpurified film and degradation products seen in the degummed film for 60 min, because proper degumming pre-processing of silk prevents the rapid biodegradation of film. Therefore, degummed for 20 min silk film has a positive effect on maintenance of rCEnCs function than unpurified silk film, and suggests that cell maintain a healthier state.

Acknowledgments: This research was supported by Brain Korea 21 PLUS Project, the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MEST) (NRF-2012M3A9C6050204).

References

1. Z. Shao and Vollrath, *Nature*, **418**, 741 (2002).
2. Y. Y. Ha, Y. W. Park, H. Y. Kweon, Y. Y. Jo, and S. G. Kim, *Macromol. Res.*, **22**, 9 (2014).
3. B. Panilaitis, G. H. Altman, J. S. Chen, H. J. Jin, V. Karageorgiou, and D. L. Kaplan, *Biomaterials*, **24**, 3079 (2003).
4. H. L. Kim, H. Yoo, H. J. Park, Y. G. Kim, D. Lee, Y. S. Kang, and G. Khang, *Polym. Korea*, **35**, 7 (2010).
5. G. H. Altman, F. Diaz, C. Jakuba, T. Calabro, R. L. Horan, J. Chen, H. Lu, J. Richmond, and D. L. Kaplan, *Biomaterials*, **24**, 401 (2003).
6. M. K. Kim and K. H. Lee, *Polym. Korea*, **38**, 1 (2014).
7. L. Meinel, S. Hofmann, V. Karageorgiou, C. K. Head, J. McCool, G. Gronowicz, L. Zichner, R. Langer, G. V. Novakovic, and D. Kaplan, *Biomaterials*, **26**, 147 (2005).
8. H. Oh, M. K. Kim, and K. H. Lee, *Macromol. Res.*, **19**, 3 (2011).
9. L. Uebersax, M. Mattotti, M. Papaloizos, P. M. Hans, and B. Gander, *Biomaterials*, **28**, 4449 (2007).
10. E. P. X. Hu, V. Finley, C. Kue, and D. Kaplan, *Macromol. Biosci.*, **13**, 3 (2013).
11. M. Lee, Y. G. Ko, J. B. Lee, W. H. Park, D. Cho, and O. H. Kwon, *Macromol. Res.*, **22**, 7 (2014).
12. N. C. Joyce, *Prog. Retin. Eye Res.*, **22**, 359 (2003).
13. J. Slingsby and S. Forstot, *Arch. Ophthalmol*., **99**, 1041 (1981).
14. M. Terry and P. Ousley, *Ophthalmology*, **110**, 755 (2003).
15. W. Wencan, Y. Mao, Y. Wentao, L. Fan, Q. Jia, W. Qinmei, and Z. Xianqtian, *Curr. Eye Res.*, **32**, 199 (2007).
16. N. Koizumi, Y. Sakamoto, N. Okumura, N. Okagara, H. Tsuchiya, R. Torii, L. Cooper, U. Ban, H. Tanioka, and S. Kinoshita, *Invest. Iphthalmol. Vis. Sci.*, **48**, 4519 (2007).
17. F. Price and M. Price, *J. Cataract. Refract. Surg.*, **32**, 411 (2006).
18. P. Madden, J. Lai, K. George, T. Giovenco, D. Harkin, and T. Chirila, *Biomaterials*, **32**, 4076 (2011).
19. C. Vepari and D. L. Kaplan, *Prog. Polym. Sci.*, **32**, 991 (2007).
20. N. Minoura, M. Tsukada, and M. Nagura, *Biomaterials*, **11**, 430 (1990).
21. T. Wang, I. Wang, S. Chen, Y. Chen, and T. Young, *Colloid Surf. B: Biointerfaces*, **90**, 236 (2012).
22. E. Y. Kim, C. H. Lee, S. Y. Kim, N. K. Jang, J. E. Song, C. K. Joo, and G. Khang, *Int. J. Tissue Reg.*, **5**, 3 (2014).
23. S. E. Lee, S. R. Cha, N. K. Jang, S. M. Kim, E. Y. Kim, J. E. Song, C. H. Park, and G. Khang, *Int. J. Tissue Reg.*, **5**, 103 (2014).
24. Y. Lee, J. Kwon, G. Khang, and D. Lee, *Tissue Eng. Part A*, **18**, 19 (2012).
25. J. H. Lee, E. Y. Kim, C. J. Lee, C. K. Joo, and G. Khang, *Int. J. Tissue Reg.*, **4**, 2 (2013).
26. H. Yoon, E. Y. Kim, H. Kim, C. H. Park, C. K. Joo, and G. Khang, *Macromol. Res.*, **22**, 3 (2014).
27. S. A. Cho, S. R. Cha, C. M. Kim, E. Y. Kim, J. E. Song, J. Gao, B. Min, and G. Khang, *Int. J. Tissue Reg.*, **5**, 46 (2014).
28. Y. J. Lee, J. Kwon, G. Khang, and D. Lee, *Tissue Eng. Part A*, **18**, 1967 (2012).
29. S. J. Lee, H. Y. Kim, S. J. Kim, J. Y. Yang, S. U. Lee, C. H. Park, C. K. Joo, and G. Khang, *Polym. Korea*, **38**, 3 (2014).
30. E. Y. Kim, N. Tripathy, J. Y. Park, S. E. Lee, C. K. Joo, and G. Khang, *Macromol. Res.*, **23**, 2 (2015).
31. M. Ho, H. Wang, and K. Lau, *Appl. Surf. Sci.*, **258**, 3948 (2012).
32. B. D. Lawrence, M. Cronin-Golomb, I. Georgakoudi, D. L. Kaplan, and F. G. Omenetto, *Biomacromolecules*, **9**, 1214 (2008).
33. M. Bhattacharjee, E. S. Thater, E. Trella, S. Das, M. Loparic, A. R. Ray, I. M. Artin, G. C. Spaqnoli, and S. Ghosh, *Biomaterials*, **34**, 8161 (2013).
34. S. W. Park and I. K. Kwon, *Int. J. Tissue Reg.*, **6**, 9 (2015).
35. T. Arai, G. Freddi, R. Innocenti, and M. Tsukada, *J. Appl. Polym. Sci.*, **91**, 2383 (2004).
36. Y. Wang, D. D. Rudym, A. Walsh, L. Abrahamsen, H. J. Kim, H. S. Kim, C. Kriker-Head, and D. L. Kaplan, *Biomaterials*, **29**, 3415 (2008).
37. P. Barfort and D. Maurice, *Exp. Eye Res.*, **19**, 11 (1974).
38. S. Hatou, M. Yamada, H. Mochizuki, A. Shiraishi, T. Joko, and T. Nishida, *Curr. Eye Res.*, **34**, 347 (2009).