**Favorable effects of superficially deacetylated chitin nanofibrils on the wound
healing process**

*Ryotaro Izumi^{a1}, Shuntaro Komada^{b1}, Kosuke Ochi^{b1}, Lisa Karasawa^b, Tomohiro Osaki^b,
Yusuke Murahata^b, Takeshi Tsuka^b, Tomohiro Imagawa^b, Norihiko Itoh^b, Yoshiharu
Okamoto^b, Hironori Izawa^a, Minoru Morimoto^c, Hiroyuki Saimoto^a, Kazuo
Azuma^{b*}, Shinsuke Ifuku^{a*}*

a: Graduate School of Engineering, Tottori University, Tottori 680-8533, Japan

b: Department of Veterinary Clinical Medicine, Tottori University, Tottori 680-8533,
Japan

c: Division of Instrumental Analysis, Research Center for Bioscience and Technology,
Tottori University, Tottori 680-8550, Japan

ABSTRACT. Previous reports indicate that the beneficial effect of chitin nanofibrils (CNFs), and chitosan nanofibrils (CSNFs) for wound healing. In this study, the wound healing effects of superficially deacetylated chitin nanofibrils (SDACNFs) were evaluated using an experimental model. In the experiments using circular excision wound model, SDACNFs induced re-epithelium and proliferation of the fibroblasts and collagen tissue. In the chitin, CNFs, and CSNFs, on the other hand, the re-epithelium and proliferation of the fibroblasts and collagen tissue were not induced perfectly compared with the SDACNFs group. In particular, re-epithelization was observed on day 4 in the only SDACNF group. Moreover, SDACNFs did not induce severe systemic inflammation in the linear incision wound model. The data indicated that SDACNFs effectively induced the proliferation and re-modeling phases compared with chitin, CNFs, and CSNFs in the wound. These data indicate that SDACNFs can be beneficial as a new biomaterial for wound healing.

KEYWORDS. superficially deacetylated chitin nanofibrils, chitin nanofibrils, wound healing, re-epithelium, biomaterials

1. Introduction

Chitin is widely distributed in nature and is the second most abundant polysaccharide after cellulose (Muzzarelli, 2011). Chitin occurs in nature as ordered macrofibrils and is the major structural component in the exoskeleton of crab and shrimp shells as well as the cell walls of fungi and yeast (Muzzarelli, 2011; Azuma, Ifuku, Osaki, Okamoto & Minami, 2014). Recently, several methods of preparing chitin or chitosan nanofibrils have been reported (Ifuku & Saimoto, 2012; Ifuku, 2014a). Ifuku et al. (2009) reported the preparation method of chitin nanofibrils (CNFs) by a simple grinding treatment under an acidic condition. **superficially deacetylated chitin nanofibrils** (SDACNFs) have also been reported (Fan, Saito & Isogai, 2010). Although the nanofibrils surface in this study was transformed into chitosan by deacetylation, the core was maintained as chitin crystals. More recently, Dutta et al. (2013b) established a method of preparing chitosan nanofibrils (CSNFs) by modifying the methods of Ifuku et al.. These nanofibrils are considered to have great potential for various applications because they have several useful properties, such as high specific surface area and high porosity.

In fact, various bioactivities of CNFs and SDACNFs were reported. CNFs suppressed clinical symptoms and colon inflammation in an experimental colitis model (Azuma et al., 2012a; Azuma et al., 2012b; Azuma et al., 2013). SDACNFs suppressed increases in body weight and serum leptin level in a model of obesity induced by high fat diets (Azuma, Ifuku, Osaki, Okamoto, & Minami, 2014). In addition, Ito et al. reported the protective effects of CNFs on skin using a 3D skin culture model with Franz cells (Ito

et al. 2014). It was indicated that the application of CNFs to skin improved the epithelial granular layer and increased granular density.

Previously, many reports have indicated that chitin and its derivatives are beneficial for wound healing (Muzzarelli, R.A.A., 2009; Jayakumar, Prabakaran, Sudheesh Kumar, Nair & Tamura, 2011; Minami, Okamoto, Saimoto, & Shigemasa, 2014). More recently, the beneficial effects of CNFs and CSNFs for wound healing were also described (Muzzarelli, R.A.A. 2012; Ding, Deng, Du, Shi, & Wang, 2014). However, there have been no reports that demonstrated the effects of SDACNFs on wound healing. The aim of this study is to evaluate the effects of SDACNFs on wound healing using an experimental model. Moreover, the differences in the wound healing effects among CNFs, SDACNFs, and CSNFs are discussed.

2. Experimental

2.1. Materials

Chitin powder from crab shells was purchased from Koyo Chemical (Tokyo, Japan). Chitosan powder was purchased from Tokyo Chemical Industry (Tokyo, Japan) and washed with water by filtration prior to use. Sodium hydroxide and acetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as received.

2.2. Preparation of CNFs, SDACNFs, and CSNFs

CNFs were prepared by following a previously reported procedure (Ifuku et al., 2009). Chitin (2.0 g) was dispersed in 200 mL of distilled water. The dispersion was roughly crushed with a grinder (MKCA6-3; Masuko Sanyo Co., Ltd.) at 1500 rpm. The crushed dispersion was passed through the Star Burst system (Star Burst Mini, HJP-25001S, Sugino Machine Co., Ltd.) equipped with a ball-collision chamber. The slurry was ejected from a small nozzle with a diameter of 100 μ m under a high pressure of 200 MPa and collided with a ceramic ball with a diameter of 12.7 mm. The suspension was passed through 30 mechanical treatments. Commercial chitin powder and thus obtained nanofibers have small portion of amino groups at the C2 position with approximately 5% degree of deacetylation. The average width of the nanofiber calculated from SPM images was 6.0 nm (Dutta et al., 2013a).

SDACNFs were prepared by following a previously reported procedure with some modifications (Fan, Saito, & Isogai, 2010). Chitin powder (30.0 g) was treated with 20% (w/w) NaOH (3.0 L) for 20 h under reflux and an argon atmosphere. After deacetylation, the supernatant was removed by decantation, and the precipitate was thoroughly washed with distilled water and 0.5 wt% aqueous acetic acid by centrifugation to remove the water-soluble products. For mechanical disintegration, the deacetylated chitin was dispersed in 4.0 L of distilled water. The dispersion was disintegrated into nanofibers using a grinder and the Star Burst system in the same manner as described above. The degree of deacetylation of the SDACNFs was 20% (Ifuku et al., 2014b).

CSNFs were prepared by following a previously reported procedure (Dutta et al., 2013b). Purified chitosan powder (Mw: 420,000, degree of deacetylation: 78%) was dispersed in distilled water at 1 wt%. The dispersion was roughly crushed with a grinder

(MKCA6-3; Masuko Sanyo Co., Ltd.) at 1500 rpm. The crushed dispersion was passed through the Star Burst system (Star Burst Mini, HJP-25001S, Sugino Machine Co., Ltd.) equipped with a ball-collision chamber. The slurry was ejected from a small nozzle with a diameter of 100 μ m under a high pressure of 245 MPa and collided with a ceramic ball with a diameter of 12.7 mm. The suspension was passed through 10 mechanical treatments. The average fiber diameter was 40.8 nm.

2.3. Animals

Wister rats (6-week-old, females) were purchased from CLEA Japan (Osaka, Japan). The animals were reared in a room with the temperature controlled at 22 ± 2 °C, humidity at $50 \pm 5\%$, with ventilation 11 times per hour and lighting set at 12:12-h light/dark cycle (light cycle, 7:00–19:00), and they were given standard chow (CE-2; Nihon Clea, Tokyo, Japan). The Animal Research Committee of Tottori University approved the care and use of the mice in this study.

2.4. Circular excision wound model

2.4.1. Study design of circular excision model

The circular excision wound model was prepared according to previous methods with slight modifications (Süntar, Baldemir, Coşkun, Keleş, & Akkol, 2011) (Figure 1). Briefly, rats were randomized into six groups: the non-treatment (NT) group, the diluted

water (water) group, the chitin group, the CNFs group, the SDACNFs group, and the CSNFs group (n = 10 in each group). The rats were anesthetized via inhalation of 2–3% isoflurane (Intervet Co. Ltd., Tokyo, Japan), and their back hairs were depilated by shaving. A circular wound was created on the dorsal region of each animal by excising the skin with an 8-mm biopsy punch (day 0; Kai Corporation and Kai Industries Co., Ltd., Tokyo, Japan). Then, full-thickness and diameter of 8 mm wounds were prepared for all animals. Samples or diluted water (0.05 mL) were applied topically on days 0, 2, 4, and 6. Animals in the non-treated (NT) group were not treated with any materials. On days 0, 4, and 8, the repair ratios of wound areas were monitored by cameras. Wound contraction ratios on days 4 and 8 were calculated as a percentage of the reduction in wound areas compared with that on day 0. On days 4 and 8, animals were killed by cervical dislocation via inhalation of 3–5% isoflurane (n = 4 in each group on day 4, and n = 6 on day 8). Skins around the wounds were harvested and subjected to histopathological examination.

2.4.2. Histopathology in the circular excision model

Samples were fixed in 10% buffered formalin, processed, and blocked with paraffin. Tissue sections (thickness, 4 µm) were prepared from each sample and stained with hematoxylin and eosin (H&E) for histopathological observation. Each section was examined microscopically and graded according to previous reports with slight modifications (Süntar, Baldemir, Coşkun, Keleş, & Akkol, 2011). Briefly, the sections were graded subjectively as none (-), mild (+), moderate (++), and severe (+++) for

epidermal or dermal re-modeling. Re-epithelization or ulcers in the epidermis, fibroblast proliferation, mononuclear and /or polymorphonuclear cells, neovascularization, and collagen deposition in the dermis were analyzed to score the epidermal or dermal re-modeling. At the end of the examination, the obtained results were combined and defined as wound healing phases, namely inflammation, proliferation, and re-modeling, in all groups.

2.4.3. Masson's trichrome staining

To determine the areas of collagen fibers in the dermis, Masson's trichrome (MT) staining was performed according to a previously reported method (Suzuki et al., 2011). Each section was examined microscopically.

2.5. Linear incision wound model

2.5.1. Study design of linear incision wound model

The linear incision wound model was also prepared according to previously described methods with slight modifications (Süntar, Baldemir, Coşkun, Keleş, & Akkol, 2011; Minagawa, Okamura, Shigemasa, Minami, & Okamoto, 2007). Rats were randomized into six groups: the non-treatment (NT) group, the diluted water (water) group, the chitin group, the CNFs group, the SDACNFs group, and the CSNFs group (n = 6 in each group). Rats were anesthetized via inhalation of 2–3% isoflurane, and their

back hairs were shaved using a shaving machine. A linear-paravertebral full-thickness skin incision of 4 cm in length was created with a sterile surgical blade (day 0). Samples or **water** (0.1 mL) were dropped into the incised wound. The incised wound was sutured by three surgical interrupted sutures placed 1 cm apart by using 4-0 surgical wire. Animals in the non-treated (NT) group were not treated with any materials. On day 7, blood collections were performed with inhalation of 2–3% isoflurane. Thereafter, rats were killed by cervical dislocation. Skin samples were taken and subjected to histopathological examination.

2.5.2. White blood cell count

Collected blood was immediately treated with ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. The number of white blood cells was counted using an auto-blood cell analyzer (Sysmex Co., Kobe, Japan).

2.5.3. Histopathological examination in linear incision wound model

Thin tissue sections (4 μ m) were prepared from each sample and stained with hematoxylin and eosin (H&E) for histological observation. Each section was examined microscopically. Five hundred cells in the vicinity of the buried agent were classified as either neutrophils, monocytes (including plasma cells and lymphocytes), or fibroblasts. The inflammatory statuses in the tissues were classified into three groups: acute inflammation, chronic inflammation, and fibrosis. Classifications were performed

according to a previously described method (Azuma et al., 2015) with slight modifications by the number of cells: 0 cells (-), 1–50 cells ($\pm$), 51–150 cells (+), 151–300 cells (++) , and over 301 cells (+++).

2.6. Statistical analysis

The data are expressed as mean $\pm$ standard deviation (S.D.). Statistical analyses were performed using Tukey-Kramer's test. A p-value of <0.05 was considered to be statistically significant.

3. Results and Discussions

Previously, some reports indicate the beneficial effects of the materials based on CNFs and CSNFs (Muzzarelli, Mehtedi, & Mattioli-Belmonte, 2014). For example, it is evaluated the efficacy, safety, and tolerability of the CNFs-derived, wound-healing technology among patients with venous leg ulcers (VLUs) (Talymed®, Marine Polymer Technologies Inc, Danvers, MA). The proportion of patients with completely healed VLUs was 45.0% (n = 9 of 20), 86.4% (n = 19 of 22), and 65.0% (n = 13 of 20) for groups receiving standard care plus Talymed® only once, every other week, and every 3 weeks, respectively, versus 45.0% (n = 9 of 20) for those receiving standard care alone after 20 weeks (Kelechi et al., 2012). Recently, chitosan-based electrospun nanofibrous materials have been developed. For example, the antibacterial activities against *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive)

were evaluated by the turbidity measurement method; and results suggest that the antibacterial effect of composite nanofibers varied on the type of bacteria. It was also indicated that the membranes were found to promote the cell attachment and proliferation murine fibroblast *in vitro* (Cai et al., 2011). To our knowledge, there is no report which describes the wound healing effects of SDACNFs. The results of the present study indicate SDACNF quickly and effectively induce both re-epithelization and proliferations of fibroblasts and collagen compared than chitin, CNFs and CSNFs.

The results of the gross findings on day 8 are shown in Figure 2, and the results of the histopathological assessments are shown in Figures 3 and 4. Re-epithelization under the scab was observed in only the SDACNF group on day 4 (Figure 3), whereas re-epithelization was not observed in the other groups on day 4. In the SDACNF group, complete re-epithelization was observed on day 8 (Figure 4). In the CNF group, adequate re-epithelization was also observed on day 8 (Figure 4). In the other groups, however, incomplete re-epithelization was observed. In the SDACNF group, fibroblast proliferation was well observed on day 8 and was superior to that of the other groups (Figure 4). Adequate collagen depositions were observed in the chitin, SDACNF, and CSNF groups on day 8 (Figure 5). Although many mononuclear cells were observed in the NT, vehicle, chitin, and CSNF groups, only a few mononuclear cells were observed in the CNF and SDACNF groups on day 8 (Figure 4). Wound healing is a specific biological process related to the general phenomenon of growth and tissue regeneration (Dai, Tanaka, Huang, & Hamblin, 2011). The wound healing process consists of a series of interdependent and overlapping stages in which a variety of cellular and matrix components act together to reestablish the integrity of damaged tissue and replacement

of lost tissue (Boateng, Matthews, & Stevens, & Eccleston, 2008). The wound healing process has been described as being comprised of 5 overlapping stages which involve complex biochemical and cellular processes. These stages are described as homeostasis, inflammation, migration, proliferation, and maturation (Beanes, Dang, Soo, & Ting, 2003). The results in this study indicate that SDACNF quickly progress the earlier stages of wound healing (homeostasis, inflammation and migration). And it is also indicated that SDACNF strongly induced the later stages of wound healing (proliferation, and maturation) than CNF, chitin and CSNF. So far, it is reported chitin and chitosan activate various cells which related wound healing, including vascular endothelium cells (Okamoto et al., 2002), lymphocytes (Nishimura, Nishi, & Tokura, 1985), and macrophages (Peluso et al., 1994). To the best of our knowledge, there is no precise study focusing on the effects of SDACNFs and CNFs for each cell. Further study must be performed for this point.

The results of MT staining on day 8 are shown in Figure 5. In the SDACNF group, many positive areas of MT staining were observed compared with those of the other groups on day 8. Following the histopathological examination, wound healing stages were summarized in each experimental group (Table 2). The wound healing process were consists of three phases, namely inflammation, proliferation, and re-modeling. As a defense mechanism of the tissue, the first response is inflammation, which provides resistance to microbial contaminations. However, a long duration in the inflammatory phase causes a delay in the healing process (Kondo, 2007). The results of the present study indicate that SDACNFs strongly induced proliferation and re-modeling phases than CNFs and CSNFs (Table 2). Especially, the sign of the proliferation was observed

in only the SDACNFs group on day 4. On the other hand, the remodeling phase was not observed in the other groups. In only the SDACNFs group, the complete regeneration of the epithelium was observed on day 8. In the CNF group, mild proliferation and re-modeling phases were observed on day 8. However, the regeneration of the epithelium was not completed (Figure 5). This result also indicates that SDACNFs quickly progress the inflammation phase and induced proliferation and re-modeling phases.

Previous reports have indicated that chitin and chitosan activated fibroblasts, which are related to the growth stage during wound healing (Okamoto et al., 2002; Mori et al., 1997; Howling et al., 2001). Moreover, it is reported chitin and chitosan induced the regeneration of epidermal keratinocytes, which is the last stage in the wound healing process (Howling et al., 2001). More recently, Ito et al. verified the effect of CNFs on skin using a three-dimensional skin culture model with Franz cells (Ito et al., 2014). The application of CNFs to skin improved the epithelial granular layer and increased granular density. It is reported that CNFs treatment causes increased expression of both α - and β -type defensins in endothelial cells and β -type defensins in keratinocytes (Linder et al., 2011). These observations indicate CNFs have beneficial effects for epidermal cells in wound healing process. Moreover, Erba, Adini, Demcheva, Valeri, & Orgill (2011) described that membranes consisting entirely of chitin nanofibers were applied to dorsal excisional wounds of db/db mice followed by application of a vacuum device. Results included significant expression of platelet-derived growth factor, transforming growth factor, epidermal growth factor, superior granulation tissue formation rich in collagen-I as well as epithelialization and wound contraction. In this study, we dropped small amount of materials for the wound. Thereby, the wound

healing effects of chitin was not enough. In the CNFs groups, enough regeneration of the epidermal was observed. However, the proliferation of fibroblast is inadequate. In CSNFs group, adequate proliferation of fibroblast was observed. But the regeneration of the epidermal was poor. In the SDACNFs group, on the other hand, well regeneration of epidermal and proliferation of fibroblast were observed. These results might be indicating SDACNFs promote wound healing with the efficiency.

In the experiment of the linear incision wound model, no changes in the number of white blood cells was observed. The only results presented as mean+standard deviation are the white blood cells on table 3. In the histopathological observation, mild acute and colonic inflammations were observed in the CNFs and SDACNF groups. On the other hand, moderate inflammation was observed in the chitin and CSNFs group. It is described the wound healing effects of chitin and chitosan, which are known as biocompatible, biodegradable, non-toxic, antimicrobial, and hydrating agents (Jayakumar, Prabakaran, Sudheesh Kumar, Nair, & Tamura, 2011). For example, we demonstrated that biological adhesions made from chitin derivatives and CNFs or SDACNFs induced inflammation locally but not systemically. However, cyanoacrylate induced strong local and systemic responses (Azuma et al., 2015). The present results indicate that CNFs and SDACNFs might be more compatible than chitin and CSNFs. If any material exhibits a combination of antioxidant, antimicrobial, and anti-inflammatory activities, it can be concluded that this material will promote wound healing and contribute to skin regeneration (Peşin Sutar, Kupeli Akkol, Yilmazer, Baykal, Alper, Kırmızıbekmez, Yeşilada, 2011). Antimicrobial activity is important during wound healing since wounds are exposed to an external environment and are more prone to

microbial attacks, usually resulting in a delay in the healing process. Lindner et al. (2011) reported that CNFs membranes promoted the expression of α - and β -defensins in endothelial cells and that of β -defensins in keratinocytes. The results also indicated that wounds infected with *S. aureus* and treated with CNFs membranes showed a significant reduction in gram-positive staining, enhancing wound healing while concomitantly limiting wound infection. Previously, the antimicrobial effects of SDACNFs were reported (Ifuku et al., 2013). Antioxidant materials provide wound healing effects via free radical scavenging activities and inhibition of lipid peroxidation, preventing cell damages (Getie Gebre, Mariam, Reitz, & Neubert, R 2002; Shetty, Udupa, & Udupa, 2008). We also reported the suppressive effects of CNFs on myeloperoxidase activation in inflammatory conditions of the colon (Azuma et al., 2012a). These evidences might indicate that SDACNFs or CNFs are efficient as new functional biomaterials for the wound healing process. However, the further study focusing on the antioxidant and antimicrobial activities of SDACNFs must be performed.

In conclusion, the data in the present study indicate that SDACNFs effectively induced the proliferation and re-modeling phases in wound healing. In particular, SDACNFs quickly promote re-epithelization and proliferation of fibroblast. Moreover, SDACNFs did not induce severe systemic inflammation. These results indicate that SDACNFs are beneficial as a new biomaterial for wound healing.

323

324 **Acknowledgements**

325 This work was financially supported by the Creating STart-ups from Advanced
326 Research and Technology (START) project of JST and KAKENHI (26708026) of JSPS.

327

328 **References**

329 Azuma, K., Ifuku, S., Osaki, T., Okamoto, Y., & Minami, S. (2014) Preparation and
330 Biomedical Applications of Chitin and Chitosan Nanofibers. *Journal of Biomedical*
331 *Nanotechnology*, *10*, 2891–2920.

332

333 Azuma, K., Nishihara, M., Shimizu, H., Itou, Y., Takashima, O., Osaki, T., Itoh, N.,
334 Imagawa, T., Murahata, Y., Tsuka, T., Izawa, H., Ifuku, S., Minami, S., Saimoto, H.,
335 Okamoto, Y., & Morimoto, M.. (2015) Biological adhesive based on carboxymethyl
336 chitin derivatives and chitin nanofibers. *Biomaterials*, *42*, 20-29.

337

338 Azuma, K., Osaki, T., Ifuku, S., Saimoto, H., Tsuka, T., Imagawa, T., Okamoto, Y., &
339 Minami, S. (2012b) α -Chitin nanofibrils improve inflammatory and fibrosis responses in
340 mice with inflammatory bowel disease. *Carbohydrate Polymers*. *90*, 197-200.

341

342 Azuma, K., Osaki, T., Ifuku, S., Saimoto, H., Tsuka, T., Imagawa, T., Okamoto, Y., &
343 Minami, S. (2013) A comparative study analysis of α -chitin and β -chitin nanofibrils by
344 using an inflammatory-bowel disease mouse model. *Journal of chitin and chitosan*
345 *science*, 1, 144-149.

346

347 Azuma, K., Osaki, T., Wakuda, T., Ifuku, S., Saimoto, H., Tsuka, T., Imagawa, T.,
348 Okamoto, Y., & Minami, S. (2012a) Beneficial and preventive effect of chitin nanofibrils
349 in a dextran sulfate sodium-induced acute ulcerative colitis model. *Carbohydrate*
350 *Polymers* 87, 1399-1403.

351

352 Cai, Z.X., Mo, X.M., Zhang, K.H., Fan, L.P., Yin, A.L., He, C.L., Wang, H.S. (2010).
353 Fabrication of chitosan/silk fibroin composite nanofibers for wound-dressing applications.
354 *International Journal of Molecular Science* 11, 3529-3539.

355

356

357 Ding, F.Y., Deng, H.B., Du, Y.M., Shi, X.W., Wang, Q. (2014).Emerging chitin and
358 chitosan nanofibrous materials for biomedical applications. *Nanoscale*, 6, 9477-9493;

359

360 Dutta, A. K, Izawa, H., Morimoto, M., Saimoto, H., & Ifuku, S. (2013a) Fiber width and
361 molecular weight studies of α -chitin nanofibers. *Journal of Chitin and Chitosan Science*,
362 1, 192-196.

363

364 Dutta, A.K., Kawamoto, N., Sugino, G., Izawa, H., Morimoto, M., Saimoto, H., & Ifuku,
365 S. (2013b). Simple preparation of chitosan nanofibers from dry chitosan powder by the
366 Star Burst system. *Carbohydrate Polymers*, 97, 363-367.

367

368 Erba, P., Adini, A., Demcheva, M., Valeri, C.R., & Orgill, D.P. (2011). Poly-N-acetyl
369 glucosamine fibers are synergistic with vacuum-assisted closure in augmenting the
370 healing response of diabetic mice. *The Journal of Trauma Injury Infection and Critical*
371 *Care*, 71, S187–S193.

372

373 Fan, Y., Saito, T., & Isogai, A. (2010) Individual chitin nano-whiskers prepared from
374 partially deacetylated α -chitin by fibril surface cationization. *Carbohydrate Polymers*, 79,
375 1046–1051.

376

377 Getie Gebre, M., Mariam, T., Reitz, R., & Neubert, R.H. (2002) Evaluation of the
378 release profiles of flavonoids from topical formulations of the crude extract of the leaves
379 of *Dodonea viscosa* (Sapindaceae). *Pharmazie*, **57**, 320–322.

380

381 Howling, G.I., Dettmar, P.W., Goddard, P.A., Hampson, F.C., Dornish, M., & Wood,
382 E.J. (2001) The effect of chitin and chitosan on the proliferation of human skin
383 fibroblasts and keratinocytes in vitro. *Biomaterials*, **22**, 2959–2966.

384

385 Ifuku S, & Saimoto H. (2012) Chitin nanofibers: preparations, modifications, and
386 applications. *Nanoscale*. **4**, 3308-3318.

387

388 Ifuku, S. (2014) Chitin and Chitosan Nanofibers: Preparation and Chemical
389 Modifications. *Molecules*. **19**, 18367-18380.

390

391 Ifuku, S., Nogi, M., Abe, K., Yoshioka, M., Morimoto, M., Saimoto, H., & Yano, H..
392 (2009). Preparation of chitin nanofibers with a uniform width as alpha-chitin from crab
393 shells. *Biomacromolecules*, **10**, 1584–1588.

394

395 Ifuku, S., Suzuki, N., Izawa, H., Morimoto, M., & Saimoto, H., (2014) Surface
396 phthaloylation of chitin nanofiber in aqueous media to improve dispersibility in aromatic
397 solvents and give thermo-responsive and ultraviolet protection properties. *RSC*
398 *Advances*, 4, 19246-19250.

399
400 Ito, I., Osaki, T., Ifuku, S., Saimoto, H., Takamori, Y., Kurozumi, S., Imagawa, T.,
401 Azuma, K., Tsuka, T., Okamoto, Y., & Minami, S. (2014) Evaluation of the effects of
402 chitin nanofibrils on skin function using skin models. *Carbohydrate Polymers*, 101, 464-
403 470.

404
405 Jayakumar, R., Prabakaran, M., Sudheesh Kumar, P.T., Nair, S.V., & Tamura, H.
406 (2011) Biomaterials based on chitin and chitosan in wound dressing applications.
407 *Biotechnology Advances*, 29, 322-337.

408
409
410 Kelechi, T.J., Mueller, M., Hankin, C.S., Bronstone, A., Samies, J., & Bonham, P.A.
411 (2012). A randomized, investigator-blinded, controlled pilot study to evaluate the safety
412 and efficacy of a poly-N-acetyl glucosamine-derived membrane material in patients with
413 venous leg ulcers. *Journal of the American Academy of Dermatology*, 66, e209-215.

414

415 Kondo, T. (2007) Timing of skin wounds. *Legal Medicine*, **9**, 109–114.

416

417 Lindner, H.B., Zhang, A., Eldridge, J., Demcheva, M., Tsihchlis, P., Seth, A., Vournakis,
418 J., & Muise-Helmericks, R.C. (2011). Anti-bacterial effects of poly-N-acetyl-
419 glucosamine nanofibers in cutaneous wound healing: requirement for Akt1. *PLoS One*.
420 **6**, e18996.

421

422 Minagawa, T., Okamura, Y., Shigemasa, Y., Minami, S., & Okamoto, Y. (2007) Effects
423 of molecular weight and deacetylation degree of chitin/chitosan on wound healing.
424 *Carbohydrate Polymers*, **67**, 640-644.

425

426 Minami, S., Okamoto, Y., Saimoto, H., & Shigemasa, Y. (2014) Effects of Chitin and Its
427 Derivatives on Wound-Healing Acceleration Mechanisms. *Journal of Chitin and*
428 *Chitosan Science*, **2**, 163-178.

429

430 Mori, T., Okumura, M., Matsuura, M., Ueno, K., Tokura, S., Okamoto, Y., Minami, S., &
431 Fijinaga, T. (1997) Effects of chitin and its derivatives on the proliferation and cytokine
432 production of fibroblasts in vitro. *Biomaterials*, **18**, 947–951.

433

434 Muzzarelli, R.A.A. (2009). Chitins and chitosans for the repair of wounded skin, 498
435 nerve, cartilage and bone. *Carbohydrate Polymers*, 76, 167-182

436

437 Muzzarelli, R. A. A. (2011). Chitin nanostructures in living organisms. In N. Gupta (Ed.),
438 *Chitin: formation and diagenesis* (pp. 1–34). Dordrecht: Springer.

439

440 Muzzarelli, R.A.A. (2012). Nanochitins and nanochitosans, paving the way to eco-
441 friendly and energy-saving exploitation of marine resources. *Polymer Science: A*
442 *Comprehensive Reference*, 10, 153-164.

443

444 Muzzarelli, R.A.A., Mehtedi, M.E., & Mattioli-Belmonte, M. (2014). Emerging Biomedical
445 Applications of Nano-Chitins and Nano-Chitosans Obtained via Advanced Eco-Friendly
446 Technologies from Marine Resources. *Marine Drugs*, 12, 5468-5502.

447

448 Nishimura, S., Nishi, N., & Tokura, S. (1985) Adhesion behaviour of murine
449 lymphocytes on the surface of fibrous chitin and its derivatives. *International Journal of*
450 *Biological Macromolecules*, 7, 100–104.

451

452 Okamoto, Y., Watanabe, M., Miyatake, K., Morimoto, M., Shigemasa, Y., & Minami, S.
453 (2002) Effects of chitin/chitosan and their oligomers/monomers on migrations of
454 fibroblasts and vascular endothelium. *Biomaterials*, **23**, 1975–1979.

455

456 Peluso, G., Petillo, O., Ranieri, M., Santin, M., Ambrosio, L., Calabró, D., *Avallone, B.*,
457 *& Balsamo, G.* (1994) Chitosan-mediated stimulation of macrophage function.
458 *Biomaterials*, **15**, 1215–1220.

459

460 Pesin Suntar, I., Kupeli Akkol, E., Yilmazer, D., Baykal, T., Alper, M., Kırmızıbekmez,
461 H., & Yesilada, E. (2010) Investigations on the in vivo wound healing potential of
462 Hypericum perforatum L. *Journal of Ethnopharmacology*, **127**, 468–477.

463

464 Shetty, S., Udupa, S., & Udupa, L. (2008) Evaluation of antioxidant and wound healing
465 effects of alcoholic and aqueous extract of Ocimum sanctum Linn in rats. *Evidence*
466 *Based Complementary and Alternative Medicine*, **5**, 95–101.

467

468 Süntar, I., Baldemir, A., Coşkun, M., Keleş, H., & Akkol, E.K. (2011) Wound healing
469 acceleration effect of endemic Ononis species growing in Turkey. *Journal of*
470 *Ethnopharmacology*, 135, 63-70.

471

472 Suzuki, K., Sun, X., Nagata, M., Kawase, T., Yamaguchi, H., Sukumaran, V., Kawauchi,
473 Y., Kawachi, H., Nishino, T., Watanabe, K., Yoneyama, H., & Asakura, H. (2011).
474 Analysis of intestinal fibrosis in chronic colitis in mice induced by dextran sulfate sodium.
475 *Pathology International*, 61, 228–238.

476

477

478

479

480

481

482

483

484

Figure legend

Figure 1. Schematic of the protocol for the circular excision wound model

Figure 2. Gross findings on days 8 in the circular excision wound model.

Data represent one rat in each group.

Figure 3. Histopathological changes on day 4 in the circular excision wound model.

All images are from one of four rats in each group on day 4. The arrows point to events during wound healing. S: scab, RE: re-epithelization, NV: neovascularization. Bar: 200 μm .

Figure 4. Histopathological changes on day 8 in the circular excision wound model.

All images are from one of four rats in each group on day 8. The arrows point to events during wound healing. S: scab, RE: re-epithelization, NV: neovascularization. Bar: 100 μm .

Figure 5. Masson's trichrome staining on day 8 in the circular excision wound model.

502 All images are from one of four rats in each group on day 8. The arrows point to events
503 during wound healing. f: fibroblast, c: collagen. Bar: 100 μ m.

504

Table 1. Wound healing processes on days 4 and 8 in the circular excision wound model

(a)

	S	U	RE	FP	CD	MNC	PMN	NV
NT	+++	+++	-	+/++	+	+++	-	++
Water	+++	+++	-	+/++	+	+++	-	+/++
Chitin	+++	+++	-	+/++	+/++	++	-	+/++
CNF	+++	+++	-	+/++	+/++	++	-	++
SDACNF	++/+++	++/+++	+	++	++	++	-	++
CSNF	+++	+++	-	++	+/++	+++	-	++

(b)

	S	U	RE	FP	CD	MNC	PMN	NV
NT	+	++	+/++	+	+/++	++	-	++/+++
Water	++	++	+	++	++	++	-	+/++
Chitin	++	++	+	++	++	++	-	+/++
CNF	++	-/+	++	++	++/+++	+	-	+
SDACNF	+	-	+++	+++	++	+	-	+
CSNF	++	+/++	+/++	++	++	++/+++	-	+/++

HE and MT stained sections on days 4 (a) and 8 (b) were scored as none (-), mild (+), moderate (++) and severe (+++) for epidermal and/or dermal re-modeling. S: scab, U: ulcers, RE: re-epithlization, FP: fibroblast proliferation, CD: collagen deposition, MNC: mononuclear cells, OMN: polymorphonuclear cells, NV: neovascularization.

Table 2. Wound healing phases on days 4 and 8 in the circular excision wound model

(a)

	I	P	R
NT	+++	+	-
Water	+++	+	-
Chitin	++/+++	++	-
CNF	++/+++	++	-
SDACNF	++	++	+
CSNF	++/+++	++	-

(b)

	I	P	R
NT	++	+	+
Water	++	++	+
Chitin	++	++	-/+
CNF	+	++	++
SDACNF	+	+++	+++
CSNF	++/+++	++/+++	+

HE and MT stained sections on days 4 (a) and 8 (b) were scored as none (-), mild (+), moderate (++) and severe (+++) for epidermal and/or dermal re-modeling. I: inflammation phase, P: proliferation phase, R: re-modeling phase

Table 3. The counts of white blood cells and histopathological findings in linear incision wound model

	WBC ($\times 10^2/\mu\text{L}$)	Histopathological changes		
		Acute inflammation	Colonic inflammation	Fibrosis
NT	120 $\pm$ 30	+	++	++
Water	100 $\pm$ 31	+	++	++
Chitin	90 $\pm$ 29	+	++	++
CNF	91 $\pm$ 19	+	+	++
SDACNF	91 $\pm$ 17	$\pm$	+	+++
CSNF	101 $\pm$ 30	+	++	+++

Data represent the mean $\pm$ standard deviation, n = 6 in each group (WBC). HE stained sections (n = 6 in each group) were scored as none (–), mild (+), moderate (++) and severe (+++).

Study design

Circular excision wound model

Dropping the sample

Figure 1

NT

Water

Chitin

CNF

SDACNF

CSNF

Figure 2

NT

Water

Chitin

CNF

SDACNF

CSNF

Figure 3

NT

Water

Chitin

CNF

SDACNF

CSNF

Figure 4

NT

Water

Chitin

CNF

SDACNF

CSNF

Figure 5