Enhancing Effects of Sericin on Corneal Wound Healing in Otsuka Long-Evans Tokushima Fatty Rats as a Model of Human Type 2 Diabetes

Noriaki NAGAI, Takatoshi MURAO,* Yoshimasa ITO,* Norio OKAMOTO,∗ and Masahiro SASAKI

*School of Pharmacy, Kinki University; †Pharmaceutical Research and Technology Institute, Kinki University; *Higashi-Osaka, Osaka 577–8502, Japan; †Department of Ophthalmology, Hyogo College of Medicine; Nishinomiya, Hyogo 663–8501, Japan; and “Technical Center, Seiren Co., Ltd.; Sakai, Fukui 913–0036, Japan.

Received April 13, 2009; accepted June 8, 2009; published online June 22, 2009

The protein sericin is the main constituent of silk. We investigated the effects of sericin on corneal wound healing in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model for human type 2 diabetes. Corneal wounds were prepared by removal of the corneal epithelium, and documented using a TRC-50X equipped with a digital camera. Sericin solutions were instilled into the eyes of rats five times a day following corneal abrasion. Plasma glucose and triglycerides were determined using an Accutrend GCT. Cholesterol and insulin were measured using a Cholesterol E-Test Kit and ELISA Insulin Kit, respectively. The plasma levels of glucose, triglycerides, cholesterol and insulin in 38-week-old OLETF rats were significantly higher than in Long-Evans Tokushima Otsuka (LETO) rats used as normal controls, and the rate of corneal wound healing in OLETF rats was slower than in LETO rats. The corneal wounds of rats instilled with saline showed almost complete healing by 72 h after corneal epithelial abrasion. On the other hand, the corneal healing rate of OLETF rats instilled with 10% sericin solution was significantly higher than that of LETO rats instilled with saline, and the wounds showed almost complete healing at 48 h after abrasion. The corneal healing rate increased with increasing sericin concentration. The present study demonstrates that the corneal wound healing rate in OLETF rat is slower than in LETO rats, and the instillation of sericin solution has a potent effect in promoting wound healing and wound-size reduction in LETO and OLETF rats.

Key words serum; cornea; type 2 diabetes mellitus; Otsuka Long-Evans Tokushima Fatty rat; corneal wound healing

Diabetes mellitus is a common metabolic disorder that affects more than 190 million people worldwide.1,2 The prevalence of type 2 diabetes mellitus is increasing rapidly, and currently affects the health of millions of humans now, and will continue to do so in the near future. Among the factors that are responsible for the increasing prevalence of this disease are obesity, the consumption of energy-dense diets and low levels of physical activity.3 Type 2 diabetes results from the failure of pancreatic beta cells to adequately compensate for obesity and insulin resistance.4

Ocular complications secondary to type 2 diabetes mellitus are a well-known cause of diabetic keratopathy.5 Diabetic keratopathy is an entity that includes slow healing or loose adhesion of the corneal epithelium after wounding in diabetic patients. Histologically, it involves a thickening of the corneal epithelial basement membrane and morphologic changes in the corneal epithelium and endothelium.6–11 Clinically, the damage to the corneal epithelium during vitreous surgery and retinal photocoagulation sometimes induces vision-threatening corneal complications, such as persistent epithelial defects in diabetic patients.12 It has been reported that such diabetic keratopathy is experienced by 50% or more of diabetic patients.13

The corneal wound repair process involves cell adhesion, migration, proliferation, matrix deposition and tissue remodeling.14 Many of these biological processes are mediated by growth factors, cytokines and other mediators released in injured tissues or cells.15 These growth factors have been recognized as important mediators of proper wound repair,16 and treatment with growth factors such as platelet-derived growth factor-BB, and recombinant human epidermal growth factor has been shown to be beneficial for patients with chronic pressure ulcers or non-healing diabetic ulcers.17–20 However, for reasons of effectiveness, safety and stable supply, a potent wound-healing agent for human corneal wounds has not yet been introduced, and the development of effective and safe corneal wound-healing drugs is highly anticipated.

Proteins such as fibroin and sericin are the main constituents of silk, with fibroin contributing 70 to 80% and sericin 20 to 30% of the total cocoon weight.21 When cocoons or raw silk are used for textiles, the sericin is mostly removed from the cocoon and disposed of unused. However, sericin has recently been investigated for its activities in biotechnological fields.22,23 We reported that sericin instillation has a potent effect in promoting wound healing and wound-size reduction in rats.24 Therefore, it is possible that sericin may be applied as eye drops for corneal wound repair in diabetic patients. In this study, we investigated the enhancing effects of sericin on corneal wound healing in the debrided corneal epithelium of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model for human type 2 diabetes.

MATERIALS AND METHODS

Animals and Reagents Male Long-Evans Tokushima Otsuka (LETO) rats as normal controls and OLETF rats aged 38 weeks were used in this study. The LETO and OLETF rats were obtained from the Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), housed under standard conditions (12 h/d fluorescent light (07:00—19:00), 25 °C room temperature) and allowed free access to a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water. All procedures were performed in accordance with the Kinki University School of Pharmacy Committee for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

© 2009 Pharmaceutical Society of Japan
Pure Sericin\textsuperscript{TM} (30 kDa) was obtained from Seiren Co., Ltd. (Fukui, Japan). All other chemicals used were of the highest purity commercially available.

**Measurement of Blood Parameters for Diabetes Mellitus** Blood was drawn from a tail vein of each rat at 9:00 a.m., and the plasma levels of glucose, triglycerides, and cholesterol were measured. Plasma glucose and triglycerides were determined by an Accutrend GCT (Roche Diagnostics, Mannheim, Germany). Plasma total cholesterol and high-density lipoprotein (HDL)-cholesterol were measured by the cholesterol oxidase method and the phosphtungstate-magnesium salt method using a Cholesterol E-Test Kit (Wako, Osaka, Japan) and HDL-Cholesterol E-Test Kit (Wako, Osaka, Japan), respectively. The plasma low-density lipoprotein (LDL)-cholesterol level was calculated by Eq. 1:

\[
\text{LDL-cholesterol} = \text{total cholesterol} - \text{HDL-cholesterol} - \text{triglycerides}/5 \tag{1}
\]

Insulin levels were measured using an ELISA Insulin Kit according to the manufacturer’s instructions (Morinaga Institute of Biological Science Inc., Kanagawa, Japan). Briefly, monoclonal antibodies specific for rat insulin were pre-coated onto microplates, standards and samples were pipetted into the wells, and the microplates were incubated at 4°C for 2 h. After washing to remove unbound materials, rat insulin antibodies were added to the wells at room temperature for 30 min. After washing, the substrates were added. The enzyme reactions yielded blue products that turned yellow when the stop solutions were added. The absorbance was measured with a microplate reader (BIO-RAD, California, U.S.A.) at 450 nm.

**Instillation of Sericin Solutions in LETO and OLETF Rats** The sericin solutions used in this study were prepared by adding Pure Sericin\textsuperscript{TM} to saline (pH 6.5—7.5) on the day of experiment. Fifty microliters of saline or sericin solution were instilled into the eyes of rats five times a day (9:00, 12:00, 15:00, 18:00, 21:00) after corneal abrasion. The eyes were kept open for about 1 min after instillation to prevent the sericin from being washed out.

**Image Analysis of Corneal Wound Healing in LETO and OLETF Rats** The debridement of corneal epithelium in LETO and OLETF rat were performed as described previously.\textsuperscript{24} LETO and OLETF rats were anesthetized with pentobarbital (30 mg/kg, i.p.), and a 3.5-mm-diameter circle was outlined in the center of the cornea with a disposable dermatological skin punch (BIOPSY PUNCH, Kai Industries Co., Ltd, Gifu, Japan). The encircled corneal epithelium was removed with a BD Micro-Sharp\textsuperscript{TM} (blade 3.5 mm, 30°, Becton Dickinson, Fukushima, Japan). The area of removed corneal epithelium was calculated by Eq. 2:

\[
\text{corneal wound healing} = \frac{(\text{wound area}_{0\text{h}} - \text{wound area}_{12-72\text{h}})}{\text{wound area}_{0\text{h}}} \times 100 \tag{2}
\]

The rate of corneal wound healing is represented by the corneal wound healing rate constant \(k_t\) (h\(^{-1}\)). The values of \(k_t\) over the period 0—72 h after corneal epithelial abrasion were calculated from the following Eq. 3:

\[
H_t = H_\infty \left(1 - e^{-k_t t}\right) \tag{3}
\]

where \(t\) is time (0—72 h) after corneal abrasion, and \(H_t\) and \(H_\infty\) are the percentages of corneal wound healing (%) at time \(\infty\) and \(t\), respectively.

**Statistical Analysis** All data are expressed as the means±standard errors (S.E.). Unpaired Student’s \(t\)-test was used to evaluate statistical differences, and multiple groups were evaluated by one-way analysis of variance followed by Dunnett’s multiple comparison. \(p\) values less than 0.05 were considered significant. The number of experiments performed in duplicate is given in the table and figure legends.

**RESULTS**

**Body Weight and Plasma Levels of Glucose, Triglycerides, Cholesterol and Insulin in LETO and OLETF Rats** Table 1 shows body weight and the plasma levels of plasma glucose, triglycerides, cholesterol and insulin in 38-week-old LETO and OLETF rats. The body weights of the OLETF rats were approximately 1.3-fold those of LETO rats. The plasma glucose and triglycerides, total cholesterol and LDL-cholesterol levels of the OLETF rats was significantly higher than that of the LETO rats. On the other hand, plasma HDL-cholesterol levels did not differ between the 38-week-old LETO and OLETF rats. The plasma insulin levels in OLETF rats were also significantly higher than in LETO rats, and a close relationship was observed between plasma insulin and glucose levels in OLETF rat (\(r = -0.51x + 335\), \(R = -0.9094, n = 4\)).

**Rate of Corneal Wound Healing in LETO and OLETF Rats** Figure 1 shows images obtained after corneal epithelium abrasion (A) and during corneal wound healing (B) in 38-week-old LETO and OLETF rat corneas. The degree of corneal wound healing was similar between LETO and

| Table 1. Body Weight and Some Blood Test Values for Diabetes Mellitus in 38-Week-Old LETO and OLETF Rats |
|-------------------------------------------------|------------|---|
| **LETO rat** | **OLETF rat** |
| **Weight (g)** | 488.8±14.2 | 621.3±19.72* |
| **Glucose (mg/dl)** | 119.3±4.8 | 213.5±15.7* |
| **Triglycerides (mg/dl)** | 128.0±9.3 | 419.8±22.3* |
| **Total cholesterol (mg/dl)** | 101.4±11.4 | 209.2±11.1* |
| **LDL-cholesterol (mg/dl)** | 43.1±3.6 | 413.±8.0 |
| **HDL-cholesterol (mg/dl)** | 33.2±7.6 | 86.0±9.0* |
| **Insulin (ng/dl)** | 104.6±11.6 | 239.3±27.0* |

The data are presented as means±S.E. of 4 independent rats. * \(p<0.05\), vs. LETO rat in each group.
Fig. 1. Corneal Wound Healing of 38-Week-Old LETO and OLETF Rat Eyes

(A) Photographs of 38-week-old LETO and OLETF rat eyes stained with fluorescein immediately (0 h) and 12, 24, 36, 48, 60 and 72 h following corneal abrasion. (B) Corneal wound healing (%) of 38-week-old LETO and OLETF rat corneas. Open circle, LETO rat; closed circle, OLETF rat. The data are presented as means±S.E. of 4 independent rats. *p<0.05 vs. LETO rat.

Fig. 2. Corneal Images of LETO (A) and OLETF (B) Rats with or without the Instillation of Sericin Solutions

The corneal epithelium was removed with a BD Micro-Sharp™, and the resulting corneal wounds were dyed with 1% fluorescein solution.
OLETF rats until 12 h after corneal epithelial abrasion. The corneal wounds in LETO rats 48 h after corneal epithelial abrasion had almost healed entirely. On the other hand, corneal wound healing in OLETF rats was slower than in LETO rats, and the corneal wounds were healed by 72 h after corneal epithelial abrasion. In addition, a close relationship was observed between the corneal wound healing rate constant (k_h) and plasma glucose levels \((r = -0.8114, n = 4)\).

**Effect of the Instillation of Sericin Solutions on Corneal Wound Healing in LETO and OLETF Rats**

Figure 2 shows images of LETO (A) and OLETF (B) rats after corneal epithelial abrasion. Figure 3 shows corneal the wound healing levels in LETO (A) and OLETF (B) rat eyes instilled with 1, 5 and 10% solutions of sericin. The corneal wounds of OLETF rats instilled with saline were approximately 30% healed at 12 h after abrasion, and approximately 40% after 24 h. The corneal wounds of rat eyes instilled with saline were almost entirely healed 72 h after corneal epithelial abrasion. On the other hand, the corneal healing rates in LETO and OLETF rat eyes instilled with sericin solutions were faster than in the case of saline instillation, and the rate constants increased with increasing sericin concentration (Table 2). The corneal wounds of OLETF rat eyes instilled with 10% sericin solution were approximately 45% healed at 12 h after abrasion, and the wounds showed almost complete healing at 48 h after abrasion. The corneal healing rate constant of rat eyes instilled with 10% sericin solution was approximate 2.5-fold that of OLETF rat eyes instilled with saline. No differences of corneal healing rate constants were observed between LETO and OLETF rats eyes instilled with 5 and 10% sericin solutions (Table 2).

**DISCUSSION**

Ocular complications secondary to type 2 diabetes are a well-known cause of diabetic keratopathy\(^5\); however, for reasons of effectiveness, safety and stable supply, a potent corneal wound-healing agent for human corneal wounds has not yet been introduced. We previously reported that the instillation of sericin has a potent effect in promoting wound healing and wound-size reduction in rats.\(^24\) Therefore, it is possible that sericin may be applied as eye drops for corneal wound repair. In this study, we investigated the enhancing effects of sericin on corneal wound healing in the debrided corneal epithelium of OLETF rats, a model for human type 2 diabetes.

In the development of corneal wound-healing drugs against type 2 diabetic mellitus, the selection of the experimental animal is very important. The OLETF rat is an established model of human type 2 diabetes.\(^26\) Nearly 100% of male OLETF rats develop a diabetic syndrome by 25 weeks of age, and hyperglycemia and hyperinsulinemia are exhibited in the early phases of the disease as a result of islet cell hyperplasia and peripheral insulin resistance.\(^27,29-30\) With continued aging, the rats eventually develop hypoinsulinemia as a result of the deterioration of islet beta cells.\(^28,30\) These changes are similar to those in GK rats, which are used as a model animal for type 2 diabetic keratopathy.\(^31\) However, while OLETF rats develop type 2 diabetic mellitus via a metabolic syndrome, GK rats develop type 2 diabetic mellitus without metabolic syndrome. Therefore, the changes in the biological characteristics of OLETF rats show an obvious correspondence to those that take place in human type 2 diabetes mellitus, indicating that OLETF rats may provide a better model than GK rats for studies to clarify the effects of corneal wound-healing drugs for type 2 diabetic mellitus.

In this study, the body weights of OLETF rats were approximately 1.3-fold higher than the LETO rats used as normal controls. Blood parameters for diabetes mellitus (glucose, triglycerides, total and LDL-cholesterol levels) in 38-week-old OLETF rats were significantly higher than in 38-week-old LETO rats. In addition, the insulin level in 38-week-old OLETF rats was also higher than in LETO rats, and a close relationship was observed between the plasma glucose and insulin levels \((r = -0.9094)\). These results show that 38-week-old OLETF rats have insulin resistance and hyperinsulinemia. Hirashima et al. reported atrophy or the disappearance of beta cells in OLETF rats older than 60 weeks of age, and found that plasma insulin levels were lower than in LETO rats.\(^32,33\) Therefore, 38-week-old...
OLETF rats, which show insulin resistance and hyperinsulinaemia, were used to compare the rates of corneal wound healing in this study. It is important that injuries to the cornea be repaired rapidly in order to re-establish function. In general, it is known that epithelial cells from the corneal surface migrate and eventually cover the wound surface. This is followed by cell proliferation to rebuild the tissues, after which tissue remodeling leads to the restoration of the stratified epithelium.34-40) The early stages of epithelial wound closure rely predominantly on cell migration rather than cell proliferation; cell proliferation starts approximately 12–24 h after corneal epithelial injury. After that, tissue remodeling to restore the stratified epithelium occurs.41,42) The corneal wounds of LETO rats 48 h after corneal epithelial abraison showed almost complete healing, while the corneal wounds of OLETF rats were repaired more slowly, with wound healing by 72 h after corneal epithelial abrasion. On the other hand, the rate of corneal wound healing in OLETF rats is similar to that in LETO rats up until 12 h after corneal epithelial abrasion. Therefore, the data suggest that cell movement, which is the main wound healing process up to 12 h after corneal epithelial abrasion, is unimpaired in OLETF rats, but rather a deficit in cell proliferation is responsible for the delay in corneal wound healing in this model.

It is important to observe the mechanisms underlying the delay in corneal wound healing in type 2 diabetic mellitus. In diabetes, the levels of glucose in the cornea and tears are increased. Glucose levels in the corneal epithelium have been reported to be 6 fold higher (1.8 to 12.2 μmol/g dry wt.) in diabetic patients than in normal controls,43) and large increases in the glucose content of tears (range 2.16—9.55 mg/dl and 14.69—27.02 mg/dl for normal and diabetic patients, respectively) have also been reported.44-46) March et al. reported that the glucose content of tears is approximately 10% the plasma glucose level, and that the glucose content of tears follows changes in plasma glucose levels.47) High glucose levels suppress the proliferation of human corneal epithelial cells.48) In addition, it has been reported that the instillation of insulin normalizes the delay in corneal wound healing in streptozotocin rats.13) These previous reports show that the decrease in corneal wound healing in diabetic keratopathy is caused by a suppression of cell proliferation due to high glucose levels in tears. In this study, a close relationship was observed between the rate of corneal wound healing and plasma glucose levels (y = —17930x + 603, R = 0.8114). This result supports the previous results for human diabetic keratopathy.49) Therefore, OLETF rats may provide a useful model for studies to develop corneal wound-healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus.

We previously reported that the instillation of 10% sercin produces no observable neovascularization or inflammation.50) In addition, the instillation of 10% sercin resulted in a significantly greater rate of corneal wound size reduction and healing than the instillation of 0.1% hyaluronic acid in Sprague-Dawley rat eyes, probably by increasing cell movement and proliferation.51) In this study, the rates of corneal wound healing in both LETO and OLETF rat eyes were faster following the instillation of sercin than in the case of saline instillation, and the rate constant increased with increasing sercin concentration. The rates of corneal wound healing in OLETF rat eyes instilled with 5 or 10% sercin solution were similar to those of LETO rats treated similarly. In addition, the instillation of sercin did not affect glucose levels in the OLETF rats (saline instillation, 213.0 ± 19.7 mg/dl; sercin instillation, 221.1 ± 13.9 mg/dl; means ± S.E. of 4 independent OLETF rats). The instillation of 5% or 10% sercin increased the amount of corneal wound healing in OLETF rats 12 h after corneal epithelial abrasion (Fig. 3). Furthermore, we previously reported that sercin increases the adhesion and proliferation of human corneal epithelial cells in in vitro experiments.52) Taken together, the instillation of sercin solution may counter the decreases in the rates of cell movement and proliferation, thus preventing the delay in corneal wound healing in OLETF rats.

In conclusion, the present study demonstrates that the rate of corneal wound healing in OLETF rats is slower than in LETO rats, probably due to the suppression of cell proliferation caused by high plasma glucose levels. The instillation of sercin solution has a potent effect in promoting wound healing and wound-size reduction in LETO and OLETF rats. We hypothesize that OLETF rats may provide a suitable animal model for diabetic keratopathy, and that sercin might provide an effective and safe drug to promote corneal wound healing in diabetic keratopathy. These findings provide information significant for designing further studies to develop potent drugs to improve the corneal wound-healing ability of diabetic patients.

REFERENCES