TOPIC HIGHLIGHT

WJD 5th Anniversary Special Issues (2): Type 2 diabetes

Therapeutic effects of sericin on diabetic keratopathy in Otsuka Long-Evans Tokushima Fatty rats

Noriaki Nagai, Yoshimasa Ito

Noriaki Nagai, Yoshimasa Ito, Faculty of Pharmacy, Kinki University, Osaka 577-8502, Japan
Author contributions: Nagai N performed the research and analyzed the data; Nagai N and Ito Y wrote the paper.
Correspondence to: Yoshimasa Ito, PhD, Faculty of Pharmacy, Kinki University, Higashi-Osaka, Osaka 577-8502, Japan. itoyoshi@phar.kindai.ac.jp
Telephone: +81-6-43073638   Fax: +81-6-43073638
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Abstract

An Otsuka Long-Evans Tokushima Fatty (OLETF) rat provides a useful model for studies to develop corneal wound healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus. We investigated the effects of sericin on corneal wound healing in OLETF rats. Corneal wounds were prepared by removal of the corneal epithelium and documented using a TRC-50X. Sericin was instilled into the eyes of rats five times a day following corneal abrasion. The plasma levels of glucose, triglycerides, cholesterol and insulin in 38 wk old OLETF rats were significantly higher than in normal control rats (LETO rats), and the rate of corneal wound healing in OLETF rats was slower than in normal rat, probably due to the suppression of cell migration and proliferation caused by high plasma glucose levels. The corneal wounds of OLETF rats instilled with saline showed almost complete healing 72 h after corneal epithelial abrasion. On the other hand, the instillation of sericin has a potent effect in promoting wound healing and wound size reduction in OLETF rats and the wounds showed almost complete healing at 48 h after abrasion. The sericin may be an effective and safe drug to promote corneal wound healing in diabetic keratopathy.

Core tip: Otsuka Long-Evans Tokushima Fatty (OLETF) rats provide a useful model for studies to develop corneal wound healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus. The present study demonstrates that the rate of corneal wound healing in OLETF rats is slower than in normal rat, probably due to the suppression of cell migration and proliferation caused by high plasma glucose levels. The instillation of sericin has a potent effect in promoting wound healing and wound size reduction in OLETF rats. The sericin may be an effective and safe drug to promote corneal wound healing in diabetic keratopathy.

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INTRODUCTION

Diabetes mellitus is a common metabolic disorder, affecting more than 190 million people worldwide[1,2]. The prevalence of type 2 diabetes mellitus is increasing rapidly, affecting the health of millions of humans and will continue to do so in the near future. Among the factors responsible for the increasing prevalence of this disease are obesity, the consumption of energy-dense diets and low levels of physical activity[3]. The development of type 2 diabetes mellitus is associated with pancreatic β-cells dysfunction occurring together with insulin resistance.

Normal β-cells can compensate for insulin resistance by increasing insulin secretion[4] but insufficient compensation leads to the onset of glucose intolerance. Once hyperglycemia becomes apparent, β-cell function progressively deteriorates, glucose-induced insulin secretion becomes further impaired and degranulation of β-cells becomes evident, often accompanied by a decrease in the number of β-cells[5-8]. Recently, it was reported that the production of reactive oxygen species (ROS) mediated by glycation reduces insulin gene transcription and decreases the number of β-cells through apoptosis[9,10]. Once β-cells are exposed to ROS under diabetic conditions they are likely to be profoundly damaged due to their low levels of antioxidant enzyme expression[11].

Ocular complications secondary to type 2 diabetes mellitus are a well known cause of diabetic keratopathy[12]. 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 morphological changes in the corneal epithelium and endothelium[13-18]. 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[19]. It has been reported that such diabetic keratopathy is experienced by 50% or more of diabetic patients[20].

The corneal wound repair process involves cell adhesion, migration, proliferation, matrix deposition and tissue remodeling[21]. Many of these biological processes are mediated by growth factors, cytokines and other mediators released in injured tissues or cells[22]. These growth factors have been recognized as important mediators of proper wound repair[23], and treatment with growth factors such as platelet-derived growth factor-BB, recombinant human epidermal growth factor and fibronectin has been shown to be beneficial for patients with chronic pressure ulcers or non-healing diabetic ulcers[24-30]. However, these autologous serum eye drops have problems in terms safety and stability. In addition, for reasons of effectiveness, safety and stable supply, a potent wound healing agent for human corneal wounds has not yet been introduced. Therefore, a potent corneal wound healing agent for human corneal wounds that avoids these problems is highly anticipated. In this study, we attempted the development of corneal wound healing drugs against type 2 diabetes mellitus.

MODEL FOR STUDY OF TYPE 2 DIABETES MELLITUS

In the development of corneal wound healing drugs against type 2 diabetes mellitus, the selection of the experimental animal is very important. Animal models used to investigate diabetes mellitus include streptozotocin-induced diabetic rats as an animal model for insulin-dependent diabetes mellitus[31,32]. Although, hyperglyce-

mia is present in streptozotocin-induced diabetic rats, the general pathophysiology of these models differs from that of non-insulin- dependent diabetes mellitus in humans. On the other hand, the general pathophysiology of Goto-Kakizaki (GK) rats, which are used as a model animal for type 2 diabetes mellitus, is similar[33]. However, GK rats develop type 2 diabetes mellitus without metabolic syndrome. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established model of human type 2 diabetes[34]. Nearly 100% of male OLETF rats develop a diabetic syndrome by 25 wk of age; hyperglycemia and hyperinsulinemia are exhibited in the early phases of the disease as a result of islet cell hyperplasia and peripheral insulin resistance[35-38], and a close relationship was observed between the plasma glucose and insulin levels ($y = -51x + 335$, $r = -0.9094$) in 38 wk old OLETF rats (Table 1)[39]. With continued aging, the rats eventually develop hypoinsulinemia as a result of the deterioration of islet beta cells (Table 1)[36,38,39]. These changes are similar to those in GK rats, which are used as a model animal for type 2 diabetes mellitus[40]. In addition, OLETF rats develop type 2 diabetes mellitus *via* a 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 provide a better model than GK rats for studies to clarify the effects of corneal wound healing drugs for type 2 diabetes mellitus[39].

KINETIC ANALYSIS OF THE RATE OF CORNEAL WOUND HEALING

The maintenance of corneal epithelial cell mass can be viewed as the result of three separate, independent phenomena[41]. Thoft and Friend have termed these: *X*, the proliferation of basal epithelial cells; *Y*, the contribution to the cell mass of the centripetal migration of peripheral cells; and *Z*, epithelial cell loss from the surface. Corneal epithelial maintenance thus can be defined by the equation $X + Y = Z$, which simply states that if the corneal epithelium is to be maintained, cell loss must be balanced by cell replacement[42]. The corneal wound healing process is divided into three sequential and partially overlapping steps: epithelial cell loss from the surface (*Z*) reduces and eventually covers the wound surface (*Y*), while cell proliferation (*X*) provides cells to rebuild the tissue and tissue remodeling to restore the stratified epithelium[33,43-48]. Therefore, it is important to develop the evaluation method of cell proliferation (*X*) and migration (*Y*) in the study for corneal wound healing using rat debrided corneal epithelium.

It has been reported that the early stages of epithelial wound closure rely predominately on cell migration rather than cell proliferation; cell proliferation starts approximately 24 h after corneal epithelial injury, after which tissue remodeling to restore the stratified epithelium occurs[49,50]. Furthermore, Zagon *et al*[49] showed that

Table 1 Body weight and some blood test values for diabetes mellitus in 38 and 60 wk old normal and Otsuka Long-Evans Tokushima Fatty rats

	38 wk old		60 wk old	
	Normal	**OLETF**	**Normal**	**OLETF**
Weight (g)	488.6 ± 14.2	621.3 ± 19.7[a]	526.3 ± 33.0	416.3 ± 17.4[c]
Glucose (mg/dL)	119.3 ± 4.9	213.5 ± 15.7[a]	140.8 ± 3.6	244.3 ± 23.9[c]
Triglycerides (mg/dL)	128.0 ± 9.3	419.8 ± 22.2[a]	150.0 ± 14.4	335.8 ± 11.0[c]
Total cholesterol (mg/dL)	101.4 ± 11.4	209.2 ± 11.1[a]	83.6 ± 14.3	274.5 ± 26.2[c]
Insulin (ng/dL)	105.5 ± 11.6	237.4 ± 26.6[a]	111.1 ± 6.7	83.0 ± 7.2[c]

The data are presented as mean ± SE of 4 independent rats. [a]$P < 0.05$ *vs* 38 wk old normal rats for each category; [c]$P < 0.05$ *vs* 60 wk old normal rats for each category. OLETF: Otsuka Long-Evans Tokushima Fatty.

the corneal wounds of Sprague-Dawley rats (250-300 g) were covered due to cell migration by 24 h after corneal epithelial injury. After that, the interior damage was healed by cell proliferation[49,51]. On the other hand, the wound surfaces in 38 wk old normal and OLETF rats were not completely covered by cell migration 24 h after corneal epithelial injury. Corneal wound healing in 38 wk old OLETF rats takes place in two clear phases. The second phase of healing, involving cell migration and proliferation, may be the source of the delay in corneal wound healing in the 38 wk old OLETF rats. Therefore, we analyzed the two phases of corneal wound healing in 38 wk old normal and OLETF rats by kinetic analysis. The rate of corneal wound healing is represented by the corneal wound healing rate constant (α and β, /h) and the corneal wound healing rate constant was calculated from the following equations (Eq.1 and Eq.2) and the iterative nonlinear least-squares regression procedure MULTI[52].

Corneal wound (%) = wound area $_{0-72\,h}$/wound area $_{0\,h}$ × 100   Eq.1

$W_t = A \bullet e^{-\alpha \bullet t} + B \bullet e^{-\beta \bullet t}$ Eq.2

where t is time (0-72 h) after corneal abrasion and W_t is the percentage of corneal wound (%) at the corresponding time. W_0 is the percentage of corneal wound (%) at time 0. α and β show the corneal wound healing rate constants in the first and second phases, respectively. A and B are the corneal wound areas (%) in the α- and β-phases, respectively. In addition, the α represents cell migration, which is the main wound healing process up to 24 h after corneal epithelial abrasion, while the β-phase, which takes place 18-72 h after corneal epithelial abrasion, represents cell proliferation[52]. The β values for 38 wk old OLETF rats were significantly lower than those for normal rats at corresponding ages. The contribution ratio A of the corneal wound healing process to the β-phase in 38 wk old normal rats was significantly higher than the ratio B of the corneal wound healing process for the second phase. The contribution ratio B of the corneal wound healing process for the -phase in 38 wk old OLETF rats tended to increase. Therefore, a deficit in cell proliferation is predominantly responsible for the delay in corneal wound healing in this model (OLETF rat). On the other hand, it was known that corneal wound healing in normal rats was delayed with

aging, and the corneal wound healing rate constant of 38 wk old normal rats was lower than that of 7 wk old normal rats. The α and β values for 38 wk old normal rats were similar and the contribution to the corneal wound healing process of ratio A for the β-phase is significantly higher than the B for the β-phase. This result suggests that the corneal wounds of old normal rats are repaired by both cell migration and proliferation, and the rate of healing wound becomes equivalent once cell proliferation begins[52].

It is important to understand the mechanisms underlying the delay in corneal wound healing in type 2 diabetes 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 *dw*) in diabetic patients than in normal controls[53] 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[54-56]. March *et al*[57] 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. High glucose levels suppress the cellular behavior (cell migration and proliferation) of human corneal epithelial cells[58]. In addition, it has been reported that the instillation of insulin normalizes the delay in corneal wound healing in streptozotocin rats[20]. Furthermore, a close relationship was observed between the α and β values and glucose levels in 38 wk old OLETF rats, unlike the progression of type 2 diabetes mellitus[52]. These reports indicate that the decrease in corneal wound healing in diabetic keratopathy is caused by a suppression of cell migration and proliferation due to high glucose levels in tears. In addition, this result supports the previous findings for human diabetic keratopathy[58].

HEALING EFFECT OF SERICIN ON DIABETIC KERATOPATHY

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[59]. When cocoons or raw silk are used for textiles, the sericin is mostly removed from the cocoon and disposed of
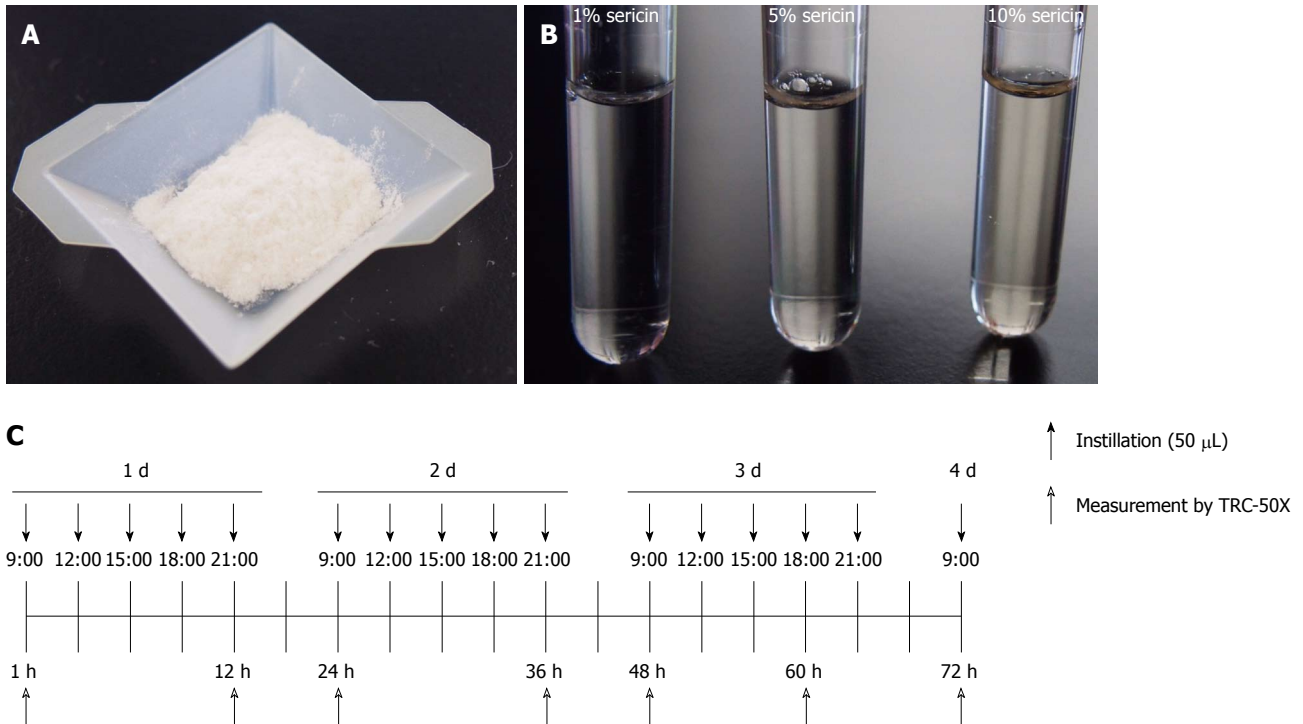
Figure 1 Picture of sericin and protocol in this study. A: Picture of sericin; B: Picture of sericin solution. The sericin solutions used in this study were prepared by adding sericin to saline (pH 6.5-7.5); C: Protocol for instillation of sericin. Saline, sericin or hyaluronic acid solutions were instilled into the eyes of rats five times a day.
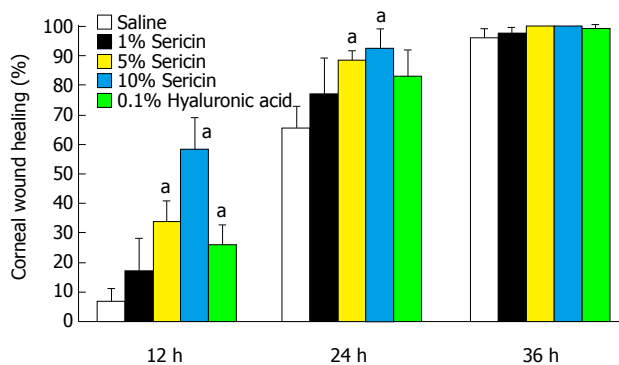

Figure 2 Corneal images of Wistar rats with or without the instillation of sericin solutions. The corneal epithelium was removed with a BD Micro-Sharp™ and the resulting corneal wounds were dyed with 1% fluorescein solution. Saline, sericin or hyaluronic acid solutions were instilled into the eyes of rats five times a day. [a]$P <$ 0.05 *vs* saline-instilled rat.

unused. However, sericin has recently been investigated for its activities in biotechnological fields (Figure 1A)[60,61]. Terada *et al*[62] found growth promotion in several human cell lines and mouse hybridomas when sericin was added to the culture media. It is possible that sericin may be applied as eye drops for corneal wound repair. The sericin solutions used in this study were prepared by adding sericin to saline (pH 6.5-7.5, Figure 1) and the instillation of 10% sericin (30 kDa, Seiren Co, Ltd, Fukui, Japan) produced no observable neovascularization or inflammation[51]. In addition, the instillation of 10% sericin resulted in a significantly greater rate of corneal wound size reduction and healing than the instillation of 0.1%

hyaluronic acid in Wistar rat eyes, probably by increasing cell migration and proliferation (Figure 2)[51]. In this study, the rates of corneal wound healing in both Long-Evans Tokushima Otsuka rats (LETO rat, normal control) and OLETF rat eyes were faster following the instillation of sericin than in the case of saline instillation, and the rate constant increased with increasing sericin concentration. The rates of corneal wound healing in OLETF rat eyes instilled with 5% or 10% sericin solution were similar to those of LETO rats treated similarly. In addition, the instillation of sericin did not affect glucose levels in the OLETF rats (saline instillation, 213.0 ± 19.7 mg/dL; sericin instillation, 221.1 ± 13.9 mg/dL; mean ± SE of 4 independent OLETF rats). The instillation of 5% or 10% sericin increased the corneal wound healing in OLETF rats 12 h after corneal epithelial abrasion (Figures 3 and 4). Furthermore, we reported that sericin increases the adhesion and proliferation[51]. Taken together, the instillation of sericin solution counters the decreases in the rates of cell migration and proliferation, thus preventing the delay in corneal wound healing in OLETF rats (Figure 5).

Next, we investigated the effects of the sericin on cell adhesion and proliferation in a human cornea epithelial cell line (HCE-T) since the effect of sericin in corneal wound healing is a result from the increase in cell migration and proliferation. The adhesion and proliferation of HCE-T cells reached a maximum when treated with 0.1%-0.2% sericin solution; the levels of adhesion and proliferation of 1.0% sericin-treated HCE-T cells did not differ significantly from those of control HCE-T cells (Figure 6). On the other hand, the instillation of
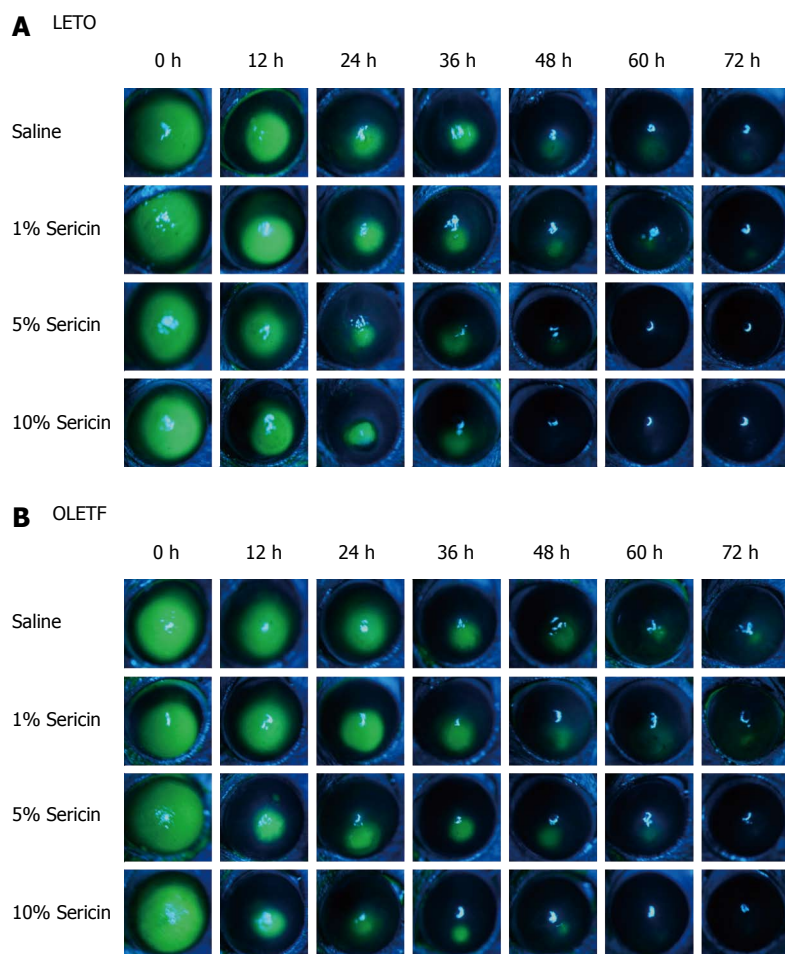
Figure 3 Corneal images. A: Long-Evans Tokushima Otsuka (LETO) rats; B: Otsuka Long-Evans Tokushima Fatty (OLETF) rats with or without the instillation of sericin solutions. The photograph was reported in reference 39. The corneal epithelium was removed with a BD Micro-Sharp™ and the resulting corneal wounds were dyed with 1% fluorescein solution. Saline or sericin solutions were instilled into the eyes of rats five times a day.
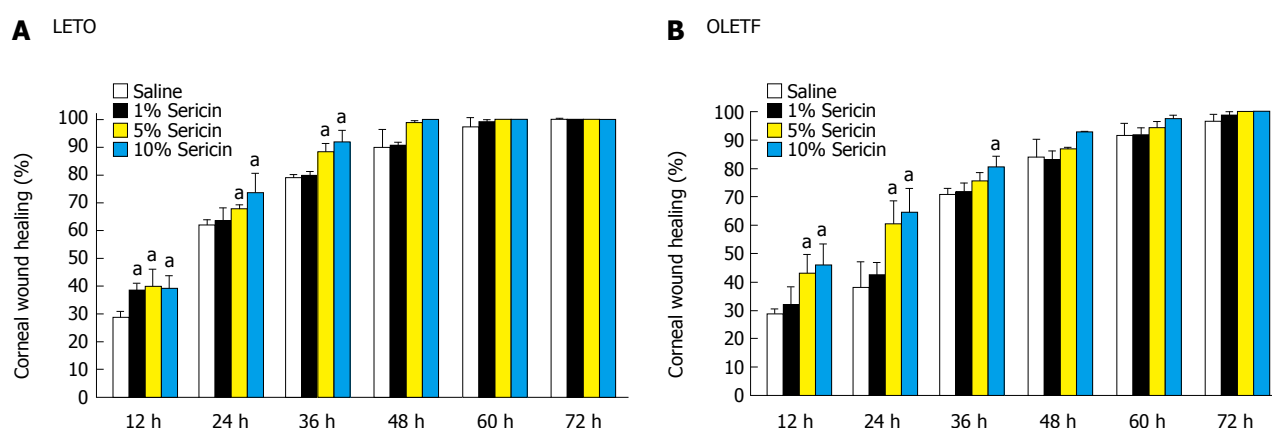

Figure 4 Effect of sericin solutions on corneal wound healing. A: Long-Evans Tokushima Otsuka (LETO) rats; B: Otsuka Long-Evans Tokushima Fatty (OLETF) rat eyes. The data were reported in reference 39. Saline or sericin solutions were instilled into the eyes of rats five times a day. The data are presented as mean ± SE of 3-5 independent rat corneas. [a]$P < 0.05$ *vs* saline-instilled rat.

high concentration sericin solutions (1%-10%) promoted enhanced wound healing in the corneal wound rat model. It is known that the concentration of drugs administered in eye drops is diluted to approximately 20% by lacrimal fluids and that the components of eye drops are excreted though the nasolacrimal duct into the mouth[34]. Thus, our findings suggest that the optimum concentration of the sericin solutions in the *in vivo* instillation experiment, which involves a short residence time, is higher than in the *in vitro* experiment. These findings
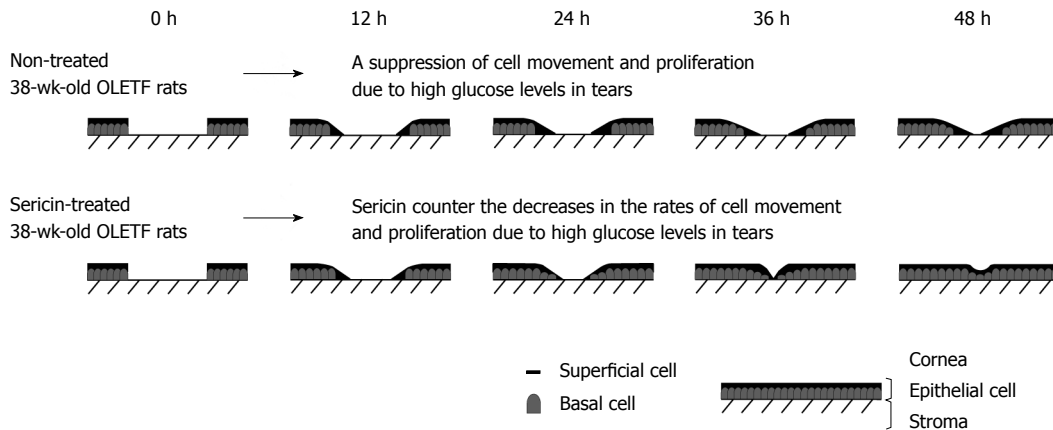
Figure 5 The function of cell migration and proliferation in corneal wound healing in 38 wk old Otsuka Long-Evans Tokushima Fatty rats with or without the instillation of sericin solutions. The movement of superficial cells shows cell migration and the number of basal cells represents cell proliferation. OLETF: Otsuka Long-Evans Tokushima Fatty.
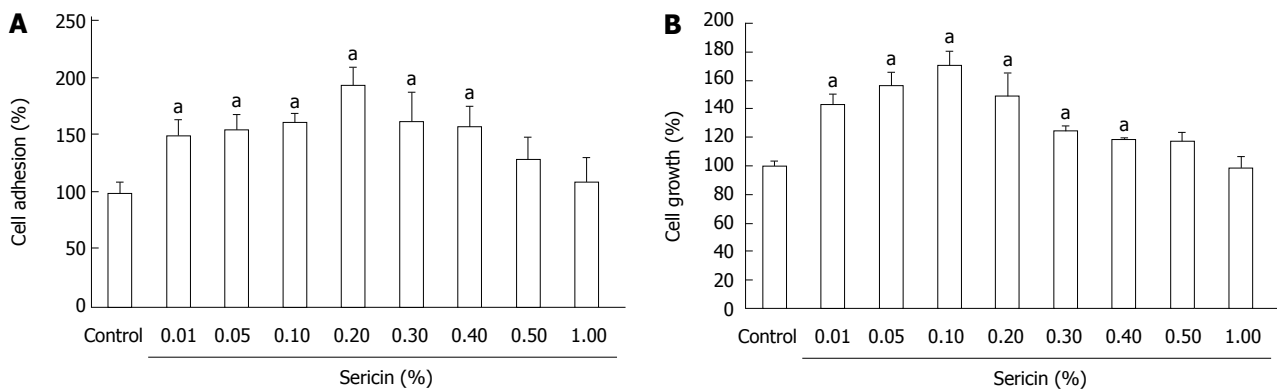

Figure 6 Effect of sericin on the adhesion (A) and growth (B) of human cornea epithelial cell line. The data were reported in reference 51. Human cornea epithelial cell line (HCE-T) cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 containing 5% (v/v) heat-inactivated fetal bovine serum, 0.1 mg/mL streptomycin and 1000 IU/mL penicillin. Cell growth was calculated by TetraColor One. The amount of cell adhesion and growth were represented by the following equation: cell adhesion or growth (%) = $Abs_{sericin\ treatment}/Abs_{control} \times 100$. The data are presented as mean ± SE of 5-25 experiments. $^{a}P < 0.05$ *vs* control HCE-T cells.

provide information significant for designing further studies to develop potent drugs to improve the corneal wound healing ability of diabetic patients.

CONCLUSION

OLETF rats provide a useful model for studies to develop corneal wound healing drugs for use in diabetic keratopathy resulting from type 2 diabetes mellitus. 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 migration and proliferation caused by high plasma glucose levels. The instillation of sericin solution has a potent effect in promoting wound healing and wound size reduction in LETO and OLETF rats. The sericin may be an effective and safe drug to promote corneal wound healing in diabetic keratopathy.

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