Original article

Type I collagen extracted from rat-tail and bovine Achilles tendon for dental application: a comparative study

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Background: Collagen has attracted great interest as a biomaterial for various dental and medical uses.
Objective: Investigate the characteristics and biocompatibility of type I collagen extracted from rat-tail tendon and bovine Achilles tendon for dental application.
Materials and methods: Type-I collagen was extracted from rat-tail and bovine Achilles tendon using pepsin. The purity of collagen extracts was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The biocompatibility with human gingival fibroblasts (HGFs) and human oral keratinocytes (HOKs) was examined using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Scanning electron microscope (SEM) illustrations of purified collagen alone and collagen with HGFs and HOKs were presented. A three-dimensional wound-healing model of fibroblast populated collagen lattice (FPCL) was used to determine the capability of both sources of collagen to induce wound healing *in vitro*. Cellular collagen lattices were fabricated to examine the contraction rate of these collagens.
Results: The average yield of collagen extracted from rat-tail and bovine Achilles tendon were 21.8±14.9% and 5.4±0.4%, respectively. The SDS-PAGE analysis showed that the extracts were composed of alpha 1, alpha 2 and beta chains with little contamination of other small proteins. The MTT assay showed good proliferation of cells cultured with each collagen extract, indicating that collagen extracts were non-toxic to the cells. SEM and the FPCL analysis showed that both types of collagen were biocompatible with both HGFs and HOKs, inducing good contraction in the *in vitro* model.
Conclusion: Type-I collagen extracted from rat-tail and bovine Achilles tendon appeared to be biocompatible with HGFs and HOKs. Both biomaterials may be of use in dental practice.

Keywords: Biocompatibility, bovine Achilles tendon, collagen, human gingival fibroblasts, human oral keratinocytes, rat tail

Collagen has attracted great interest as a biomaterial for various medical uses and as a substrate for tissue engineering. It has been widely employed as a surgical dressing, surgical suture, hemostatic material, and membranes for guided tissue regeneration (GTR) or guided bone regeneration (GBR) for dental use. It has also been used in an *in vitro* model for wound healing and biocompatibility studies [1].

Type I collagen is the most abundant type of collagen used in biomedical studies and its use is widely documented [2]. It is a natural material with good biological compatibility and low antigenicity [3]. It is found in skin, bone, tendon, ligament, and cornea of animals. The main sources of type I collagen for biomedical use are from animal skin and tendon, such as rat-tail tendon [4], bovine tendon [5], porcine tendon [6], or equine tendon [7]. Collagen from different species has their own unique chemical, physical, and biological properties [7, 8]. Rat-tail tendon is among the original sources of type I collagen extracts [4, 9, 10]. Bovine Achilles tendon is also a source of type I collagen in dental application especially in implant dentistry.

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In Thailand, collagen products used in biomedical application are mainly imported, but the raw materials for collagen extraction can be obtained domestically. The extraction protocol of type I collagen from bovine Achilles tendon is widely documented [6, 7, 11]. It is an urgent task to develop collagen products from these domestic sources to reduce cost.

Up to now, there are limited numbers of comparative study on the species-related properties of type I collagen. In addition, the studies regarding the effect of collagen extracts on oral cells are restricted. In this study, we aimed to obtain the basic knowledge about collagen properties and biocompatibility to find a good source of type I collagen that can be developed for the use in dental research.

Materials and methods

This study was approved by the Committee on Human Rights Related to Human Experimental of the Mahidol University.

Type I collagen was extracted from rat-tail and bovine Achilles tendon. Rat tails were obtained from the National Laboratory Animal Center, Mahidol University. Bovine Achilles tendon was purchased from a local market. All materials were kept at -20°C until they were extracted. Type I collagen was first extracted using the modified method by Huang et al. [6], lyophilized, and stored at -20°C for further application. Collagen solution (0.5% w/v in 17 mM acetic acid) were sterilized by dialysis against sterile 17 mM acetic acid followed by 1% chloroform as described by Rajan et al. [12].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Collagen extracts from different sources were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis according to the method by Laemmli [13] using 6% separating gels. The gel was then stained overnight with 0.017% (w/v) Coomassie blue R-250 (Bio-Rad®, Hercules, USA) in 38.8% methanol and 6.8% acetic acid. Subsequently, each gel was destained with 5% methanol and 5% acetic acid for 48 hours.

Cell culture

Human gingival fibroblasts (HGFs) were derived from gingiva received from gingival surgery. Cells were seeded and cultivated in Dulbecco-MEM (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 95% humidified air with 5% CO_2. The HOK cell line was established by Piboonniyom et al. [14]. The cells were established from normal oral keratinocyte immortalized with a retrovirus containing *H-TERT* and grown in keratinocyte serum free medium (GIBCO®, New York, USA) supplemented with epidermal growth factor and bovine pituitary extract and incubated at 37°C in 95% humidified air with 5% CO_2. Culture media were changed at selected time interval (every two to three days). After reaching confluence, adherent cells were enzymatically detached by 0.1% trypsin-EDTA (GIBCO®, New York, USA) and subcultured.

Scanning electron microscope (SEM) analysis of collagen scaffolds

One hundred microlitres of sterile 0.5% (w/v) collagen solutions in 17 mM acetic acid were pipetted on the sterile cover slips and frozen at -20°C for 24 hours. Subsequently, they were lyophilized for six hours to generate collagen scaffold and microstructure of the lyophilized scaffolds was visualized by SEM (JEOL JSM-5410LV, Tokyo, Japan) at the magnifications of 200 and 500. The pore size and area of porosity was determined using the ImagePro Plus Software program Version 3.0 for Windows (Media Cybernectics, Gorgia, USA). Sixty representative pores were randomly selected to evaluate the mean diameter of the pores from each scaffold. Based on each representative pore, we measured six straight lines that passed through the centric position.

Cell morphology after day one, three, and five were also investigated using SEM at a magnification of 1000 or 1500. HGFs were plated at a density of 2×10^4 cells per well in 24-well plates containing test scaffolds or cover slip. For HOK, a density of 4×10^4 cells per well was used and similar experiment was performed.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide) assay of collagen scaffolds and oral cells

MTT assay was used to estimate cell attachment and cell proliferation [15]. HGFs were plated at a density of 2×10^4 cells per well in 24-well plates containing each type of collagen scaffold or cover slip alone as a control. For HOK, a similar set of experiments were performed. After cells were plated, all of the culture media were pipetted off and cells were rinsed with phosphate buffer saline solution

(PBS) after two hours and on days 1, 3, and 5. Five hundred μL of MTT (0.5 mg/mL, Sigma®, St. Louis, USA) were added into each well and incubated for two hours following by elution with 500 μL per well of dimethylsulfoxide (DMSO, Sigma®, St. Louis, USA). The absorbance was measured by spectrophotometer at 540 nm with DMSO as blank. The optical densities were calculated and presented in mean±standard deviation (SD). Data was derived from triplicate wells for each assay point, and all samples were performed in triplicate.

In vitro wound healing assay model and collagen contraction rate [4]

Fibroblast populated collagen lattices (FPCLs) of rat-tail were fabricated according to O'Leary et al. [4]. For bovine Achilles tendon collagen lattices, 11 mL of the following solution was prepared and pipetted into each plate: 3 mL 2x DMEM; 1 mL fetal bovine serum (FBS); 5 mL 0.5% (w/v) bovine collagen solution; 1.5 mL 0.1 M NaOH; 0.5 mL $1x10^6$ cells/mL HGFs in 1x DMEM. For epidermal equivalent, HOKs were laid on the surface of polymerized FPCL at a concentration of $5x10^5$ cells/mL. On the following day, the lattices were detached from the dishes with a 23-gauge needle and allowed to contract until they reached approximately 10-30% of an initial diameter. The lattices were wounded by a 5 mm punch biopsy then transferred to an acellular collagen lattice which was prepared identically to the method for FPCL above without HGFs and 150 μL of collagen solution was applied to act as a glue between the two lattices. Afterwards, culture medium was changed every two days. In the positive control group, cells were treated with EGF at a concentration of 5 ng/mL. Defect repopulation was measured by counting the number of cells that migrate from the cut edge of the lattice at three to seven days post wounding. Diameters of FPCLs from dermal and epidermal equivalents were also recorded after the lattices were detached from the plate in order to study the collagen contraction rate.

Data collection and statistical analysis

Data were analyzed using the statistical package SPSS for Windows version 14. The data was checked by the Komogolov-Smirnov test for normal distribution. One-way analysis of variance (ANOVA) or Kruskal-Wallis test was applied to detect any difference among groups after test of homogeniety of variance had been done by Levene median test. Multiple comparisons, using Mann-Whitney test with Bonferroni correction, were done. The level of significance was determined at $p < 0.05$.

Results
Collagen characteristics

The yield of extraction was calculated as % yield by comparing the final weight of lyophilized collagen to the original weight of minced tendons. The average yield±SD of extraction from rat-tail and bovine Achilles tendon was 21.8%±14.9% and 5.4%±0.4%, respectively. The isolated type I collagen from rat-tail and bovine Achilles tendon were subsequently subjected to SDS-PAGE analysis. **Figure 1** shows that both collagen extracts contained two main α-chains ($α_1$ and $α_2$) and one β chain with very little contamination of other proteins. This suggested that both collagen extracts were pure with very little protein degradation product or other protein contamination.

Morphology of collagen scaffolds under SEM

After test scaffolds were fabricated and visualized, SEM images of both groups revealed porous collagen scaffold with interconnected network as shown in **Figure 2**.

Table 1 shows distribution of pore sizes in collagen scaffolds. We noted that both collagen scaffolds had a high proportion of the pore diameters in the range of 30 to 50 μm and 50 to 100 μm after the diameters of the selected pores were sorted. Only a small portion of the pore size was smaller than 30 or exceeded 100 μm.

The effect of collagen scaffolds on oral cell attachment and proliferation

The biocompatibility of collagen with oral cells is shown in **Figure 3**. Two hours after HGFs were seeded and cultured on the test scaffolds, the viability of HGFs on the rat-tail scaffold was highest. Cell viability was also significantly higher in the collagen scaffold groups compared to the control. This suggested that HGFs could attach on the collagen scaffolds better than on the cover slip alone and the attachment of HGFs on rat-tail collagen was better than that of bovine collagen scaffold. On day 1, cell viability was found to be highest in the bovine collagen test scaffold group. On days 3 and 5, viability of cells was highest in the control group. These suggested

that within two hours, cells could attach on the collagen scaffolds better than on the cover slips. On day 1, it seemed likely that there were more cells in the group with bovine collagen scaffold. On days 3 and 5, cells could proliferate better on the cover slip compared to the collagen scaffolds.
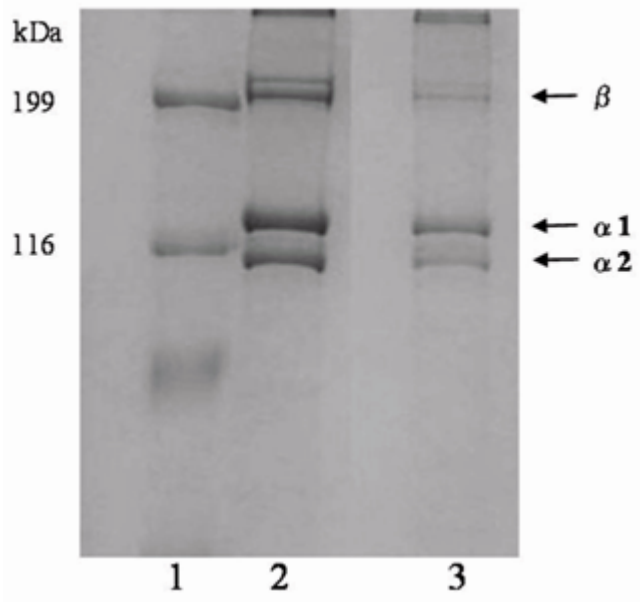

Figure 1. SDS-PAGE images of type I collagen using six percent polyacrylamide gel to examine the purification of collagen extracts. Lane **1**; molecular marker, lane **2**; rat-tail collagen and lane **3**; bovine collagen.
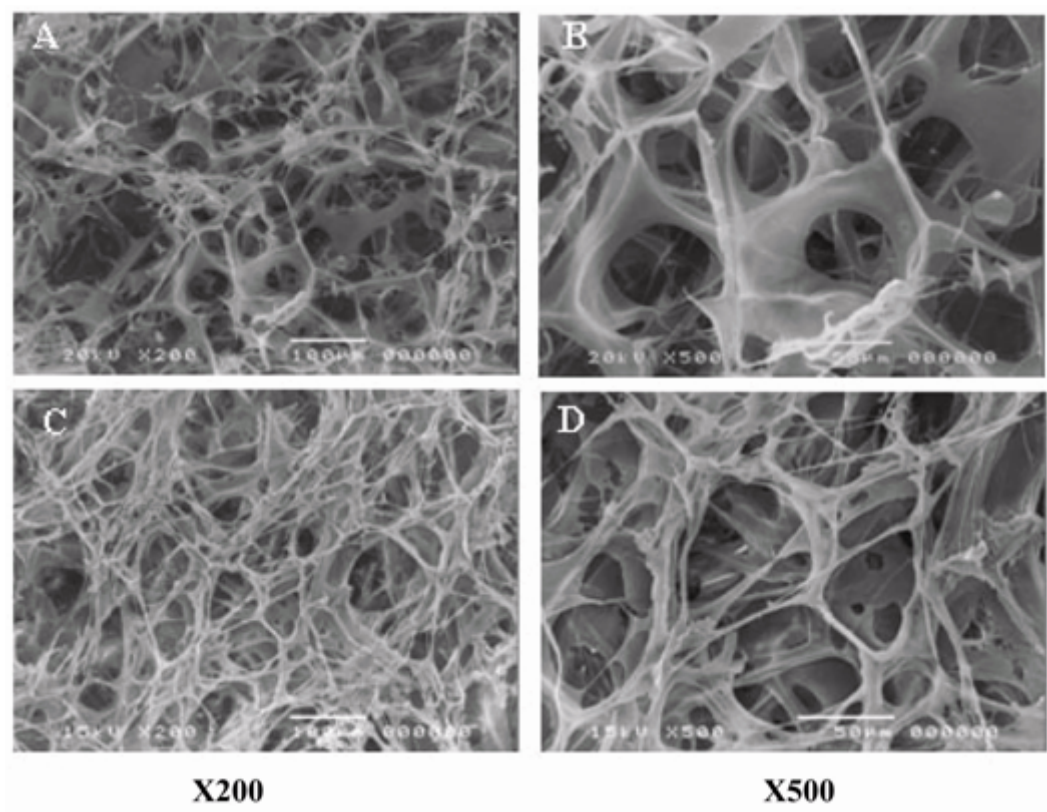

Figure 2. SEM images of collagen scaffolds (x200 and x500). **A** and **B:** rat collagen scaffolds. **C** and **D:** bovine collagen scaffolds.

Table 1. Distribution of pore sizes in diameter and mean diameter of collagen scaffolds

Groups of collagen scaffold	Pore size in diameter (% from 60 pores of two representative views)				Mean diameter (mean±SD) (μm)
	< 30 μm	30-50 μm	>50-100 μm	>100 μm	
Rat	1.67	30	63.33	5	64.191 ± 22.533
Bovine	5	25	65	5	65.283 ± 21.538
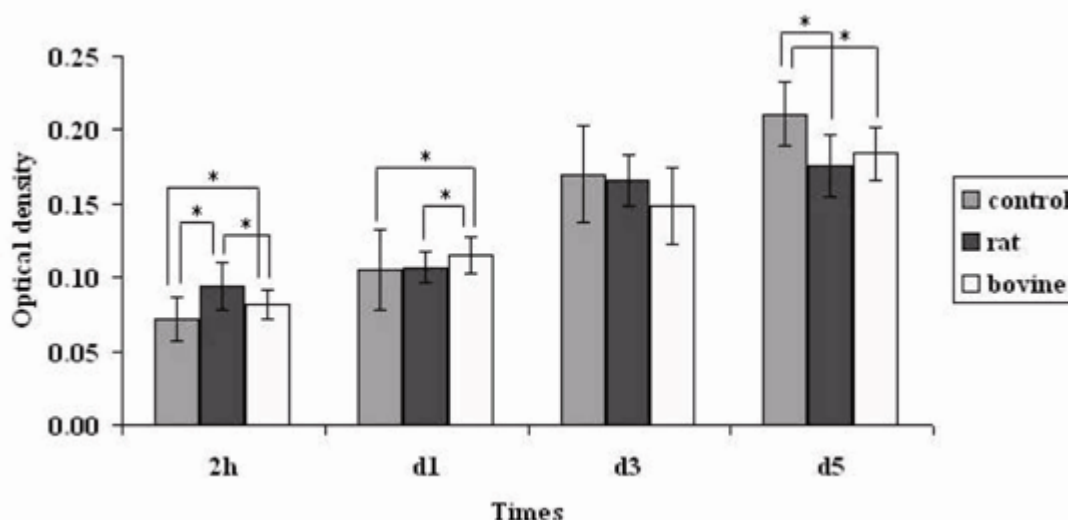
Figure 3. The absorbance of MTT formazan formation of HGFs on collagen scaffolds. Each value was calculated from nine samples and presented as mean±SD. Statistical significance was accepted at p-value <0.05. *statistically significant difference.

In HOKs, a similar trend to HGFs was observed. At two hours, cells could attach well on the collagen scaffolds. On days 1 and 3, no significant difference in the viability of cells was observed among the 3 groups and on day 5, the viability of cells was highest in the control group (**Figure 4**). These results implied that, at two hours, there were more cells in the collagen scaffold groups compared to the control group. On days 3 and 5, there were more cells in the control group compared to that of test scaffold groups.
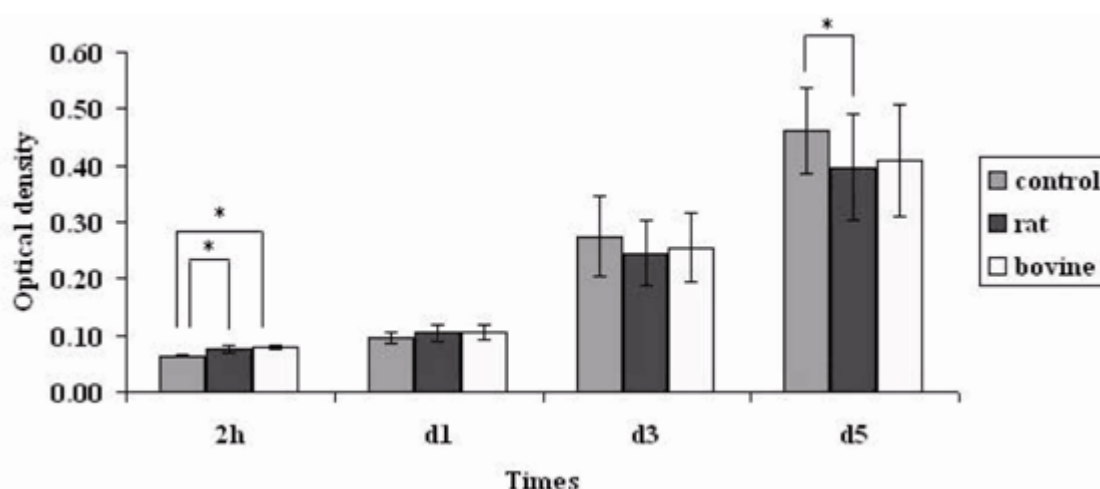

Figure 4. The absorbance of MTT formazan formation of HOKs on collagen scaffolds. Each value was calculated from nine samples and presented as mean±SD. Statistical significance was accepted at *p-value* <0.05. *statistically significant difference.

Overall results indicated that all collagen extracts did not induce cytotoxicity to both HGFs and HOKs although the proliferation rate of cells in the test groups was slightly lower than that of the control group.

Oral cell morphology on collagen scaffolds

The representative pictures of cells grown on cover slips alone and on collagen scaffolds are depicted in **Figures 5** and **6.**
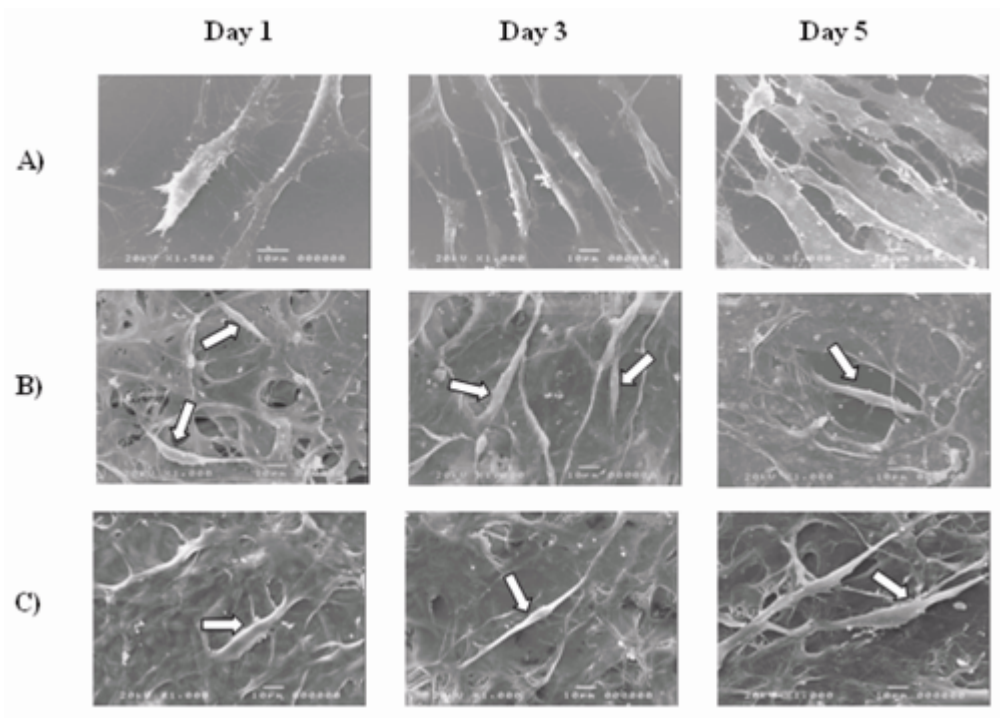

Figure 5. Morphology of HGFs on control and test scaffolds (x1000, except for the picture of cover slip group on day 1 that is x1500). **A**: cover slips, **B**: rat collagen, **C:** bovine collagen. White arrows indicate HGF attachment on collagen scaffolds.
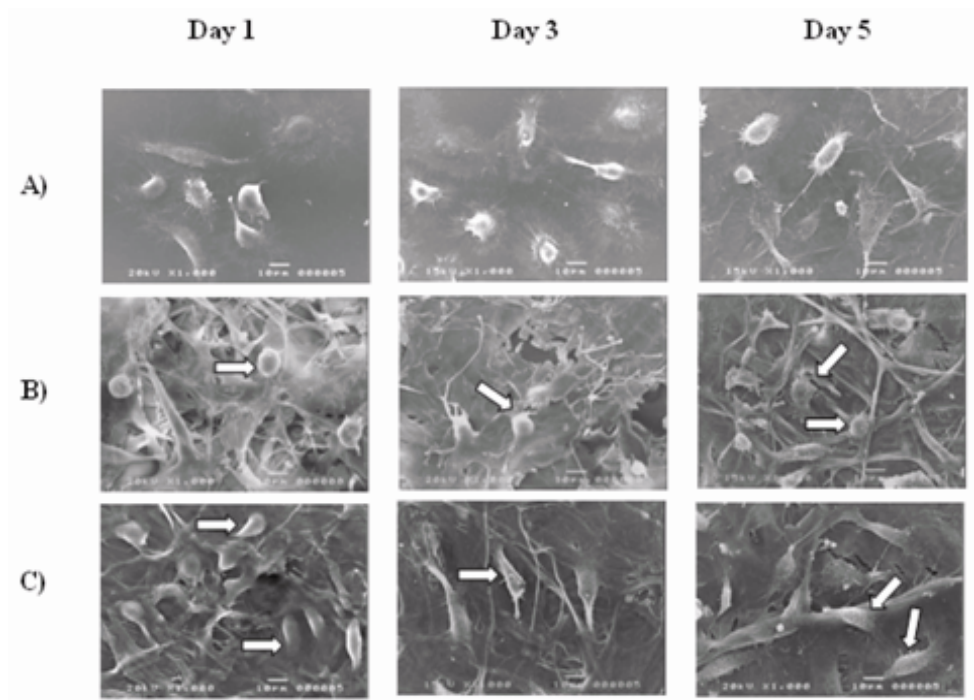

Figure 6. Morphology of HOKs on control and test scaffolds (x1000). **A:** cover slips, **B:** rat collagen, **C:** bovine collagen. White arrows indicate HOK attachment on collagen scaffold.

SEM images showed that HGFs possessed a typical elongated spindle-shaped morphology with numerous cytoplasmic extensions and filipodia in close contact with collagen fibrils. Several cell-cell as well as fibroblast-collagen contacts could be identified, as shown in **Figure 5**. Although HGFs cultured on cover slips demonstrated cytoplasmic process and cell-cell contacts, their morphology was flattened. These findings indicated that both collagen scaffolds were biocompatible with HGFs. In addition to HGFs, HOKs also attached well on the surface of both collagen scaffolds with round and elliptical shapes. They spread and extended their cytoplasmic processes and showed some cell-cell contacts on both collagen scaffolds and cover slips. Similar to HGFs, this suggested that all collagen scaffolds were biocompatible with HOKs, as shown in **Figure 6**.

In vitro wound healing assays
Dermal equivalent

FPCLs were fabricated by growing HGFs in a reconstituted collagen matrix using rat or bovine collagen extract with or without EGF. After wounding with a 5 mm punch biopsy, HGF cells were allowed to migrate into the wounded area and photographed for the appearance of cells repopulating at three and seven days after wounding. Both bovine acellular and cellular collagen lattices showed a very cloudy appearance. In some images, HGFs were vaguely observed at the edge of the wound. In rat-tail collagen lattices on day 3, HGFs were seen to distribute randomly and on day 7, HGFs directly migrated into the center of the wound (**Figure 7**).
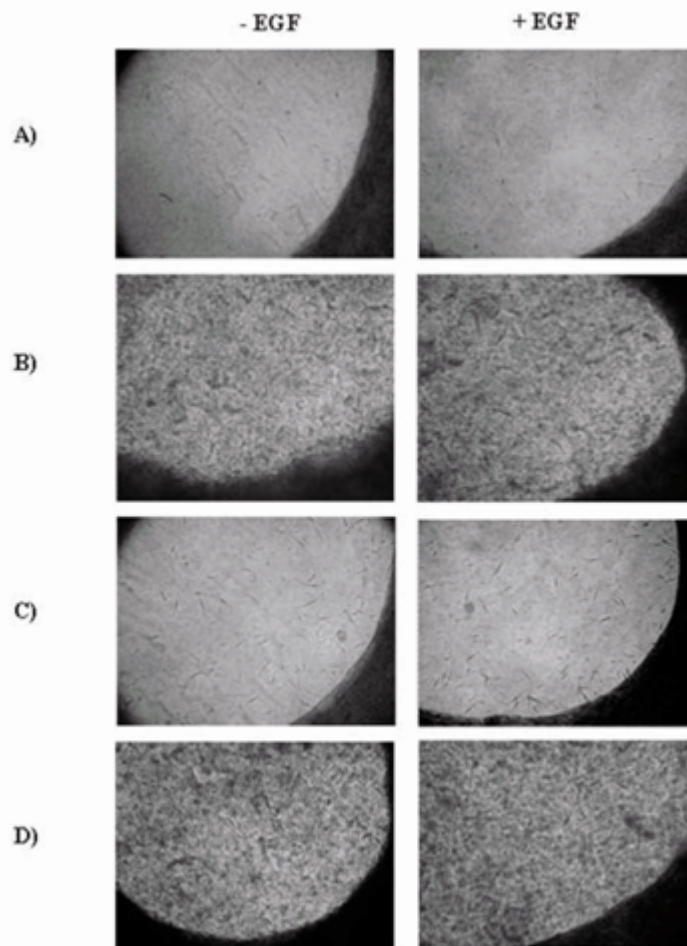

Figure 7. Dermal equivalent wound healing assay on days 3 and 7 after wounding. **A:** rat collagen lattice, day 3. **B:** bovine collagen lattice, day 3. **C:** rat collagen lattice, day 7. **D:** bovine collagen lattice, day 7.

Epidermal equivalent

HOKs were laid on FPCL after polymerization and allowed to contract for two days. Then, the lattices were wounded. On day 3 after wounding, HOKs started to be obviously revealed in the wound defect of rat collagen lattice. In bovine collagen lattices, HOKs were hardly detected. On day 7, in rat collagen lattice, there was a few cells migrated into the wound defect. As in **Figure 8**, on bovine collagen lattice, some sheaths of epithelium-like structure were observed on day 7, but the edge of cell migration could not be clearly identified.

Collagen lattice contraction

The diameters of the FPCLs fabricated in wound healing assays were recorded to study the contraction rate during the period of nine days. The diameters of the lattices were recorded and presented in

Figure 9. Clearly, the rat collagen lattice had the lower contraction rate compared to that of the bovine collagen lattice. Moreover, addition of EGF in rat and bovine collagen lattices did not affect the contraction rate.

Discussion

Collagen provides a support and framework for cells, giving strength and resiliency to body structure. In dental practice, collagen has been used as oral wound dressings and as a biomaterial for guided tissue regeneration (GTR) in periodontal surgery. In this study, we extracted type I collagen from rat-tail and bovine Achilles tendon, and investigated their characteristics utilizing SEM and the biocompatibility using MTT assays. In addition, the effect of collagen extracts from different species in promoting wound contraction was studied. By examining the yield of
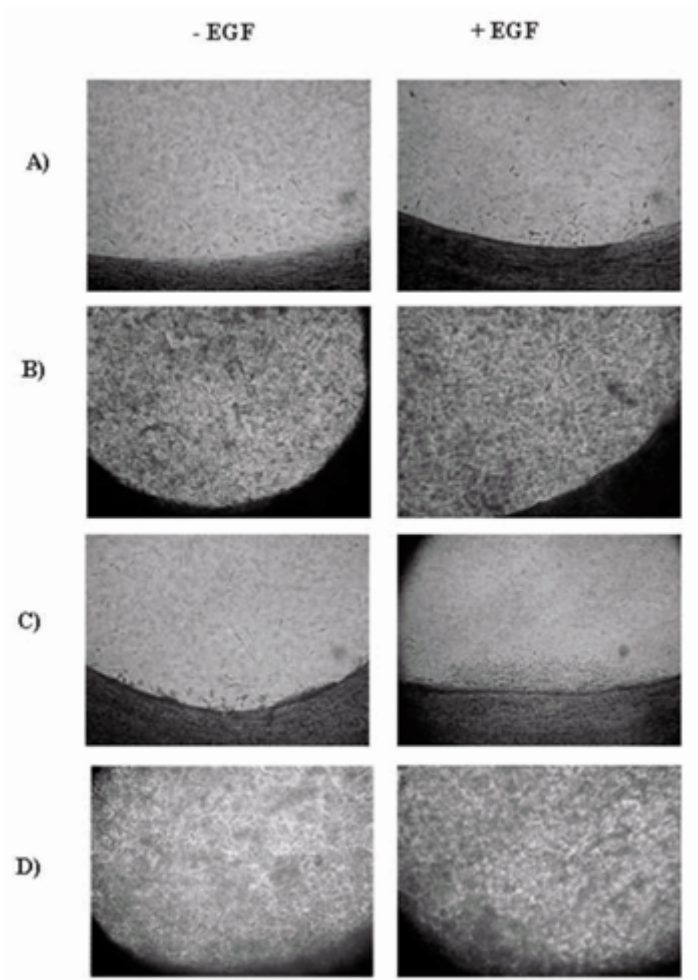

Figure 8. Epidermal equivalent wound healing assay on day 3 and 7 after wounding. **A:** rat collagen lattice, day 3. **B:** bovine collagen lattice, day 3. **C:** rat collagen lattice, day 7. **D:** bovine collagen lattice, day 7.
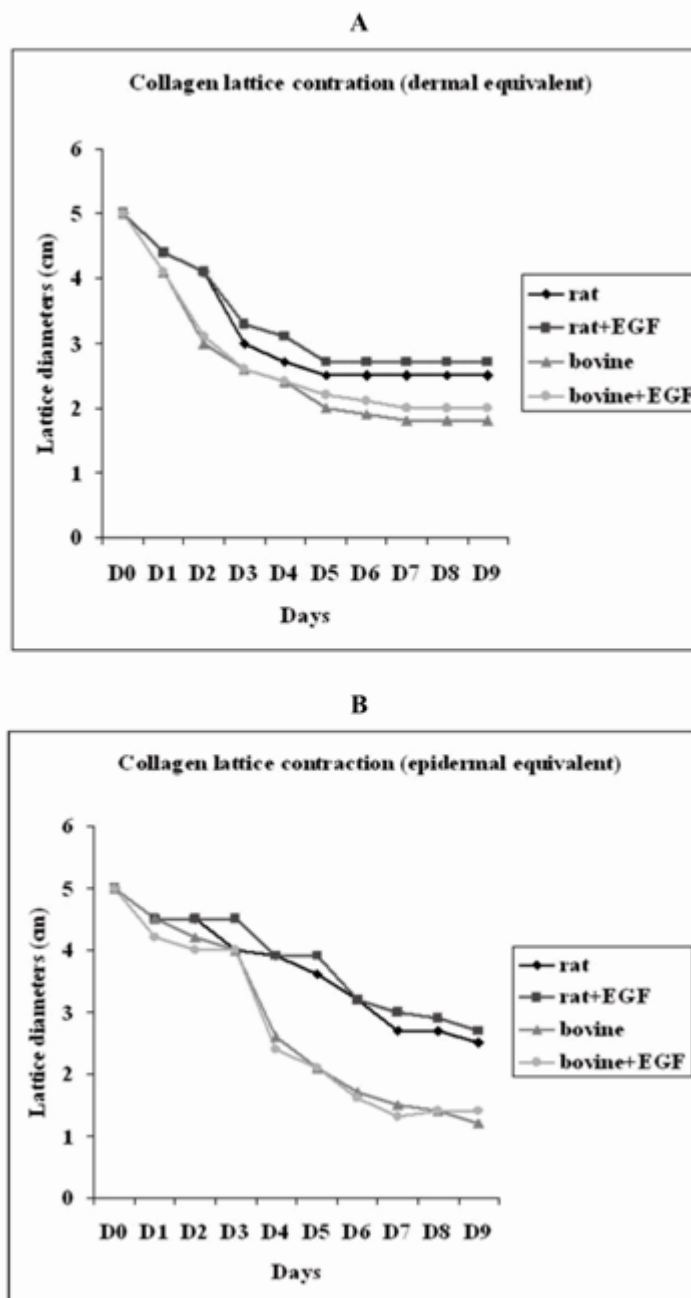
Figure 9. Lattice diameter (cm) in dermal (**A**) and epidermal (**B**) equivalent during the period of nine days after FPCLs polymerized and were detached from culture dishes.

extraction from each source of collagen, we showed that rat-tail gave a higher yield compared to bovine Achilles tendon. The purity of collagen extracts was subsequently determined by SDS-PAGE analysis. It was found that both collagen extracts showed two α-chains (α1 and α2), which are the characteristic of type I collagen (**Figure 1**). In some study, contamination of other proteins or degradation of collagen products could be found during SDS-PAGE

analysis [16]. According to our result in electrophoretic pattern, no low molecular weight fragments were found. Therefore, it can be assumed that both collagen extracts were not degraded by pepsin treatment during the extraction process.

Many constraints must be satisfied to create the biologically active scaffold to promote cell adhesion and growth. One of them is the mean pore size that must be large enough for cells to migrate through the

pores and small enough to retain a critical total surface area for appropriate cell binding. The optimal pore size that allows maximal entry of cells, cell adhesion and matrix deposition has been shown to vary with different cell types [17]. However, it has been reported that the optimal pore size providing appropriate space for cellular infiltration and proliferation ranges between 50 and 150 μm [18]. In our study, this optimal pore size was obtained in all collagen scaffolds with the mean diameter of 64.19 μm in rat collagen and 65.28 μm in bovine collagen. The most abundant pore size of both collagens was also in the range of 50 to 100 μm. The pore size diameter can be regulated by many factors. Freezing temperature also affects the pore size [17, 19, 20]. In the study by Lee et al. [19], type I collagen was extracted from calf skin by pepsin treatment. Lyophilization process was performed at different freezing temperature including -20, -70, and -196°C, to make a porous collagen membrane. The mean pore diameter of porous collagen membrane prepared with freezing temperature of -20°C was 196.9 μm, which was larger than that in our study. The decrease in the freezing temperature resulted in decreased pore size. In the study by Huang et al. [6], the bi-layer three dimensional collagen scaffold was fabricated from porcine Achilles tendon collagen which was extracted by the same method as in our study. The freezing temperatures of -196°C to create the upper dense layer for primary keratinocyte culture and -20°C in the lower loose layer for dermal fibroblast culture were used. The result demonstrated that the mean pore diameter in the lower loose layer with freezing temperature of -20°C was 100 to 200 μm, while those of inner pore of the upper layer and the surface of the upper layer with freezing temperature of -196°C were 10 to 30 μm and 1 to 5 μm, respectively.

In our experiment, we used HGFs and HOKs to investigate the biocompatibility and biological performance of both collagen extracts. The absence of cytotoxicity of both collagen extracts was established by MTT assays and their properties to function as substrates for culturing HGFs and HOKs were additionally examined. Both collagen extracts had no toxicity to both cell types according to the increase of the cell number of HGFs and HOKs in MTT assays, as shown in **Figures 3** and **4**. In addition, cell viability on these collagen scaffolds was also presented in the SEM images, as shown in **Figures 5** and **6**. In MTT assays of HGFs, there

was no significant difference in the optical density between both collagen test scaffolds on day 5. Nevertheless, the optical density of control group was significantly higher than both collagen test scaffolds on day 5 (**Figure 3**). This might be due to the difference in material surface for cell growth between collagen scaffold and cover slip. The result of MTT assays in HOKs demonstrated a similar trend in that on day 5, and HOKs could proliferate better in the control group compared to the collagen scaffold groups. These results indicated that both collagen extracts were biocompatible with HGFs and HOKs.

Another factor to influence the biocompatibility of collagen and cells is the pore size of collagen. O'Brien et al. [21] investigated the effect of scaffold pore size on MC3T3-E1 mouse clonal osteogenic cells attachment at 24 and 48 hours post seeding. The mean pore size of the scaffold had significant effect on cell attachment both at 24 and 48 hours. The smallest mean pore size (95.9 μm) showed the highest percent cell attachment accounted for over 40% of the remaining viable cells, while the largest mean pore size (150.5 μm) showed only 20% remaining cells. No significant difference in cell attachment was found between the intermediate scaffolds (109.5 and 121 μm). In our study, the mean pore size of rat and bovine collagen scaffolds was 64.19 and 65.28 μm, respectively. The result from MTT assays of HGF and HOK showed no significant difference in the optical densities between different collagen scaffolds at two hours, day 1, and day 5 although there were slightly different mean pore size between two collagen scaffolds. This indicates that in this present study, collagen pore size did not have any effect on cell attachment and cell proliferation.

Oral cell morphology under SEM showed that HGFs and HOKs could attach well on both collagen scaffolds with normal cell morphology. The HGF spindle shape and bipolar morphologies with cytoplasmic processes attached on collagen scaffolds were achieved from both collagen scaffolds. Differently, in the control group, HGFs showed the flattened cell shape with stress fibers and focal adhesions (**Figures 5** and **6**). These findings correlate with the cell mechanics in three dimensional matrices as previously described by Rhee and Grinnell [22]. They suggested that cells interacting with collagen matrices exhibit distinct patterns of signaling and migration and remodel matrices locally and globally to achieve tensional homeostasis. With cells growing

on cover slips, the high-tension state and formation of stress fibers and focal adhesion occur, while low-tension state and dendritic/bipolar morphologies take place with cells growing on relaxed collagen matrix.

According to the method for the fabrication of collagen lattices previously described, bovine collagen lattice could not be observed for their ability to promote wound healing from this experiment due to its cloudy appearance. However, the cloudy appearance did not affect the biocompatibility of the collagen since MTT assay did not show significant difference cell viability between rat and bovine collagen scaffolds. The cloudy appearance might affect the ability to use three dimensional wound healing model to investigate the capability of the collagen to promote wound healing.

Dermal and epidermal equivalent collagen lattices were fabricated to study the behavior of collagen lattice contraction. Rat collagen lattices showed lower contraction rate compared to bovine collagen lattices both in dermal and epidermal models. The addition of EGF did not seem to accelerate the lattice contraction when compared to the group without EGF. However, Bell et al. [9] suggested that the factors influence the rate of lattice contraction are the protein content of hydrated lattice or the percentage of collagen in the gel and the number of cells incorporated into the lattice. The rate of lattice contraction varies inversely with the gel protein concentration while proportionally to the number of cells and dependent on the presence of serum and the integrity of the cytoskeleton [23].

In conclusion, our collagen extracts from two different species were biocompatible to HGFs and HOKs *in vitro*. Further study should be done to improve the physical properties of these collagens. It is necessary to understand biological effects of the immune response to these collagen extracts via animal studies to develop these collagen extracts to be used as a biomaterial in human dental practice.

Acknowledgement

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