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Erythrocytes Inhibit Ligament Fibroblast Proliferation in a Collagen Scaffold

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Abstract

In this work, we hypothesized that the concentration of erythrocytes in a provisional scaffold would have a significant effect on three of the major biological processes occurring in early wound healing. ACL fibroblast proliferation, collagen production, and scaffold contraction were measured in collagen gels containing fibroblasts and erythrocytes in subphysiologic (1×10^8 erythrocytes/ml), physiologic (1×10^9 erythrocytes/ml), and supraphysiologic (1×10^{10} erythrocytes/ml) concentrations. Fibroblast-seeded gels containing only platelet-poor plasma were used as a control group. All gels were cultured for 1, 14, and 21 days. DNA, ELISA for procollagen and scaffold size measurements were used to quantify the three above parameters of wound healing. Samples with concentrations of erythrocytes lower than that in whole blood stimulated greater fibroblast proliferation and scaffold contraction than those with erythrocyte concentrations similar to that in whole blood ($p < 0.027$; $p < 0.03$). Increasing the erythrocyte concentration over that in the whole blood stimulated fibroblast collagen production ($p < 0.009$) and limited scaffold contraction ($p < 0.031$). Further work examining the role of the erythrocyte in the early provisional scaffold is warranted.

Keywords

erythrocyte; ACL; fibroblast; proliferation; collagen; contraction

Anterior cruciate ligament (ACL) repair techniques have recently become of interest as less invasive treatment for ACL injuries are sought. In order to facilitate ACL repair, a provisional scaffold must persist in the wound site and become populated by productive fibroblasts. Previous studies have revealed that the presence of such a provisional scaffold is a primary difference between MCL and ACL healing profiles.¹ In connective tissues that heal successfully (MCL), the provisional scaffold (clot) contains platelets, erythrocytes, and white blood cells (WBC). In contrast, in unsuccessful healing, as in the ACL, no clot is sustained in the wound site, so these potentially important healing components are absent. While there is an emerging body of work surrounding the role of the platelet in the provisional scaffold, there is less known about the role of the erythrocytes.

There have been several studies completed recently that show that the presence of erythrocytes affects cell proliferation in vitro. Fredriksson et al.² determined that proliferation of human lung fibroblasts was significantly inhibited when cultured with erythrocyte concentrations above 5×10^5 and 5×10^8 erythrocytes/ml, according to WST-1 (Water Soluble Tetrazolium) staining and DNA incorporation, respectively. Additionally,

Jacobson et al.³ determined that human ACL fibroblast proliferation was significantly inhibited in a 3D collagen–platelet hydrogel with 1.5×10^9 erythrocytes/ml when compared to the control collagen–platelet hydrogel with no erythrocytes.

In this work, we hypothesized that the concentration of erythrocytes in a provisional scaffold would have a significant effect on three of the major biological processes occurring in early wound healing, namely, cellular proliferation, collagen production, and scaffold contraction. We were most interested in testing erythrocyte concentrations in the range of their physiologic concentration in whole blood, which typically lies between 4.5 and 6.0×10^9 erythrocytes/ml in humans^{4,5} and 5.0 and 8.0×10^9 erythrocytes/ml in porcine models.^{6,7} In addition, we aimed to isolate the effect of erythrocytes by eliminating platelets and WBCs and studying a range of erythrocyte concentrations in platelet-poor plasma (PPP). We measured changes in DNA content, procollagen expression, and gel contraction over 3 weeks in collagen gels containing subphysiologic (SUB; 1×10^8 erythrocytes/ml), physiologic (PHYS; 1×10^9 erythrocytes/ml), and supraphysiologic (SUPRA; 1×10^{10} erythrocytes/ml) concentrations of erythrocytes.

METHODS

Gels were prepared by combining PPP containing the specified concentrations of erythrocytes with a bovine collagen slurry and porcine fibroblasts. The four experimental groups were PPP (no erythrocytes), SUB (1×10^8 erythrocytes/ml), PHYS (1×10^9 erythrocytes/ml), and SUPRA (10×10^9 erythrocytes/ml). The gels were cultured in media containing ascorbic acid for 3 weeks. On days 1, 14, and 21 DNA, procollagen, and contraction were measured.

Blood Fraction Preparation

Blood was drawn from a Yorkshire pig into a 500 cc blood-bag containing 10% by volume acid-citrate dextrose (ACD) by Animal Resources at Children's Hospital (ARCH, Boston, MA) from an animal undergoing another IACUC approved study. Blood was centrifuged (GH 3.8 rotor, Beckman GS-6 Centrifuge, Fullerton, CA) at 2,500 rpm for 10 min. Care was taken to not disrupt sediment layers when removing from centrifuge. A syringe was used to remove 20 cc of erythrocyte-concentrate from the bottom of the bag, which was then dispensed into a sterile graduated plastic tube. Two 60 cc syringes were used to remove the top layer of plasma and transfer to sterile 50 cc tubes. The plasma was centrifuged again at 2,500 rpm for 10 min in the tubes to sediment any remaining cells. The resultant clear supernatant was transferred to a fresh graduated plastic tube and designated PPP.

The cell number in the erythrocyte concentrate was 1.12×10^{10} erythrocytes/ml. Supraphysiologic, physiologic, and subphysiologic hematocrit blood fractions were prepared by creating $\times 1$, $\times 10$, and $\times 100$ dilutions of the erythrocyte-concentrate in PPP, to achieve final concentrations of 11×10^9 erythrocytes/ml, 1.1×10^9 erythrocytes/ml, and 0.11×10^9 erythrocytes/ml, respectively. The blood fractions were tested for leukodepletion using a CD45 antibody (a fluorescent marker detected by FACS) (FACS Aria, BD Biosciences, San Jose, CA). The marker identified 0.8% nonerythrocyte cells, which is below the threshold for leukodepletion. In addition, platelet counts were below the lower detection limit of the CBC counter (Abaxis Vetscan HM2).

Fibroblast Cell Growth

Primary outgrowth porcine ACL fibroblasts were cultured in complete media containing Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech, Inc., Cat. #10-013-CV, Herndon, VA), 10% FBS (HyClone, Inc., Cat. # 16777-006, South Logan, UT), and 1% AB/

AM. When cells reached >80% confluency they were passaged. Cells were used at the third passage.

Preparation of Gel Molds

Silicone tubing (1 cm diameter) was cut into 35 mm pieces. Two length-wise slices were made approximately 1 cm apart to remove one-third of the tubing. Aquarium sealant (DAP, Inc., Baltimore, MD) was added to the resulting U-shaped ends of the construct to create a closed well. Before complete drying occurred, a 4 mm × 6 mm rectangular piece of polyethylene mesh was inserted on the inside of both ends of the construct (to later anchor the collagen gel). The completed constructs were sterilized in an autoclave and then secured into six-well plates, one per well.

Acid-soluble, Type I collagen slurry was made by sterilely harvesting bovine fascia which was solubilized in an acidic pepsin solution to a final concentration of 14 mg/ml at pH 2. The collagen was mixed 1:1 with a neutralizing buffer containing HEPES and ×5 PBS resulting in a slurry with pH 7.0 and collagen concentration of 7 mg/ml.

After the third passage, the porcine fibroblasts were trypsinized, counted, and resuspended in the prepared erythrocyte fractions to achieve final cell count of 1.0×10^6 cells/ml, verified by hemocytometer. Then 1.5 ml of the blood-cell mixtures were added to 3.0 ml of the neutralized collagen slurry, resulting in a final collagen concentration of 4.67 mg/ml.

Aliquots of 0.5 ml were transferred into the silicone constructs using a repeat pipettor. Six gels were prepared for each group at each time point. The gels were incubated at 37°C for 1 h, until the gel solidified. Afterwards, 6–7 ml of complete media (same composition as in fibroblast cell growth section) containing 0.25 mg/ml ascorbic acid (Wako, Osaka, Japan, Cat.# 013-12061) was added to each well to cover the composites. The concentration of ascorbic acid used was above that shown to enhance collagen production by ACL fibroblasts by Fermor et al.⁸ The plates were stored in a 37°C CO₂ humidified incubator until designated time points during which the ascorbic acid media was changed two times per week. After 1, 14, or 21 days the gels were gently dislodged from the mesh ends of the construct using a sterile spatula and transferred to 1.5 ml cryovial tubes. The gels were weighed and stored in a –80°C freezer.

DNA Measurement

Gels were digested in 1 ml of papain digest (100 mM sodium phosphate buffer/10 mM Na₂ EDTA/10 mM L-cysteine/0.125 mg/ml papain) at 60°C for 4 h. The amount of DNA in each sample was determined fluorometrically using the Pico Green assay (Quant-iT Pico-Green assay, Molecular Probes, Eugene, OR). DNA content was normalized by the wet-weight of each gel.

Procollagen Measurement

Aliquots were used according to the commercially available Procollagen Type I C-Peptide (PIP) EIA Kit (Takara Bio, Inc., Otsu, Shiga, Japan) protocol. The measured procollagen was divided by the amount of measured DNA for each sample.

Contraction Measurement

At days 1, 14, and 21, before removing the gels from the constructs, the six-well plates containing the gels were placed on a lighted stage (The Back Light Hall Productions, San Luis Obispo, CA) and each pair of wells was photographed from directly above the plate. Contraction was determined by dividing the narrowest width of the gel by the original width of the gel. Only days 14 and 21 were used for statistical analysis.

RBC Lysis Assay

Additional SUB ($n = 6$) and SUPRA ($n = 6$) collagen–erythrocyte gels were also made without fibroblasts and cultured for 21 days. Media surrounding the 12 gels was removed at Days 4, 6, 8, 11, 14, 18, and 21 and replaced with fresh media. The removed media was centrifuged at 1,000 rpm for 10 min to remove any cell debris. The absorbance of the supernatant was read at 405 nm using a microplate reader to quantify hemoglobin content, as previously described.^{9,10} In addition, six SUPRA and six SUB gels were prepared for each of seven time points of culture. At each time point 12 gels ($n = 6$ for both groups) were fixed in formalin, embedded in paraffin, sectioned at 7 μm , and stained with hematoxylin and eosin.

Statistical Evaluation

A linear regression model with the PHYS group as the baseline was built taking into account the different days and individual groups for all DNA, procollagen, and contraction assays. Regression analysis was also used to compare hemoglobin content over time between groups in the RBC lysis assay. An α of 0.05 was considered significant. All calculations were done using Stata10 (College Station, TX).

RESULTS

DNA

The amount of DNA/gel wet-weight in the PPP and SUB samples increased over time (Table 1). The PHYS samples did not show an increase in DNA/gel wet-weight over time, but remained relatively constant throughout the 21 days. Statistically, the DNA concentration in the PPP and SUB samples increased $\times 14$ and $\times 20$ between days 1 and 21, which was significantly more than in the PHYS samples ($p < 0.027$ and $p < 0.004$, respectively). Finally, the SUPRA samples decreased slightly over time, but there was no significant difference between the changes in DNA concentration over time in the PHYS and SUPRA samples ($p < 0.916$).

Procollagen

The amount of procollagen/DNA in the gels increased from days 1 to 14 in the PPP, SUB, and PHYS groups, but no change in procollagen/DNA was seen in these three groups between days 14 and 21 (Table 1). According to the applied linear regression model, there was no significant difference between the increase over time of procollagen/DNA levels when comparing the PHYS and PPP groups ($p < 0.207$). The increase over time of procollagen/DNA levels in the SUB samples was slightly less than the increase over time of procollagen/DNA levels in the PHYS samples by an amount that approached but did not reach statistical significance ($p < 0.096$) (Table 1).

The procollagen/DNA in the SUPRA samples did not level off at day 14, as seen in the other three groups, but rather continued to show an increase through day 21. On average, the procollagen/DNA in the SUPRA samples increased more than twice as quickly ($p < 0.009$, 95% CI 110–143%) when compared to the PHYS samples (Fig. 1).

Contraction

There was no visible contraction in any sample on day 1. The PPP and SUB samples showed a similar trend in contraction over the 21 days and the PHYS samples contracted less than the PPP and SUB samples on days 14 and 21 (Table 1 and Fig. 2). The amount of contraction measured for PPP and SUB samples over the 21 days was 71% ($p < 0.031$) and 312% ($p < 0.006$) greater than the PHYS samples, respectively. There was no contraction in

the SUPRA samples on day 14 and no significant difference between the measured contraction per day of samples in the PHYS and SUPRA groups.

RBC Lysis Assay

There was a significantly greater release of hemoglobin from the SUPRA gels than from the SUB gels over time ($p < 0.0001$, Fig. 3) with the greatest release from the SUPRA gels occurring between 8 and 11 days after gel formation. There was little difference between the hemoglobin release from SUPRA and SUB gels at day 4, and after day 14 (Fig. 3).

Histologic analysis revealed a steady loss of hemoglobin and intact RBCs from the SUPRA gels, particularly over the first week of culture. In addition, images of both the SUPRA and SUB groups showed few intact RBCs present in the gels at the 14 day time point (Fig. 4).

DISCUSSION

Erythrocyte concentration had a significant effect on ACL fibroblasts within a 3D scaffold. The samples with an erythrocyte concentration at or above physiologic concentration showed a suppression of cell proliferation over time when compared to those with lower erythrocyte concentrations. Interestingly, even with lower cell proliferation, the samples with supraphysiologic erythrocyte concentration showed a significantly higher rate of procollagen/DNA production over 21 days than the samples with physiologic or lower concentration of erythrocytes. In addition, erythrocyte concentration at or above physiologic levels caused a slower rate of scaffold contraction in the collagen gels.

The results in the study support the findings previously reported by Fredriksson et al.² and Jacobson et al.³ regarding erythrocyte suppression of fibroblast proliferation. Specifically, Fredriksson et al. determined that cell proliferation of human lung fibroblasts was significantly inhibited when cultured with erythrocytes above concentrations 5×10^5 and 5×10^8 erythrocyte/ml DMEM (2). Similarly, Jacobson et al.³ determined that cell proliferation was significantly less in a 3D collagen–platelet hydrogel with 1.5×10^9 erythrocyte/ml than the control collagen–platelet hydrogel with no erythrocytes. Here, in a 3D collagen gel containing PPP and varying concentrations of erythrocytes, cellular proliferation was significantly suppressed in the two groups at or above 1×10^9 erythrocyte/ml. The gels used in the Jacobson et al. study match our physiologic gels both in erythrocyte concentration and fibroblast seeding density, which serves to support the validity of our findings for that group. Our model expands on the Jacobson et al. study to show the proliferation response of the same gels with higher and lower erythrocyte concentrations as well, which suggests the concentration of erythrocytes in whole blood may be a threshold value below which cell proliferation is not suppressed in a 3D collagen gel. We see that in both a 2D and 3D model in the presence and absence of other blood components, erythrocytes at or above physiologic concentrations suppress fibroblast proliferation.

The procollagen/DNA production over time by the fibroblasts was highest in the supraphysiologic samples. Linear regression analysis revealed that only 6% of the change in procollagen over time could be predicted by DNA levels. Since fibroblast concentration was consistent between groups at the time of gel seeding and no significant levels of other blood components were present in the gels, it is likely that the high erythrocyte concentration was a major factor in causing this effect. Accumulation of procollagen is often attributed to ascorbic acid deficiency,¹¹ but in this study media containing 1% ascorbic acid was replenished for all samples every 3–4 days. Nonetheless, our study shows that although cell proliferation was suppressed in the supraphysiologic gels, the remaining fibroblasts in those samples produced the highest levels of procollagen.

From our DNA measurements, it appears that fibroblast proliferation in the PPP and SUB groups began between days 1 and 14 and was accelerated between days 14 and 21. In contrast we observed a maintenance and decline in fibroblast count in the PHYS and SUPRA gels, respectively, during those time frames. We hypothesize that the high levels of hemoglobin released between days 4 and 11 in the PHYS and SUPRA gels result in damage or death of the fibroblasts resulting in suppression of further proliferation. One of the main products of hemoglobin degradation is bilirubin. Previous studies by Chuniaud et al.¹² have shown that fibroblasts are subject to mitochondrial activity suppression and cell lysis in the presence of increasing concentrations of bilirubin. Such adverse effects begin within 24 h of exposure and result in near complete elimination of activity and viability within 48 h. The higher concentrations of erythrocytes in the SUPRA and PHYS groups compared to SUB and PPP groups may have resulted in higher concentrations of hemoglobin release after lysis, which then may have resulted in high concentrations of biproducts (bilirubin plus perhaps others) toxic to fibroblasts.

Interestingly, a suppression of procollagen release from fibroblasts in the SUPRA gels was not observed even as fibroblast proliferation was inhibited. We hypothesize that this result could also be related to the large amounts of hemoglobin released from lysed erythrocytes in the SUPRA gels. In a series of studies, Stamler et al. began to elucidate the mechanism by which hemoglobin, in addition to binding oxygen, also binds and releases nitric oxide (NO) in order to regulate vasodilation in vivo.^{13,14} Through an oxygen-dependant allosteric transition, the position and reactivity of the heme and cysteine residues within the hemoglobin molecule are changed in order to allow different binding conformations of NO. In the presence of oxygen, NO is bound to the cysteine residue and remains active, whereas in a deoxygenated state hemoglobin scavenges free NO through binding to the heme residues, inactivating NO.¹³ Furthermore, several studies have shown that increased levels of NO stimulate collagen synthesis by fibroblasts in vitro.^{15,16} Therefore, we postulate that the increase in procollagen levels in the SUPRA group after day 14 may be related to the high levels of hemoglobin release from lysed erythrocytes through day 11. NO specifically bound to the cysteine residue of released hemoglobin molecules could stimulate procollagen synthesis from remaining viable fibroblasts within the gel. Further studies measuring NO within the gels and correlation with collagen production are needed to validate this hypothesis.

In the current study, contraction over time was suppressed in samples with physiologic and higher erythrocyte concentrations over 21 days. This is in contrast to another study by Fredriksson et al.¹⁷ which showed an increase in contraction in fibroblast-containing collagen gels with addition of erythrocytes in a time and concentration-dependent manner. Significant increase was shown compared to gels without erythrocytes on all days between 1 and 5, and the augmentation was dependent on the number of erythrocytes added. In the Fredriksson study, the gels were free-floating at the time of measurement, while the gels in the current study were anchored. In addition, we did not view any gel contraction until the day 14 time point, while they observed contraction after 24 h.

Gel contraction has previously been found to be a function of whether the gels are attached, or anchored, to stable points. One study supportive of the differences in time of contraction observed between the current study and the Fredriksson study was done by Galois et al.¹⁸ They found that free-floating collagen gels seeded with chondrocytes contracted more readily than anchored gels over 12 days. Furthermore, in an interesting series of studies, Grinnell et al. determined that fibroblasts in anchored collagen gels develop isometric tension after several hours of culture, whereas fibroblasts in floating gels remain mechanically unloaded. That work also suggested that gels in both models will eventually contract the same amount, but the contraction occurs via different mechanisms and at

different times. The floating gels contract immediately while the anchored gels contract upon release from the anchoring surface as cell extensions collapse.¹⁹ In our experiment, the collagen gels were firmly anchored with mesh on two ends, but likely also experienced temporary tension from the silicon tubing along the other two long edges. As a result, the measured contraction was delayed until the gels were released from the construct along the long edges. It is possible that further contraction would have continued if the gels were released from the mesh anchors.

One limitation to this study is the use of animal models. As it is very difficult to obtain ACL cells from a patient without an ACL injury or osteoarthritis, we worked to mitigate this limitation by using an animal model that is thought to most closely approach the human condition from a biomechanical and wound healing standpoint. The same animal model (porcine) was used to obtain both the fibroblasts and blood components used in the study to minimize any cross-species complications. The porcine model is commonly used for wound healing studies²⁰ and also has similar anatomy and biomechanics to the human knee.²¹ The fibroblasts used in this study were passaged twice prior to the experiment. We chose a low passage because it has been shown that late passaged fibroblasts display an increased nuclear size, which correlates with slow and nondividing cells and thus, higher passage cells may be a less accurate representation of the in vivo condition.^{22,23}

The chief limitation of this study is its in vitro nature. The environment of the culture dish is very different from that of the in vivo environment, and although the in vitro model is useful to begin to understand intrinsic ACL cell migration behaviors, there are likely many other cell types involved in governing the wound response, including inflammatory cells found in the blood. These cells are excluded from this in vitro assay, and therefore future studies should include in vivo studies where the effects of the intrinsic ACL cells are more difficult to isolate and ascertain, but the cumulative invasion characteristics of all the cells in the in vivo wound environment can be determined as a function of erythrocyte concentration.

In summary, the inclusion of physiologic or supraphysiologic concentrations of erythrocytes in a simulated 3D provisional scaffold resulted in protection of the scaffold during the first few weeks of remodeling. Procollagen expression was increased and the scaffold had significantly less contraction. These features suggest the presence of erythrocytes in a tissue-engineered construct may be beneficial in maintaining a scaffold in a wound site. Additional in vivo studies are warranted to study this hypothesis.

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Figure 1.

Average procollagen/DNA produced by fibroblasts in the four groups (each $n = 6$) with SEM bars. The levels of procollagen/DNA in the PPP, SUB, and PHYS groups increased from days 1–14 and remained constant through day 21, with no significant differences between groups. The procollagen/DNA increased through day 21 in the SUPRA samples, resulting in significantly more procollagen/DNA than the PHYS group, denoted by * ($p < 0.009$).

Figure 2.

Average measured contraction (% of original width) of the gels in each of the four groups (each $n = 6$) on days 1, 14, and 21 with SEM bars. The PPP and SUB samples showed a similar trend of contraction, both contracting significantly more than the PHYS samples, denoted by * ($p < 0.031$ and $p < 0.006$, respectively). The SUPRA samples contracted the smallest amount, but not significantly less than the PHYS samples.

Figure 3.

Hemoglobin content in the media surrounding SUPRA and SUB gels (each $n = 6$) with no fibroblasts including SEM bars. Absorbance at 405 nm was measured at 4, 8, 11, 14, 18, and 21 days after gel formation. There was a significantly greater release of hemoglobin from the SUPRA gels than from the SUB gels over time ($p < 0.0001$) with the greatest release from the SUPRA gels occurring between 8 and 11 days after gel formation. There was little difference between the hemoglobin release from SUPRA and SUB gels at day 4, and after day 14.

Figure 4.

Histologic changes in collagen-RBC hydrogels over 2 weeks. Note the steady loss of hemoglobin and intact RBCs, particularly over the first week of culture (hematoxylin and eosin, $\times 100$). Clockwise from top left: SUPRA day 4, SUPRA day 6, SUPRA day 8, SUPRA days 11–21. SUPRA days 18 and 21, as well as SUB days 4–21 were similar in appearance to SUPRA Day 11.

Table 1

The Mean and Standard Deviation of DNA/Gel Wet-Weight, Procollagen/DNA, and Contraction for All Groups Over 21 Days

	PPP	SUB	PHYS	SUPRA
DNA/wet-weight of gel $\times 10^4$ (pg/g); $n = 6$				
Day 1	2.03 $\pm$ 0.09	1.86 $\pm$ 0.13	2.10 $\pm$ 0.12	1.74 $\pm$ 0.29
Day 14	3.30 $\pm$ 0.80	5.52 $\pm$ 2.44	1.12 $\pm$ 0.12	0.87 $\pm$ 0.05
Day 21	30.6 $\pm$ 1.10	38.6 $\pm$ 10.4	1.50 $\pm$ 0.35	0.65 $\pm$ 0.11
Procollagen/DNA (pg/ml); $n = 6$				
Day 1	1.65 $\pm$ 0.28	0.66 $\pm$ 0.18	0.00 $\pm$ 0.09	0.80 $\pm$ 0.03
Day 14	8.94 $\pm$ 1.59	8.38 $\pm$ 2.19	14.04 $\pm$ 1.90	12.23 $\pm$ 1.22
Day 21	8.71 $\pm$ 2.34	7.51 $\pm$ 1.78	13.57 $\pm$ 2.06	32.29 $\pm$ 4.50
Contraction (% of original gel width); $n = 6$				
Day 14	72 $\pm$ 11	67 $\pm$ 13	94 $\pm$ 4	100 $\pm$ 0
Day 21	41 $\pm$ 19	17 $\pm$ 4	70 $\pm$ 9	81 $\pm$ 10

The mean and standard deviation of DNA/gel wet-weight and procollagen/DNA measured on days 1, 14, and 21 and contraction measured on Days 14 and 21 for all groups. DNA was measured using the PicoGreen DNA assay and wet-weights were determined when the gels were removed from the silicone constructs. Procollagen levels were determined using a Procollagen Type I C-Peptide (PIP) EIA kit. The contraction was calculated by dividing the narrowest width of the gel at each time point by the original width.