Published in final edited form as:

J Biomed Mater Res A. 2010 June 1; 93(3): 1132–1139. doi:10.1002/jbm.a.32606.

Effects of cell concentration and collagen concentration on contraction kinetics and mechanical properties in a bone marrow stromal cell-collagen construct

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Abstract

A cell-collagen construct is commonly used to investigate the phenomenon of wound healing and to estimate the variables for tissue engineering. The purpose of this study was to assess the effects of cell concentration and collagen concentration on the contraction kinetics and mechanical properties of bone marrow stromal cell (BMSC) seeded collagen lattices. To investigate the effects of both variables on the contraction kinetics, the construct contraction was monitored up to 13 days. Incremental stress–relaxation tests were carried out after a 2-week incubation to obtain the stress–strain profiles, which were subsequently assessed in a quasilinear viscoelastic (QLV) model. During contraction, aligned BMSCs were observed first in the interior portion of the ring, followed by the middle portion and finally in the exterior portion. Constructs seeded with a higher initial cell concentration (higher than 1×10^5 cells/mL) or lower initial collagen concentration (lower than 2 mg/mL) exhibited faster contraction, higher ultimate stress, and superior elasticity and reduced relaxation behavior ($p < 0.05$). The cell-collagen model was successfully used to yield information regarding the initial cell concentration and the initial collagen concentration on contraction kinetics and mechanical behavior, which may have possible application in tissue engineering.

Keywords

collagen structure; stem cell; mechanical properties; *in vitro* test; tendon

INTRODUCTION

Tissue engineering offers the possibility of replacing damaged ligament or tendon with a functional soft collagenous load-bearing structure.¹ One of the major aspects of tissue engineering is the ability to mimic extracellular matrix (ECM), serving to organize cells and regulating their behavior. Among all the components of ECM, type I collagen is the most abundant component within tendon tissues.² To understand the mechanism of wound healing and explore the application of tissue engineering, the 3D cell-populated collagen

lattice model has long been used to investigate how cells respond to the local chemical and mechanical microenvironment.^{3,4} It has been demonstrated, that the structure and properties of the collagenous network of the ECM are altered through remodeling because of the traction exerted during cell locomotion, which can concomitantly compact the surrounding network, driven by cells exhibiting little migration.⁵

Butler and coworkers found that bone marrow stromal cells (BMSCs) can condense a hydrated collagen lattice to a tendon-like structure, and the contraction kinetics was significantly affected by cell density and cell to collagen ratio.^{6,7} Their results showed that the average maximum force and maximum stress of the repairs increased at significantly higher rates than natural repairs over time by comparing the BMSC-collagen composites with natural repairs. However, parameters such as contents of matrices, cell type, cell concentration, and growth factors, that regulate gel contraction and matrix synthesis in the BMSC seeded 3-D matrix, as well as the mechanical behavior of BMSCs populated collagen matrix, have not been fully studied. Determination of such fundamental structural-mechanical properties is necessary for estimating optimal parameters of tissue equivalents. In the present study, the effects of cell concentration and collagen concentration on the contraction kinetics and mechanical properties of BMSCs seeded collagen lattices were investigated. Incremental stress-relaxation test was used to investigate the equilibrium elastic stress-strain properties and the viscoelastic stress relaxation behavior in the collagen constructs. Mechanical properties were calculated by fitting with quasi-linear viscoelastic (QLV) model, which improves the imperfection that the linear viscoelasticity could not be able to adequately model the observed nonlinear tissue behaviors, and has subsequently been used extensively to determine relaxation behavior in association with various fabricating parameters, collagen concentration and cell density.

MATERIALS AND METHODS

Harvesting and culturing BMSCs

BMSCs were isolated and processed as previously described.⁸ Six mixed-breed dogs (average weight, 27.4 kg), euthanized for other IACUC approved studies, were used in this study. Immediately after euthanasia, 4.0 mL of bone marrow was aspirated from the proximal tibiae using a 20 mL syringe containing 1.0 mL heparin solution (Heparin sodium injection, Baxter Healthcare Corporation, Deerfield, IL). Bone marrow containing heparin solution was added into 5.0 mL phosphate-buffered saline (PBS), and centrifuged at 1500 rpm for 5 min at room temperature. The supernatant was discarded and bone marrow cells were resuspended and incubated in minimal essential medium (MEM) with Earle's salts (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 5% antibiotics (Antibiotic-Antimycotic, GIBCO) at 37°C, 5% CO₂ humidified atmosphere. Cells were used for experiments between passages 2 and 4.

Preparation of BMSCs populated collagen gel ring

BMSC-populated collagen gel rings were prepared as previous studies.^{9,10} Briefly, 10 mL of sterile type I bovine dermal collagen (Cohesion Technologies, Palo Alto, CA) was mixed with 3 mL of sterile 5 × MEM, 1.05 mL of sterile 0.167M NaOH and 0.95 mL distilled water to make 15 mL temporary collagen/MEM solution on ice. The resulting concentration of collagen and FBS were 2.0 mg/mL and 10%, respectively, at a pH of 7.0. For the first aim of the current study, four collagen gel concentrations were evaluated (0.5, 1.0, 1.5, and 2.0 mg/mL), all with a cell concentration of 5×10^5 cells/mL. For the second aim to evaluate the effect of cell concentration, four BMSCs concentrations (0.1, 0.25, 0.5, and 1.0×10^6 cells/mL) were studied with a collagen concentration of 1.5 mg/mL. A total of 2 mL aliquots of cell-seeded collagen gel was added to each mold, which was prepared by centered sterile

small cloning rings, outer diameter of 8 mm and height of 8 mm, on each well in 6-well bacteriologic culture plate. After incubation at 37°C in 5% CO₂ humidified incubator for one-hour gelation, cell-seeded gels were physically detached from the well to remain free-floating in the culture medium. Subsequently, standard BMSCs growth medium was added, and incubated at 37°C, 5% CO₂ humidified atmosphere. The medium was changed every other day until the decided time points.

Quantification of gel contraction

The progress of the gel contraction was monitored by measuring the surface area of the gels every day after gelation.^{3,7,11–13} The cell culture dish and cell-seeded gel was photographed every day using a digital camera with resolution of 2560 × 1920 pixels. The digital images of the surface were analyzed and calculated using Scion Image Beta 4.03 (Scion Corporation, Frederick, MD). The contracted surface area was expressed as a percentage of the initial area. The day on which the contracted surface area was less than 5% of the initial area was defined as the day when complete collagen gel contraction.

Staining of BMSCs populated collagen gels for confocal microscopy

To investigate the morphology and alignment of BMSCs during collagen contraction, the BMSC-seeded collagen lattices were simultaneously stained with Hoechst 33258 and Alexa Fluor 488 conjugated to phalloidin (Molecular Probes; Eugene, OR), and observed at 12, 24, 48 hr after gelation. Collagen gels were fixed with 3% paraformaldehyde/PBS for 15 min and solubilized for 5 min with 0.5% Triton X100/PBS. The nonspecific binding sites were blocked by incubating the substrata in 1% BSA/PBS for 30 min. Second antibodies diluted in 1% BSA/PBS were added to the gels to visualize the actin cytoskeleton and nuclear morphological changes, and gels were incubated for 30 min and were extensively washed, mounted in VECTASHIELD (Vector laboratories, Burlingame, CA) and examined with Zeiss LSM 510 laser confocal scanning microscope.

MECHANICAL MEASUREMENT

Incremental stress–relaxation test

After 2 weeks of incubation, all lattices had contracted into ring shapes with inner diameters of 8 mm. Uniaxial stepwise stress-relaxation tests were conducted to determine the effect of collagen concentration and cell concentration on mechanical properties of BMSCs populated collagen gel constructs with a custom-built micro-tester system, composed of a 50 g load transducer (Model GSO-50, Transducer Techniques, CA), a miniature linear stage (MX80L, Parker Hannifin Corporation, Daedal Division, PA), and an inverted light microscope (CKX41, Olympus America) (Fig. 1). The collagen rings were set up on two horizontal 0.6 mm diameter glass posts, mounted on the testing machine. The rings were prestretched to a length of 11.62 mm, the length equaled to half the net circumference between the tube and the glass hooks, in a rate of 0.05 mm/s. Before mechanical testing, the mean diameter of the rings (measured under 40× magnification) was calculated from 10 different locations around the circumference. In mechanical testing period, the specimens were immersed in a MEM solution at room temperature. Incremental stress–relaxation tests were performed with incremental displacement of 1 mm, which equaled to strain of 8.6%. After each strain increment, the specimen was held for 90 s. These steps were repeated until failure. Force and stretch displacement of the ring were recorded at a frequency of 10 Hz. Peak stress and equilibrium stress were obtained at each strain increment. In this study, the lattices were assumed to have a circular cross-section. From the $\sigma - \epsilon$ result in each test, all stress–strain curves exhibited three regions, toe region, linear region, and failure region. The strain and stress of each incremental-relaxation curve in the linear region were computed into the QLV model introduced by Fung¹⁴ for subsequent calculation.

Quasilinear viscoelastic theory

The QLV model introduced by Fung was used to describe viscous and elastic parameters from stress relaxation experiments.¹⁴ The QLV theory assumes that the stress relaxation behavior of soft-tissue is related to both strain and time, expressed as Eq. (1).

$$\sigma(\varepsilon, t) = G(t)\sigma^e(\varepsilon) \quad (1)$$

where $G(t)$ is the reduced relaxation function, and $\sigma^e(\varepsilon)$ is the instantaneous elastic response. The theory states that the stress, $\sigma(t)$, in response to the strain history at time, t , can be described as the convolution integral between $G(t)$ and $\sigma^e(\varepsilon)$:

$$\sigma(t) = \int_0^t G(t - \tau) \frac{\partial \sigma^e(\varepsilon)}{\partial \varepsilon} \frac{\partial \varepsilon}{\partial t} d\tau \quad (2)$$

The concept of the expression for $G(t)$ was based on a continuous spectrum of relaxation:

$$G(t) = \frac{1 + C(E_1(t/\tau_2) - E_1(t/\tau_1))}{1 + C \ln(\tau_2/\tau_1)} \quad (3)$$

where C , τ_1 , and τ_2 are material constants describing the relaxation characteristics of the

material and $E_1(y) = \int_0^\infty \frac{e^{-t}}{t} dt$ is the exponential integral. The elastic stress response can be given by an exponential approximation:

$$\sigma^e(\varepsilon) = A(e^{B\varepsilon} - 1) \quad (4)$$

where A and B are material constants describing the elastic characteristics of the material.

A , B , C , τ_1 , and τ_2 are material parameters obtained by curve-fitting of the stress–relaxation data to Fung's QLV model using the least-squares method in a custom MATLAB software. The initial guess for each constant determined from a preliminary analysis was multiplied by decades ranging from 0.01 to 100 to ensure algorithm convergence. The algorithm consistently converged to a unique solution, suggesting the global minimum was reached.

Statistical analysis

The sample size was estimated from a pilot study and data published in several related studies.^{11,12,16} The outcome measurements were analyzed using one way ANOVA, followed by the Tukey's Studentized Range (HSD) post hoc test; a p -value of 0.05 or less was chosen to indicate significant difference between groups.

RESULTS

Cell morphology and alignment

Images of cellular distribution on the superficial area of the ring at different time points showed that the gel contraction pattern were similar with different collagen concentrations. Gel contraction occurred in all conditions after incubating 12 h, with dense BMSCs in parallel alignment observed on the interior, but not on the middle or exterior regions (Fig. 2). Cells in the middle portion were aligned in a circumferential direction after 24 h of incubation.

Contraction rate

The rate of contraction was dependent on the concentration of cells incorporated into the collagen lattices (Fig. 3). Contraction occurred in the first day for all four levels of cell concentration. Higher contraction rates were observed with the higher cell-seeded densities. The contraction rates for the collagen lattices with the concentration of 0.5×10^6 and 1.0×10^6 cells/mL were significantly higher than those of either of the two lower cell concentration levels. The time from the start of detectable contraction to final contraction was 11 days for 0.1×10^6 cells/mL, 9 days for 0.25×10^6 cells/mL, 8 days for 0.5×10^6 cells/mL, and 2 days for 1×10^6 cells/mL.

The contraction rate varied inversely with the initial collagen concentration (Fig. 4). The cell-seeded collagen lattices with an initial collagen concentration of 0.5 mg/mL contracted faster than that of 1.0, 1.5, and 2.0 mg/mL concentrations. All lattices contracted to an area less than 5% of initial area after 4 days of incubation.

Mechanical properties

In the incremental stress relaxation test, all collagen gel constructs exhibited similar stress-strain relationship showed identifiable toe, linear, and failure regions (Fig. 5). Mechanical properties of 3D BMSCs populated collagen gel constructs were found to be related to both the initial cell concentration and collagen concentration. Increasing the initial cell concentration led to an increase in ultimate stress of the ring (Fig. 6). The ultimate stress for cell concentration of 1.0×10^6 cells/mL significantly increased in comparison with that of cell concentrations of 0.1, 0.25, and 0.5×10^6 cells/mL. The parameters describing the instantaneous elastic response for BMSCs-seeded collagen gel with various cell concentrations are summarized in Table I, and a typical curve fit is presented in Figure 7. C , τ_1 , and τ_2 are material constants describing the relaxation characteristics of the material. The parameter combination $A \times B$ determined the initial slope of the elastic stress-strain curve, and B influenced its nonlinearity. The constant A , B , C and the combination of constants $A \times B$ were greater for cell-seeded collagen lattices with cell concentrations higher than 0.5×10^6 cells/mL compared with those for cell concentration of 0.1×10^6 and 0.25×10^6 cells/mL. There were no statistical differences for time constant τ_1 or τ_2 between all the conditions.

The variations of mechanical properties with collagen concentrations of 0.5, 1.0, 1.5, and 2.0 mg/mL are shown in Figure 8 and Table II. A higher ultimate stress was observed in the collagen concentration of 0.5 mg/mL, than that of 1.0 mg/mL (Fig. 8). No significant difference in the ultimate stress of collagen gel was observed between collagen concentrations of 1.5 mg/mL and 2.0 mg/mL. The QLV parameters of the rings with various collagen concentrations are shown in Table II. The constant A and C were greatest for the rings with collagen concentrations of 0.5 mg/mL, followed by 1.0 mg/mL, and were least in the concentrations of 1.5 mg/mL and 2.0 mg/mL. The combination constants $A \times B$ decreased with higher collagen concentration. No significant differences were found in constant B and time constants, τ_1 and τ_2 .

DISCUSSION

Cell-collagen lattice compaction is driven by the traction exerted by cells,⁵ which may result in rearrangement of the noncovalent crosslinks in the fibrillar network, the contacts between cells, and eventually, the establishment of a three-dimensional cellular network.¹⁶ Besides, it was shown that cells and collagen align along the axis of greatest tension in the developing construct.¹⁷ From another view point, collagen orientation was found to appear similar to the cell orientation with parallel unconstrained boundaries.⁴ Barocas and Tranquillo

concluded that lattice geometries and boundary conditions could influence cell alignment in cell populated collagen lattices.¹⁸ In the current study, cells aligned concentrically around the circular central tube, parallel to the free boundary, which is consistent with previous studies.^{4,17,18} The contraction was first observed in the interior portion of the ring, followed by middle portion and finally in the exterior portion (Fig. 2). This phenomenon is consistent with the model established by L'Heureux et al.,¹⁹ indicating that circumferential alignment of the fibrils and cells was observed with either tissue engineered tubes with a free-slip mandrel or an adherent mandrel. However, in this study, the dominant factor resulting in the cell-collagen compaction was not able to be assessed, as the direction of greatest tension in this study was typically parallel to the free boundary of the gel.

It is reported that the rate of collagen contraction depends on several factors, such as cell density, collagen concentration, cell-to-collagen ratio, cell type, and biomolecules.^{3,10,20} The rate of gel contraction correlated positively with cell concentration and negatively with collagen concentration.^{3,13,21} Nirmalandhan et al. indicated that above a threshold value of cell density, the contribution of reductions in collagen concentration on contraction kinetics was more than that due to increases in cell density.⁷ Our results are consistent with these previous studies. We observed that the initial cell concentration had a positive effect on the contraction rate of BMSCs populated collagen lattices; the gel contraction exhibited inverse correlation with the initial collagen concentration.

The stress-strain curve shown in Figure 5 represents the overall mechanical behavior of the 3-D collagen gel constructs. The curves for collagen gel constructs are similar in shape to those of other intact collagenous tissues,²² although the overall mechanical property in this study resulted from not only oriented cells/collagen fibers but also contractile force generated by cells, different from those of tendon specimens. Herein, comparisons of ultimate tensile stress on the parameters of cell concentration and collagen concentration revealed that the ultimate stress increased with higher cell concentration and lower collagen concentration. On one hand, higher cell density can generate greater contractile forces in the gel construct and on the other, with the same amount of cells, gel constructs with higher collagen concentration were less condensed, which resulted in less ultimate stress. In addition, it has been seen in previous studies that cells can rearrange the alignment of collagen fibers and the degree of cell alignment will also affect the mechanical behavior. Seliktar et al. showed that cell/collagen vessels with circumferentially oriented cells revealed considerably higher strength and stiffness in the direction parallel to the preferred cell orientation than the unconditioned vessels with randomly oriented cells.²³ The nonlinear elastic properties, represented by the constants A, B and $A \times B$ in the stress-strain relationship, revealed a higher modulus for the collagen gel constructs with higher cell concentration and lower collagen concentration. In particular, the difference in $A \times B$, which indicated the initial slope of the instantaneous elastic response, suggested differences in the composition and structure of tissue substitutes among different conditions. Further, the collagen lattices with either higher cell concentration or lower collagen concentration had larger a viscous response, as shown by increasing values of constant C, resulting in increased stress relaxation. Thus, collagen gel constructs with either higher cell concentration or lower collagen concentration should dissipate more energy and have a longer recovery time upon the removal of load.

In a previous study, Shi and Vesely demonstrated that the mechanical strength of the cell-collagen constructs increased with the fiber density and culture duration.¹³ The stiffness modulus of fibroblast populated collagen lattices was reported to increase linearly with the initial cell number, but exhibit a logarithmic decrease with an increase of initial collagen concentration.²¹ Apart from cell density and collagen concentration, the differentiation of BMSCs, variations in the content of the ECM may also influence mechanical behaviors. It is

well known that mechanical stimulation may lead BMSCs to differentiate into certain adult cells. Omae et al. reported that BMSCs could survive and organized along the collagen fibers on the tendon slices, and expressed a marker of tendon phenotype, tenomodulin.⁸ In addition, after mechanical stimulation for 7 and 14 days, type I collagen expression significantly increased in a BMSCs seeded collagen sponge.²⁴ During the contraction process, tensile force in the direction parallel to the free outer boundary, and compressed force in the radial direction may have changed the status of BMSCs in such collagen constructs. Our results indicated that collagen gel constructs with higher cell concentration or lower collagen concentration exhibited more elastic properties and superior relaxation behavior suggesting that the cell-to-collagen ratio is an important factor affecting the mechanical properties of the collagen gel constructs. However, this study failed to report the influence of cell-to-collagen ratio. Therefore, it is needed to clarify the influences of cell-to-collagen ratio, differentiation status of BMSCs in type I collagen constructs and the amount of secreted proteins on the mechanical properties of the BMSCs seeded collagen gel construct to fully understand the formation of a collagenous tissue substitute.

CONCLUSIONS

This study provided a simplified model of a BMSC-collagen construct to assess the effect of variables on tissue equivalent functions *in vitro*. We demonstrated that higher initial cell concentration positively correlated with contraction rate of BMSCs populated collagen lattices and mechanical properties. Additionally, the lattices with lower initial collagen concentration exhibited a higher gel contraction rate, greater elastic modulus and superior relaxation behavior. We believe this simplified model can also yield valuable information regarding other factors, such as effects of growth factors, the differentiation of BMSCs, cell-to-collagen ratio, and proteoglycans, on the mechanical behavior of such constructs, and therefore will provide a useful tool for tissue engineering studies.

Acknowledgments

Contract grant sponsors: Mayo Foundation; National Science Council, Taiwan (R.O.C)

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Figure 1. Schematic of the uniaxial micro-tester system. The BMSCs populated collagen ring, mounted on the glass hooks, was connected to the load transducer and a motor that controlled the strain of the specimen.

Figure 2.

The morphology and distribution of BMSCs in collagen gel (collagen concentration = 1.0 mg/mL; cell concentration = 1.0×10^6 cells/mL) at different portions after 12, 24, and 48 h of incubation. (a–f: $\times 100$, scale bar = 100 μm ; j–r: $\times 400$, scale bar = 20 μm). Nuclear were stained with Hoechst 33,258 (blue) and the cytoskeletal filamentous actin network was stained with Alexa Fluor 488 conjugated to phalloidin (green). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 3.

Effect of cell concentrations (0.1×10^6 , 0.25×10^6 , 0.5×10^6 and 1.0×10^6 cells/mL) on the contracted area of collagen lattice was shown with collagen concentration of 1.5 mg/mL.

Figure 4. Effect of collagen concentrations (0.5, 1.0, 1.5, and 2.0 mg/mL) on the contracted area of collagen lattice was shown with cell concentration of 0.5×10^6 cells/mL.

Figure 5. Representative stress-strain curve of a collagen matrix. Stress-strain curve exhibited three regions, toe region, linear region, and failure region.

Figure 6. Ultimate stress of BMSCs populated collagen lattices with different cell concentrations (0.1×10^6 , 0.25×10^6 , 0.5×10^6 , and 1.0×10^6 cells/mL), all lattices with same collagen concentration of 1.5 mg/mL.

Figure 7.
A typical stress-relaxation response and QLV curve fit result.

Figure 8. Ultimate stress of BMSCs-collagen lattices of various collagen concentrations (0.5, 1.0, 1.5, and 2.0 mg/mL) with cell concentration of 0.5×10^6 cells/mL.

TABLE I

QLV Parameters for Various Cell Concentrations: Mean (std. dev.)

Cell Concentration	A (kPa)	B	A × B (kPa)	C	τ_1 (ms)	τ_2 (s)
0.1×10^6 cells/mL	4.4974 (0.8122)	0.010000 (0.000000)	0.044975 (0.008122)	0.2538 (0.0590)	0.1038 (0.0039)	100.0331 (0.1459)
0.25×10^6 cells/mL	4.9317 (0.8586)	0.010000 (0.000000)	0.049318 (0.008586)	0.2856 (0.0618)	0.1063 (0.0100)	100.1287 (0.4054)
0.5×10^6 cells/mL	6.6013 (1.1922)	0.010000 (0.000000)	0.066016 (0.011923)	0.4065 (0.0865)	0.1064 (0.0105)	100.0497 (0.2515)
1.0×10^6 cells/mL	9.5977 (2.6489)	0.010000 (0.000000)	0.095979 (0.026491)	0.6257 (0.1928)	0.1066 (0.0133)	100.0125 (0.0390)
<i>p</i> value	0.0008	–	<0.0005	0.0006	0.485	0.119
Post hoc Comparisons ($p < 0.05$)	1.0×10^6 cells/mL>		1.0×10^6 cells/mL>	1.0×10^6 cells/mL>		
	0.5×10^6 cells/mL>		0.5×10^6 cells/mL>	0.5×10^6 cells/mL>		
	0.25×10^6 cells/mL and		0.25×10^6 cells/mL,	0.25×10^6 cells/mL,		
	0.1×10^6 cells/mL		0.1×10^6 cells/mL	0.1×10^6 cells/mL		

TABLE II

QLV Parameters for Various Collagen Concentrations: Mean (std. dev.)

Collagen Concentration	A (kPa)	B	A × B (kPa)	C	τ_1 (ms)	τ_2 (s)
0.5 mg/mL	6.0810 (1.4042)	0.010003 (0.0000001)	0.060818 (0.014025)	0.3757 (0.0899)	0.2279 (0.50560)	101.9441 (6.55171)
1.0 mg/mL	3.3864 (0.9053)	0.010004 (0.0000000)	0.033877 (0.009057)	0.1826 (0.0641)	0.3328 (0.38632)	105.5281 (14.70972)
1.5 mg/mL	2.2881 (0.3560)	0.010003 (0.0000000)	0.022887 (0.003562)	0.0952 (0.0245)	0.1878 (0.22314)	102.1117 (7.02161)
2.0 mg/mL	2.2184 (0.3460)	0.010003 (0.0000000)	0.022190 (0.003460)	0.0889 (0.0248)	0.1248 (0.04087)	100.0672 (0.11619)
<i>p</i> value	<0.0001	0.86	<0.0001	<0.0001	0.071	0.069
Post hoc comparisons (<i>p</i> <0.05)	0.5 mg/mL >		0.5 mg/mL,	0.5 mg/mL >		
	1.0 mg/mL >		1.0 mg/mL >	1.0 mg/mL >		
	1.5 mg/mL,		1.5 mg/mL >	1.5 mg/mL,		
	2.0 mg/mL		2.0 mg/mL	2.0 mg/mL		