Article

The Susceptibility of Tenocytes from Different Ages of Donors Towards Dexamethasone and Ascorbic Acid can be Screened in a Microfluidic Platform

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Abstract: Hamstring tendon is one of the best graft choices for anterior cruciate ligament reconstruction. The upper age limit of reconstruction is not determined because tenocytes from old individuals have less proliferative ability than young ones. Dexamethasone is commonly used to deal with musculoskeletal disorder with dose-dependent cytotoxicity toward tenocytes. Ascorbic acid is essential for tenocytes culture and collagen secretion and can alleviate the cytotoxicity of dexamethasone. In the current study, a microfluidic platform was used to screen the best dexamethasone and ascorbic acid combination treatment for tenocytes from young and old donors because it has been proven to provide a high throughput analysis platform. Comparison of their proliferation under three concentrations of ascorbic acid and dexamethasone was performed. Tenocytes proliferation among young and old donors was also measured when exposed to nine combinations of ascorbic acid and dexamethasone. The result confirmed the differences in cells proliferation when hamstring tenocytes from different ages of donors are exposed to different concentrations of dexamethasone and ascorbic acid. Tenocytes from old donors are not always more susceptible to dexamethasone and ascorbic acid. An optimal dose of ascorbic acid in decreasing the cytotoxic effect of dexamethasone can be screened by a high throughput microfluidic platform.

Keywords: ascorbic acid; dexamethasone; microfluidic system; hamstring; anterior cruciate ligament (ACL); age; tenocytes

1. Introduction

The incidence of anterior cruciate ligament (ACL) tear ranges from 30 to 52 per 100,000 people in different countries [1–3]. It commonly occurs from a noncontact twisting injury of the knee during sports activity. Once torn, the ACL does not heal spontaneously by itself because of poor vascularization of the ruptured ligament and an unfavorable intra-articular environment [4]. Therefore, ACL reconstruction (ACLR) is the golden standard to deal with ACL tear with a success rate up to 90% [5,6]. Plenty of graft choices are reported from literature, such as hamstring, bone-patellar tendon-bone, and quadriceps autografts and allografts. However, there is still a lack of agreement regarding the effect of graft source on the functional outcome of surgery [7]. On the other hand, there is still controversy regarding the upper age limit of ACLR. Brown et al. published a systemic review

describing that ACLRs in patients aged 40 years and older with functional instability could produce satisfactory results [8]. However, ACLRs in elderly donors have more comorbidity and readmission within 90 days of surgery [9]. Furthermore, cells from elderly donors have slower cell metabolism, which may be responsible for a weaker tendon-bone healing [10], leading to graft failure. In a rat model, Nakano et al. demonstrated that ACL-derived cells from younger donors enhanced early bone-tendon healing in an immunodeficient rat model of ACLR. Surgeons should consider a donor's age before ACLR and try to avoid any condition that jeopardizes tenocytes proliferation to achieve a high success rate of surgery.

Corticosteroids are, in many cases, the primary nonsurgical option for treating painful and inflammatory musculoskeletal pathologies. However, they are associated with significant side effects and often lead to tissue degeneration [11]. The use of corticosteroids has been confirmed to reduce tendon strength and suppress proliferation and collagen synthesis of tenocytes derived from animal tendons [12,13]. Tenocytes treated with dexamethasone, one of the commonly used corticosteroids, at a range of doses (0.1–1000 nM) decreases cell proliferation in a dose-dependent manner [14]. Dexamethasone has also been reported to induce adipogenic differentiation [15–17] and adipogenic with chondrogenic differentiation in tendon stem cells [18], which decrease the mechanical properties of the reconstructed ligament such as ACLR using hamstring tendon graft. On the other hand, Zhang et al. proved that dexamethasone treatment stimulated cell proliferation at lower concentrations (<1000 nM), whereas a high concentration (>1000 nM) decreased cell proliferation [19]. Therefore, the dosage of dexamethasone plays an important role in tenocytes studies.

Ascorbic acid (vitamin C) is a cheap and well-characterized antioxidant known to promote collagen biosynthesis and prevent free radical formation [20]. Poulsen et al. showed its efficacy in protecting hamstring-derived tenocytes from oxidative stress and proposed it as an additive to dexamethasone injections [21]. Chiu et al. proved the decrease in cytotoxic effects of anesthetics and non-steroid anti-inflammatory drugs (NSAIDs) towards tenocytes from rotator cuff tendon when treated together with ascorbic acid in a microfluidic platform [22]. However, ascorbic acid had its cytotoxicity in relative high concentration. Hakimi et al. described that ascorbic acid was strongly toxic to cells in concentrations above 10 mM and negatively affected cell morphology at concentrations from 1 mM [23]. Therefore, the optimal dose of ascorbic acid should be determined before clinical application.

There are not much data about the effects of different doses of dexamethasone and ascorbic acid when treated together in tenocytes derived from human hamstring tendon, which is commonly used for ACLR. Additionally, there are differences in cell proliferation between tenocytes from young and old donors. We hypothesized that human tenocytes isolated from the hamstring tendon of old donors would have more susceptibility towards dexamethasone and ascorbic acid. An optimal dose of ascorbic acid in decreasing the cytotoxic effect of dexamethasone could be screened by high throughput microfluidic platforms.

2. Materials and Methods

2.1. Isolation of Human Tenocytes

Human tenocytes were isolated from the hamstring tendon of one 20-year-old and one 41-year-old male donor receiving arthroscopic ACLR (Figure 1a,b), which was approved by the Institutional Review Board at the hospital of the first author. Tendon samples and cells seeding were treated as previously described [22]. Only the first three passages of tenocytes were used in this study.

2.2. Microfluidic System

The microfluidic system used in the current study was the xCELLigence system (Roche/ACEA Biosciences, San Diego, CA, USA). It is designed to allow for continuous real-time monitoring of cellular adhesion properties in vitro in a non-invasive, label-free manner (Figure 1c).

Figure 1. Harvest human hamstring tenocytes. (a) Tenocytes were isolated from hamstring tendon during anterior cruciate ligament (ACL) reconstruction. (b) Cell seeding in culture medium. (c) An xCelligence system was used for real-time tenocytes proliferation monitoring.

2.3. Ascorbic Acid and Dexamethasone Preparation

Three different concentrations of ascorbic acid and dexamethasone were added alone or together with tenocytes 24 hours after seeding. For ascorbic acid (Vitacicol Inj. Taiwan Biotec Co., Taoyuan, Taiwan) treatment, concentrations of 50, 250, and 500 $\mu\text{g/mL}$ were applied in a volume of 10 μL . For dexamethasone (Standard Chem & Pharm Co., Tainan, Taiwan), concentrations of 0.5, 2.5, and 5 mg/mL (10%, 50%, and 100% of clinical dosage) were used in a volume of 10 μL . Control cultures were exposed to saline solution under the same conditions.

2.4. Interactions of Ascorbic Acid Against Dexamethasone

To test the cytotoxicity of ascorbic acid and dexamethasone against tenocytes, 10 μL of 50, 250, or 500 $\mu\text{g/mL}$ ascorbic acid was applied in the culture well with 10 μL dexamethasone with concentrations of 0.5, 2.5, or 5 mg/mL . All conditions are shown in Figure 2. The whole procedure lasted for 190 hours.

Figure 2. Flowchart of the study.

2.5. Tenocytes Proliferation and xCELLigence Software Data Plotting

xCELLigence software version 1.2.1 was used in this experiment to provide an electronic record of the experimental details. The Cell Index (CI) represents the measure of cellular adhesion across each individual well. In the absence of living cells (media only) or with a suspension of dead cells, the CI values are close to zero. Upon attachment of tenocytes onto the electrode, the measured signal correlates linearly with the cell number throughout the experiment with sufficient accuracy.

2.6. Statistical Analysis

Each experiment was performed six times. To compare CI among different culture conditions, analysis of variance (ANOVA) followed by Bonferroni post hoc test was used. A p -value of <0.05 was considered significant. All statistical analyses were performed with SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Tenocytes Proliferation in xCELLigence System

3.1.1. Control

After 190 h of tenocyte proliferation, the CIs were 2.21 ± 0.22 and 2.47 ± 0.32 in young and old donors (Table 1). There was no significant difference between tenocytes proliferation in young and old donors ($p = 1$).

3.1.2. Ascorbic Acid

CIs in the young donor at 190 h were 2.5 ± 0.08 , 1.52 ± 0.17 , and 0.61 ± 0.36 , respectively, when they were exposed to 50, 250, and 500 $\mu\text{g/mL}$ ascorbic acid (Figure 3a). They were 2.46 ± 0.12 , 1.94 ± 0.34 , and 0.67 ± 0.3 in the old donor (Figure 3b). When compared to the control, tenocytes in the young donor experienced 250 and 500 $\mu\text{g/mL}$ ascorbic acid and revealed significantly decreased CI ($p = 0$), which implied the cytotoxicity of a larger dose of ascorbic acid. The same situation was observed in tenocytes from the old donor. No significant difference was found between the same treatment condition (Table 1).

Figure 3. Real-time changes of cell index (CI) when tenocytes were exposed to different concentrations of ascorbic acid. (a) Cell index when tenocytes were exposed to 50, 250, and 500 $\mu\text{g/mL}$ ascorbic acid in the 20-year-old patient. (b) Cell index when tenocytes were exposed to 50, 250, and 500 $\mu\text{g/mL}$ ascorbic acid in the 41-year-old patient. * $p < 0.05$.

3.1.3. Dexamethasone

CI_s at 190 h in the young donor were 2.62 ± 0.28 , 0.48 ± 0.05 , and -0.14 ± 0.05 when tenocytes were exposed to 0.5, 2.5, and 5 mg/mL dexamethasone, respectively (Figure 4a). They were 2.82 ± 0.1 , 0.54 ± 0.06 , and -0.17 ± 0.05 in the old donor (Figure 4b). Both donors presented better tenocytes proliferation when they were exposed to 0.5 mg/mL dexamethasone ($p < 0.05$), which confirmed the stimulating effect of dexamethasone at lower concentrations. When tenocytes were exposed to 2.5 and 5 mg/mL dexamethasone, the CI decreased significantly ($p < 0.05$) and proved a high concentration of dexamethasone decreased cell proliferation. There were no significant differences between donors' tenocytes proliferation when they were exposed to three concentrations of dexamethasone (Table 1).

Figure 4. Real-time changes of cell index when tenocytes were exposed to different concentrations of dexamethasone. (a) Cell index when tenocytes were exposed to 0.5, 2.5, and 5 mg/mL dexamethasone in the 20-year-old patient. (b) Cell index when tenocytes were exposed to 0.5, 2.5, and 5 mg/mL dexamethasone in the 41-year-old patient. * $p < 0.05$.

Table 1. Cell index at 190 h when tenocytes were exposed to ascorbic acid and dexamethasone in young and old donors.

	Control	Ascorbic Acid			Dexamethasone		
		50 μ g/mL	250 μ g/mL	500 μ g/mL	0.5 mg/mL	2.5 mg/mL	5 mg/mL
Young Donor	2.21 ± 0.22	2.5 ± 0.08	1.52 ± 0.17	0.61 ± 0.36	2.62 ± 0.28	0.47 ± 0.05	-0.14 ± 0.05
Old Donor	2.47 ± 0.31	2.46 ± 0.12	1.94 ± 0.34	0.67 ± 0.3	2.82 ± 0.1	0.54 ± 0.06	-0.17 ± 0.05
<i>p</i> Values	1	1	0.2	1	1	1	1

3.1.4. Interaction Between Ascorbic Acid and Dexamethasone

Three concentrations of dexamethasone and ascorbic acid were cross-matched to see their effects on tenocytes proliferation among donors.

Dexamethasone 0.5 mg/mL and Three Concentrations of Ascorbic Acid

CI increased significantly when 50 and 250 $\mu\text{g/mL}$ ascorbic acid were used in combination with 0.5 mg/mL dexamethasone ($p = 0$) in both the young and the old donor's tenocytes (Figure 5a,b). They failed to demonstrate any benefit in proliferation when 500 $\mu\text{g/mL}$ ascorbic acid and 0.5 mg/mL dexamethasone were used together ($p = 1$ for young and 0.75 for old tenocytes) (Figure 5a,b). When the same conditions were compared between donors, tenocytes from the old donor demonstrated significantly better proliferation in 0.5 mg/mL dexamethasone with 250 $\mu\text{g/mL}$ ascorbic acid and 0.5 mg/mL dexamethasone with 500 $\mu\text{g/mL}$ ascorbic acid group compared to the young donor ($p = 0$ and $p = 0.03$) (Table 2).

Figure 5. Real-time changes of cell index when tenocytes were exposed to 0.5 mg/mL of dexamethasone and different concentrations of ascorbic acid. (a) Cell index when tenocytes were exposed to 50, 250, and 500 $\mu\text{g/mL}$ ascorbic acid in the 20-year-old patient. (b) Cell index when tenocytes were exposed to 50, 250, and 500 $\mu\text{g/mL}$ ascorbic acid in the 41-year-old patient. * $p < 0.05$.

Table 2. Cell index at 190 h when tenocytes from young and old donors were exposed to dexamethasone and ascorbic acids cotreatment.

Dexamethasone	Ascorbic Acid					
	Young donor			Old donor		
	50 $\mu\text{g/mL}$	250 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	250 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$
0.5 mg/mL	3.48 ± 0.44	4.3 ± 0.18	2.4 ± 0.25	3.75 ± 0.45	5.67 ± 0.78	3.23 ± 0.31
2.5 mg/mL	2.23 ± 0.25	2.38 ± 0.1	2.22 ± 0.35	2.47 ± 0.29	2.63 ± 0.34	1.77 ± 0.54
5 mg/mL	0.34 ± 0.11	0.47 ± 0.15	0.38 ± 0.17	0.48 ± 0.12	0.47 ± 0.15	0.44 ± 0.08

Dexamethasone 2.5 mg/mL and Three Concentrations of Ascorbic Acid

In the young donor, tenocytes proliferation was not significantly different between three groups ($p = 1$) (Figure 6a). This was also observed when tenocytes from the old donor were exposed to 50 and 250 $\mu\text{g/mL}$ ascorbic acid ($p = 1$) (Figure 6b). Both results implied the reduced cytotoxic effect of 50

and 250 µg/mL ascorbic acid when used with 2.5 mg/mL dexamethasone. The lowest proliferation was observed in 2.5 mg/mL dexamethasone with 500 µg/mL ascorbic acid group ($p = 0.02$), which confirmed the hypothesis that tenocytes from the old donor had more susceptibility towards a specific concentration of dexamethasone and ascorbic acid (Figure 6b).

Figure 6. Real-time changes of cell index when tenocytes were exposed to 2.5 mg/mL of dexamethasone and different concentrations of ascorbic acid. (a) Cell index when tenocytes were exposed to 50, 250, and 500 µg/mL ascorbic acid in the 20-year-old patient. (b) Cell index when tenocytes were exposed to 50, 250, and 500 µg/mL ascorbic acid in the 41-year-old patient. * $p < 0.05$.

Dexamethasone 5 mg/mL and Three Concentrations of Ascorbic Acid

All tenocytes proliferations were close to zero. This implied the cytotoxicity of 5 mg/mL dexamethasone could not be alleviated by all three concentrations of ascorbic acid in both donors (Figure 7a,b). No significant difference was found between groups ($p = 1$).

Figure 7. Real-time changes of cell index when tenocytes were exposed to 5 mg/mL of dexamethasone and different concentrations of ascorbic acid. (a) Cell index when tenocytes were exposed to 50, 250, and 500 µg/mL ascorbic acid in the 20-year-old patient. (b) Cell index when tenocytes were exposed to 50, 250, and 500 µg/mL ascorbic acid in the 41-year-old patient. * $p < 0.05$.

4. Discussion

The result confirmed that there are differences in cell proliferation when hamstring tenocytes from different ages of donors are exposed to different concentrations of dexamethasone and ascorbic acid. Hamstring tenocytes from an old donor are not always more susceptible to dexamethasone and ascorbic acid treatment. An optimal dose of ascorbic acid in decreasing the cytotoxic effect of dexamethasone can be screened by a high throughput microfluidic platform. Tenocytes from an old donor have the best proliferation when they are exposed to 0.5 mg/mL dexamethasone and 250 µg/mL ascorbic acid, while tenocytes from a young donor have more resistance toward the cytotoxicity of 2.5 mg/mL dexamethasone when they are treated along with 500 µg/mL ascorbic acid.

Hamstring tendon is commonly used in ACLR. Quadrupled hamstring autograft is believed to provide the best overall strength when compared to other types of autograft [24,25]. However, there is still controversy regarding the best graft choice in older patients, because Hasegawa et al. proved cell density and proliferation potential decrease with age [26]. On the other hand, hamstring tendonitis are commonly treated by corticosteroid injection [27], which is believed to reduce tenocytes proliferation, viability, and collagen synthesis as well as to increase markers of apoptosis [27,28]. Among them, a high concentration of dexamethasone depletes the pool of human tendon stem cells, suppresses type I collagen, and enhances fatty and cartilage-like tissue changes that can lead to tendon ruptures, while low concentrations of dexamethasone stimulate cell proliferation [19].

Ascorbic acid is critical in collagen secretion and biosynthesis. The standard growth medium with fetal bovine serum containing 25 µg/mL ascorbic acid was found to be the most suitable formulation [29]. Additionally, 1250 µg/mL ascorbic acid played an important role during cell culture [30], and 50 µg/mL ascorbic acid is believed to support proper collagen synthesis [31]. Furthermore, cotreatment of tenocytes with ascorbic acid protects against the glucocorticoid-induced inhibition of ERK and Akt

activation and the reduction in cell number induced by chemical inhibition of ERK, and, to a lesser extent, inhibits Akt signaling [21]. Therefore, the current study used three concentrations (50, 250, and 500 µg/mL) of ascorbic acid to see the effects in reducing the cytotoxicity of dexamethasone. The objective was to determine the optimal concentrations and the formulation of ascorbic acid when used with dexamethasone and their different effects on tenocytes from young and old donors.

To screen the best treatment combination, conventionally, large numbers of cells and large volumes of reagents are needed. However, harvested cells are sometimes limited because most of them should be used in reconstruction. With the development of integrated microfluidic devices, precise, rapid, and reproducible measurements on small reagent volumes can be achieved [32]. Chiu et al. published their result that ascorbic acid reduces the cytotoxic effects of analgesics and NSAIDs [22]. In current study, the reduction of the cytotoxic effect of dexamethasone with cotreatment with ascorbic acid in a specific concentration was also observed in the microfluidic platform. In addition, tenocytes performed differently in young and old donors. The microfluidic platform provides an opportunity for rapid and low-cost screening of the best treatment protocol among each individual, making personalized medicine possible.

There are still limitations about this study. Firstly, we used relatively healthy hamstring tendons from donors rather than from the torn ACL itself. Therefore, the in vitro best treatment combination may not correspond to the real condition in which the ACL is already torn, as adolescent patients commonly exhibited a higher proliferation and multilineage differentiation potential compared to older donors [33]. To achieve the goal of optimized personal medicine, the next step of the study will be the confirmation of different ages, genders, and timing of injury as well as their effects on real-time tenocytes responses toward different stimuli in the microfluidic platform. Secondly, how old is old and where the elderly age group begins are questions with inconclusive answers. Klatte-Schulz et al. demonstrated cells of female donors older than 65 years old showed less cell count, type I collagen protein synthesis, and potential for self-renewal compared to the tenocytes of older male donors [34]. The “old” tenocytes from this study were from a 41-year-old male patient, which may not have been old enough to achieve biological differences. However, people older than 65 years old seldom receive an ACLR. We can only conclude in our study that tenocytes from older donors will have more susceptibility toward dexamethasone and ascorbic acid in a specific concentration compared to younger donors.

5. Conclusions

Hamstring tenocytes from different ages of donors respond differently to a certain concentration of dexamethasone and ascorbic acid. Older tenocytes are not always more susceptible to dexamethasone and ascorbic acid treatment. An optimal dose of ascorbic acid in decreasing the cytotoxic effect of dexamethasone can be screened by the high throughput microfluidic platform.

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References

1. Janssen, K.W.; Orchard, J.W.; Driscoll, T.R.; van Mechelen, W. High incidence and costs for anterior cruciate ligament reconstructions performed in Australia from 2003–2004 to 2007–2008: Time for an anterior cruciate ligament register by Scandinavian model? *Scand. J. Med. Sci. Sports* **2012**, *22*, 495–501. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Csintalan, R.P.; Inacio, M.C.; Funahashi, T.T. Incidence rate of anterior cruciate ligament reconstructions. *Perm. J.* **2008**, *12*, 17–21. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Granan, L.P.; Bahr, R.; Lie, S.A.; Engebretsen, L. Timing of anterior cruciate ligament reconstructive surgery and risk of cartilage lesions and meniscal tears: A cohort study based on the Norwegian National Knee Ligament Registry. *Am. J. Sports Med.* **2009**, *37*, 955–961. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Murray, M.M.; Martin, S.D.; Martin, T.L.; Spector, M. Histological changes in the human anterior cruciate ligament after rupture. *J. Bone Jt. Surg. Am.* **2000**, *82*, 1387–1397. [\[CrossRef\]](#)
5. Griffin, L.Y.; Albohm, M.J.; Arendt, E.A.; Bahr, R.; Beynon, B.D.; Demaio, M.; Dick, R.W.; Engebretsen, L.; Garrett, W.E., Jr.; Hannafin, J.A.; et al. Understanding and preventing noncontact anterior cruciate ligament injuries: A review of the Hunt Valley II meeting, January 2005. *Am. J. Sports Med.* **2006**, *34*, 1512–1532. [\[CrossRef\]](#)
6. Larsen, M.W.; Garrett, W.E., Jr.; Delee, J.C.; Moorman, C.T., 3rd. Surgical management of anterior cruciate ligament injuries in patients with open physes. *J. Am. Acad. Orthop. Surg.* **2006**, *14*, 736–744. [\[CrossRef\]](#)
7. Foster, T.E.; Wolfe, B.L.; Ryan, S.; Silvestri, L.; Kaye, E.K. Does the graft source really matter in the outcome of patients undergoing anterior cruciate ligament reconstruction? An evaluation of autograft versus allograft reconstruction results: A systematic review. *Am. J. Sports Med.* **2010**, *38*, 189–199. [\[CrossRef\]](#)
8. Brown, C.A.; McAdams, T.R.; Harris, A.H.; Maffulli, N.; Safran, M.R. ACL reconstruction in patients aged 40 years and older: A systematic review and introduction of a new methodology score for ACL studies. *Am. J. Sports Med.* **2013**, *41*, 2181–2190. [\[CrossRef\]](#)
9. Lyman, S.; Koulouvaris, P.; Sherman, S.; Do, H.; Mandl, L.A.; Marx, R.G. Epidemiology of anterior cruciate ligament reconstruction: Trends, readmissions, and subsequent knee surgery. *J. Bone Jt. Surg. Am.* **2009**, *91*, 2321–2328. [\[CrossRef\]](#)
10. Klatte-Schulz, F.; Pauly, S.; Scheibel, M.; Greiner, S.; Gerhardt, C.; Schmidmaier, G.; Wildemann, B. Influence of age on the cell biological characteristics and the stimulation potential of male human tenocyte-like cells. *Eur. Cells Mater.* **2012**, *24*, 74–89. [\[CrossRef\]](#)
11. Garbe, E.; LeLorier, J.; Boivin, J.F.; Suissa, S. Inhaled and nasal glucocorticoids and the risks of ocular hypertension or open-angle glaucoma. *JAMA* **1997**, *277*, 722–727. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Mikolyzk, D.K.; Wei, A.S.; Tonino, P.; Marra, G.; Williams, D.A.; Himes, R.D.; Wezeman, F.H.; Callaci, J.J. Effect of corticosteroids on the biomechanical strength of rat rotator cuff tendon. *J. Bone Jt. Surg. Am.* **2009**, *91*, 1172–1180. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Torricelli, P.; Fini, M.; Giavaresi, G.; Carpi, A.; Nicolini, A.; Giardino, R. Effects of systemic glucocorticoid administration on tenocytes. *Biomed. Pharmacother.* **2006**, *60*, 380–385. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Scutt, N.; Rolf, C.G.; Scutt, A. Glucocorticoids inhibit tenocyte proliferation and tendon progenitor cell recruitment. *J. Orthop. Res.* **2006**, *24*, 173–182. [\[CrossRef\]](#)
15. Pittenger, M.F.; Mackay, A.M.; Beck, S.C.; Jaiswal, R.K.; Douglas, R.; Mosca, J.D.; Moorman, M.A.; Simonetti, D.W.; Craig, S.; Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **1999**, *284*, 143–147. [\[CrossRef\]](#)
16. Gregoire, F.M.; Smas, C.M.; Sul, H.S. Understanding adipocyte differentiation. *Physiol. Rev.* **1998**, *78*, 783–809. [\[CrossRef\]](#)
17. Arutyunyan, I.V.; Rzhabinova, A.A.; Volkov, A.V.; Goldstein, D.V. Effect of dexamethasone on differentiation of multipotent stromal cells from human adipose tissue. *Bull. Exp. Biol. Med.* **2009**, *147*, 503–508. [\[CrossRef\]](#)
18. Rui, Y.F.; Lui, P.P.; Li, G.; Fu, S.C.; Lee, Y.W.; Chan, K.M. Isolation and characterization of multipotent rat tendon-derived stem cells. *Tissue Eng. Part A* **2010**, *16*, 1549–1558. [\[CrossRef\]](#)
19. Zhang, J.; Keenan, C.; Wang, J.H. The effects of dexamethasone on human patellar tendon stem cells: Implications for dexamethasone treatment of tendon injury. *J. Orthop. Res.* **2013**, *31*, 105–110. [\[CrossRef\]](#)
20. Frei, B.; England, L.; Ames, B.N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6377–6381. [\[CrossRef\]](#)

21. Poulsen, R.C.; Carr, A.J.; Hulley, P.A. Protection against glucocorticoid-induced damage in human tenocytes by modulation of ERK, Akt, and forkhead signaling. *Endocrinology* **2011**, *152*, 503–514. [[CrossRef](#)] [[PubMed](#)]
22. Chiu, C.H.; Chen, P.; Chen, A.C.; Chan, Y.S.; Hsu, K.Y.; Rei, H.; Lei, K.F. Real-time monitoring of ascorbic acid-mediated reduction of cytotoxic effects of analgesics and NSAIDs on tenocytes proliferation. *Dose Response* **2019**, *17*, 1559325819832143. [[CrossRef](#)] [[PubMed](#)]
23. Hakimi, O.; Poulsen, R.; Thakkar, D.; Yapp, C.; Carr, A. Ascorbic acid is essential for significant collagen deposition by human tenocytes in vitro. *Oxid. Antioxid. Med. Sci.* **2014**, *3*, 119–127. [[CrossRef](#)]
24. Weiler, A.; Hoffmann, R.F.; Stahelin, A.C.; Bail, H.J.; Siepe, C.J.; Sudkamp, N.P. Hamstring tendon fixation using interference screws: A biomechanical study in calf tibial bone. *Arthroscopy* **1998**, *14*, 29–37. [[CrossRef](#)]
25. Wilson, T.W.; Zafuta, M.P.; Zobitz, M. A biomechanical analysis of matched bone-patellar tendon-bone and double-looped semitendinosus and gracilis tendon grafts. *Am. J. Sports Med.* **1999**, *27*, 202–207. [[CrossRef](#)]
26. Hasegawa, A.; Nakahara, H.; Kinoshita, M.; Asahara, H.; Koziol, J.; Lotz, M.K. Cellular and extracellular matrix changes in anterior cruciate ligaments during human knee aging and osteoarthritis. *Arthritis Res. Ther.* **2013**, *15*, R29. [[CrossRef](#)]
27. Sarifakioglu, B.; Afsar, S.I.; Yalbuздag, S.A.; Ustaomer, K.; Bayramoglu, M. Comparison of the efficacy of physical therapy and corticosteroid injection in the treatment of pes anserine tendino-bursitis. *J. Phys. Sci.* **2016**, *28*, 1993–1997. [[CrossRef](#)]
28. Wong, M.W.; Tang, Y.Y.; Lee, S.K.; Fu, B.S.; Chan, B.P.; Chan, C.K. Effect of dexamethasone on cultured human tenocytes and its reversibility by platelet-derived growth factor. *J. Bone Jt. Surg. Am.* **2003**, *85*, 1914–1920. [[CrossRef](#)]
29. Cooper, J.O.; Bumgardner, J.D.; Cole, J.A.; Smith, R.A.; Haggard, W.O. Co-cultured tissue-specific scaffolds for tendon/bone interface engineering. *J. Tissue Eng.* **2014**, *5*, 2041731414542294. [[CrossRef](#)]
30. Pietschmann, M.F.; Wagenhauser, M.U.; Gulecyuz, M.F.; Ficklscherer, A.; Jansson, V.; Muller, P.E. The long head of the biceps tendon is a suitable cell source for tendon tissue regeneration. *Arch. Med. Sci.* **2014**, *10*, 587–596. [[CrossRef](#)]
31. Fredriksson, M.; Li, Y.; Stalman, A.; Haldosen, L.A.; Fellander-Tsai, L. Diclofenac and triamcinolone acetone impair tenocytic differentiation and promote adipocytic differentiation of mesenchymal stem cells. *J. Orthop. Surg. Res.* **2013**, *8*, 30. [[CrossRef](#)] [[PubMed](#)]
32. Pavesi, A.; Adriani, G.; Rasponi, M.; Zervantonakis, I.K.; Fiore, G.B.; Kamm, R.D. Controlled electromechanical cell stimulation on-a-chip. *Sci. Rep.* **2015**, *5*, 11800. [[CrossRef](#)] [[PubMed](#)]
33. Uefuji, A.; Matsumoto, T.; Matsushita, T.; Ueha, T.; Zhang, S.; Kurosaka, M.; Kuroda, R. Age-related differences in anterior cruciate ligament remnant vascular-derived cells. *Am. J. Sports Med.* **2014**, *42*, 1478–1486. [[CrossRef](#)] [[PubMed](#)]
34. Klatte-Schulz, F.; Pauly, S.; Scheibel, M.; Greiner, S.; Gerhardt, C.; Hartwig, J.; Schmidmaier, G.; Wildemann, B. Characteristics and stimulation potential with BMP-2 and BMP-7 of tenocyte-like cells isolated from the rotator cuff of female donors. *PLoS ONE* **2013**, *8*, e67209. [[CrossRef](#)]