

ORIGINAL ARTICLE

Ascorbic acid is essential for significant collagen deposition by human tenocytes *in vitro*

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Keywords

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Abstract

Objective: We sought to compare L-ascorbic acid (AA), the natural form of vitamin C, and magnesium ascorbyl phosphate (MAP), a stable derivative, for their effect on cell proliferation, culture collagen content and collagen expression in tendon-derived cells.

Methods: Tenocytes received these two ascorbate formulations at concentrations between 0.01 mM and 2 M. The effect of supplementation was evaluated in terms of cell growth, collagen expression, collagen secretion, cell morphology and collagen localization.

Results: MAP supplementation resulted in dose-dependent increase in growth rates, whilst AA supplementation was toxic to tendon-derived cells from as low as 1 mM. Collagen expression was only mildly modulated by ascorbic acid, but collagen accumulation was significantly increased, supporting the hypothesis of its possible role in secretion. Immunofluorescence staining revealed differential localization of collagen I *in vitro* with collagen localized outside cells in the presence of ascorbic acid. Multi-photon microscopy further demonstrated that collagen fibrils were only formed in the presence of ascorbic acid.

Conclusions: Taken together, these results suggest that ascorbic acid, preferably the magnesium ascorbyl phosphate formulation, should be routinely used as a supplement to tendon cell culture when collagen fibrils are required, especially in the context of tissue engineering and scaffold development.

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INTRODUCTION

Non-healing tendons, especially in the rotator cuff, are a challenging clinical problem, and there is a consensus in the literature that current strategies for surgical repair are insufficient [1]. Ascorbic acid (vitamin C) is a cheap, and well-characterized antioxidant, known to promote collagen biosynthesis and prevent free radical formation [2]. There is evidence to suggest that ascorbic acid could help tendons to heal, and a number of studies have proposed using it as a supplement to various tendon treatments. For example, Omeroglu *et al* [3] have shown that it improved the rate and quality of rat Achilles repair, possibly due to improved angiogenesis, and proposed high-dose ascorbic acid injections at 2 day intervals. Poulsen *et al* [4] have shown that ascorbic acid can protect hamstring-derived tenocytes from oxidative stress, and proposed it as an additive to glucocorticoid injections. Moreover, it has been shown as early as the seventies that ascorbic acid stabilizes the differentiation of primary avian tendon cells by helping to restore collagen production *in vitro* [5]. Since driving and maintaining cell differentiation has emerged as one of the main strategies of tendon regeneration [6-8], there exists the motivation to determine the optimal concentrations and formulation of ascorbic acid for tendon cell culture.

Ascorbic acid is available in its native form (L-ascorbic acid, AA), as well as in chemically modified forms, such as the more stable derivatives ascorbyl tetraisopalmitate (ATIP) and magnesium ascorbyl phosphate (MAP). Because of its stability and hydrophilic nature [9], MAP has been extensively used in the cosmetics industry, and its effect on skin fibroblasts has been studied *in vitro* and *in vivo* [10]. Table 1 summarizes the properties of AA and MAP in terms of stability and activity.

A number of published studies have mentioned the supplementation of tendon cell growth media with the addition of between 0.5 and 50 µg/ml ascorbic acid [11-13], but equally, in many studies no such supplementation is noted [14-17]. Moreover, in many other studies where tenocytes were expanded *in vitro* with ascorbic acid, the authors have not specified which formulation has been used, leaving the reader to assume that the native form (AA; see formula in Fig. 1a) has been used.

The overall aim of this study was to determine the optimum dose of vitamin C for tendon cell culture supplementation. With a view to achieving that, we tested the effect of two different ascorbic acid formulations at a very wide range of concentrations on human tendon-derived fibroblasts in terms of

proliferation rate, culture collagen content, and collagen expression. Cells used in this study were derived from torn, human rotator cuff, and are therefore likely to represent an example of the cellular population that may require such supplementation.

MATERIALS AND METHODS

Human tendon material, donor demographics and clinical data

Tendon tissue was obtained from the Oxford Musculoskeletal Biobank, with informed donor consent in full compliance with the National and Institutional ethical requirements and the United Kingdom Human Tissue Act (HTA).

Supraspinatus tendon samples were collected from patients with chronic degenerative rotator cuff tendinopathy and partial/full thickness supraspinatus tears. All patients were undergoing surgery for rotator cuff repair or subacromial decompression, during which tendon tissue was resected from the distal torn edge of the tendon and transferred immediately into a sterile tube containing Dulbecco's Modified Eagle's Medium (DME) and Ham's F12 Nutrient Mixture (DMEM:F12; Lonza, UK). Explantation was as previously described [18]. Briefly, tissue was diced and incubated in high serum (50%) DMEM:F12 for 1-2 weeks, until cell migrated out, to be expanded in DMEM:F12 with 10% fetal bovine serum (FBS). No collagenase was used in this process.

Tendon-derived cells for this study were obtained from 1 male and 4 female donors aged 52-79 years (mean age 67.6). Cells from each donor were used individually.

Cell metabolism assay

To monitor the effect of vitamin C supplementation on cell growth, alamarBlue® (AbD Serotec, UK) was used to monitor changes in metabolism as a surrogate for viable biomass. 15.6×10^3 cells per cm^2 were seeded in 96 well plates. Cells were then fed DMEM:F12 media supplemented with 5% FBS (Biosera, UK), 1% pen/strep (Lonza) and different concentrations (0, 0.01, 0.1, 1, 10, 100, 1000 or 2000 mM) of either AA or MAP (both Sigma-Aldrich, UK). For pulsed-supplementation, media was changed every 2 days. The concentration of FBS chosen for the assay (5%) was based on a previous study of the growth of primary human tenocytes in different serum concentrations, showing concentrations under 5% to be insufficient without growth factors supplementation [19].

The viable biomass in each well was measured using 5% alamarBlue in complete media over 2 h. To calculate the relative growth (or change in biomass per cell population), the metabolic rate after supplementation was divided by the metabolic rate before supplementation in the following equation:

$$\text{Relative growth} = \frac{\text{Cell metabolism at endpoint}}{\text{Cell metabolism at time zero}}$$

Fluorescence microscopy of cells and collagen localization

To visualise the appearance of cells receiving AA and MAP, tendon derived cells from 2 donors at a concentration of 5×10^4 cells per plate were seeded onto glass-bottom plates (FluoroDish, World Precision Instruments Inc, PR China) in triplicates. One plate from each donor was supplemented with 2 mM MAP, a second plate with 2 mM AA and a third received standard complete tenocyte media. Media was refreshed and supplemented every 2-3 days. After 7 days, plates were fixed in 10% formalin (Fisher Scientific) for 5 min and permeabilised using 0.1% Triton-X (Sigma-Aldrich) for 5 min. Consequently,

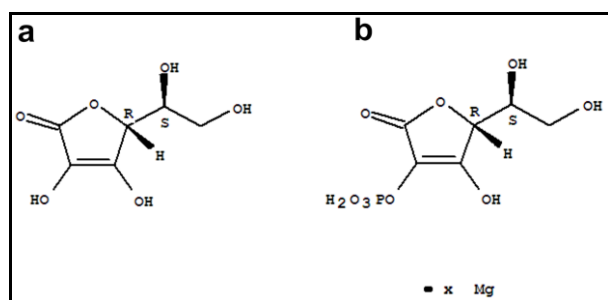


Figure 1. Formulas of, (a) ascorbic acid (AA), and (b) magnesium ascorbyl phosphate (MAP).

Table 1. The Stability and anti-oxidant activity of L-ascorbic acid and magnesium ascorbyl phosphate.

	L-Ascorbic acid	Magnesium ascorbyl phosphate
Stability [9, 20]	-Extremely unstable over time in room temperature and with light exposure -Non-detectable after 3 days in cell culture media	-Improved stability is achieved by introducing a phosphate group protects the molecule from hydrolysis -Good stability in room temperature over long periods of time -Over 95% still present after 7 days in cell culture media
Anti-oxidant activity <i>in vitro</i> [10]	-Good antioxidant activity in aqueous system	-Relatively low antioxidant activity in aqueous system

cells were stained using ActiGreen to enable visualization of the actin cytoskeleton (Invitrogen, UK), and 4',6-diamidino-2-phenylindole (DAPI) to visualize the cell nuclei according to manufacturer's instructions (Molecular Probes). Samples were visualized using a fluorescence microscope (eclipse TE300, Nikon).

To visualize the localization of collagen, cells were seeded at a density of 5×10^4 cells per ml onto 1 cm diameter cover glass slides in 24-well plates and allowed to adhere overnight. Cells were then cultured in 5% FBS/DMEM:F12 with or without 1 mM MAP for 6 days at 37°C in 95% CO₂. Cell media was changed and fresh MAP added after 3 days of culture. Media was then removed and cells washed twice with PBS before fixing for 5 min in 100% acetone. Cells were then washed with PBS and incubated for 1 h at room temperature with 3% horse serum in PBS, followed by an 18 h incubation with rabbit anti-human collagen I (Bio-Rad Laboratories Inc, Kidlington, UK) diluted 1:100 in PBS at 4°C in a humidified chamber. Secondary antibody was Dylight-488 anti-rabbit secondary antibody (Thermo-Fisher Scientific Ltd, Rockford, IL, USA) diluted 1:100 with PBS for 45 min at room temperature. Consequently, cells were stained with DAPI before mounting in VectaShield (Vector Laboratories Inc, Burlingame, CA, USA) and imaging on an Olympus BX40 microscope using an Olympus DP70 camera (Olympus, Southend-on-Sea, UK). IgG and secondary antibody only controls were used to verify specificity of the primary antibody.

pH

The pH of the growth medium supplemented with vitamins was measured using a Seven Easy (Mettler Toledo) pH meter.

Collagen quantification

A hydroxyproline assay [21] was used to quantify the total collagen content of cell populations in 10 cm dishes. Briefly, 400 µl of scraped monolayer samples as well as a collagen standard were hydrolyzed in 6 M hydrochloric acid for 20 h at 95°C. After hydrolysis, sample was centrifuged in a vacuum desiccator and dry samples were dissolved in a citric acid assay buffer. Samples were then oxidized by freshly prepared chloramine-T reagent for 20 min at room temperature on a shaker, before 75 µl of aldehyde perchloric acid was added to each sample. The mixture was left to incubate at 60°C for 1 h, placed on ice to cool to room temperature and samples were read at 560 nm in a SpectramaxPlus 384 plate reader (Molecular Devices).

RNA expression

To measure mRNA expression, tendon-derived cells in passage 2 were plated in 96-well plates (Corning) at a density of 6×10^3 cells per well and allowed to attach

overnight. The following day, cell media was replaced with serum-free media for a period of 24 h, after which the cell medium was replaced with DMEM:F12 supplemented with 0, 0.01, 0.1 or 1 mM of MAP or AA. After 5 days, RNA was extracted and converted to cDNA using the cells to cDNA kit (Ambion) according to manufacturer instructions. Real time quantitative polymerase chain reaction (qPCR) was performed using a ViiA™ 7 (Life Technologies) with software version 1.2 (Applied Biosystems, USA), using Power SYBR® Green (Applied Biosystems) and QuantiTect primer assays according to manufacturer instructions (Qiagen). Cycling conditions were default parameters for relative quantification using Sybr green. Untreated cells were used as controls and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Data was analyzed in terms of relative expression.

Multiphoton microscopy (MPM)

To view collagen fibril formation, cell populations were prepared as for fluorescence microscopy. After 21 days, collagen fibers and cells were imaged by second harmonic generation (SHG) and two-photon excitation fluorescence, respectively. The imaging system consisted of a Zeiss LSM710 NLO (Carl Zeiss, UK) multiphoton microscope coupled to a tunable Ti:Sapphire Chameleon Vision II laser (Coherent, UK) tuned to 860 nm. Images were acquired using a 40 x 1.2 N.A. C-Apochromat water immersion lens. We used the microscope's spectral detectors in lambda scan mode to identify a second harmonic signal at approximately 430 nm (half the wavelength of the laser), which suggests the presence of collagen.

Experimental design and statistical analysis

All experiments with cells were carried out using cells from at least 2 donors as biological replicates, except collagen accumulation which was only performed with cells from one donor, as it requires a large population of cells in early passage. Results were analysed by two-way ANOVA test with a Bonferroni multiple comparison post test, using Prism version 5. Results are expressed as mean ± SD and P values were annotated on the graph as follows: *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

RESULTS

As can be seen in Fig.2, cells receiving > 100 mM of MAP showed consistently increased growth rates compared to other groups. However, cell growth rates varied depending on the point of monitoring. In the shortest time point of 48 h (Fig.2a), there was a nearly linear increase in cell growth rate with both MAP and AA, up to 100 mM. At higher concentrations, cell growth rate continued to increase with increase in MAP

concentrations, but was reduced with > 100mM of AA. A more moderate increase in cell growth rate as a response to one-off supplementation of MAP was observed after 72 h (Fig.2b) and 120 h (Fig.2c). The flattening of the curve may suggest cells reaching confluence. An additional explanation for that flattening is that much of the supplemented ascorbic acid was consumed. In all experiments, cells exposed to 100 mM or more of AA showed a reduction in cell growth. More frequent supplementation over 144 h (Fig.2d) further confirmed this differential effect, where a repeatedly refreshed concentration of as high as 1 M or 2 M MAP increased cell growth rate, whilst a repeatedly refreshed concentration of 1 M or 2 M AA caused a sharp decline in growth rates. This was further confirmed by visualizations of cell populations

receiving 2 mM MAP or AA for 7 days. As can be seen in Fig.3, cells exposed to MAP showed higher confluence than control. Cells receiving AA showed reduced confluence as well as rounded morphology and higher incidence of round cells and cell aggregates, suggesting the toxic effect of AA was induced in concentrations as low as 2 mM.

Decline in cell growth at very high concentrations (> 100 mM) of AA could be easily attributed to pH levels of the supplemented media (Fig.4), which were slightly acidic at 10 mM (around pH 6.71) and highly acidic at 100 mM (pH 3.75). However, pH results do not explain the toxic effect observed at around 2 mM AA in the imaging. Moreover, the addition of MAP did not significantly change the pH, and no adverse effect to cells was observed.

Figure 2.
The effect of a single media supplementation with ascorbic acid (AA) and magnesium ascorbyl phosphate (MAP) on metabolism, measured by alamarBlue after 48 hours (a), 72 hours (b), 120 hours (c), or over 144 hours, where vitamin was supplemented repeatedly every 48 hour (d).

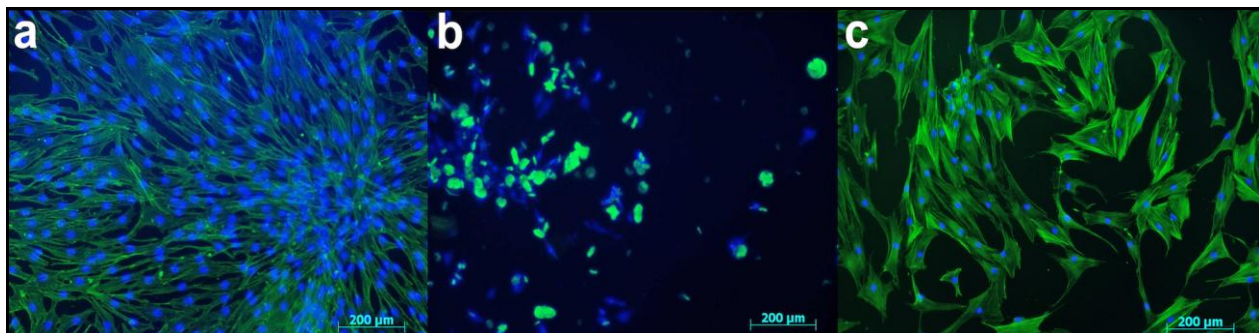
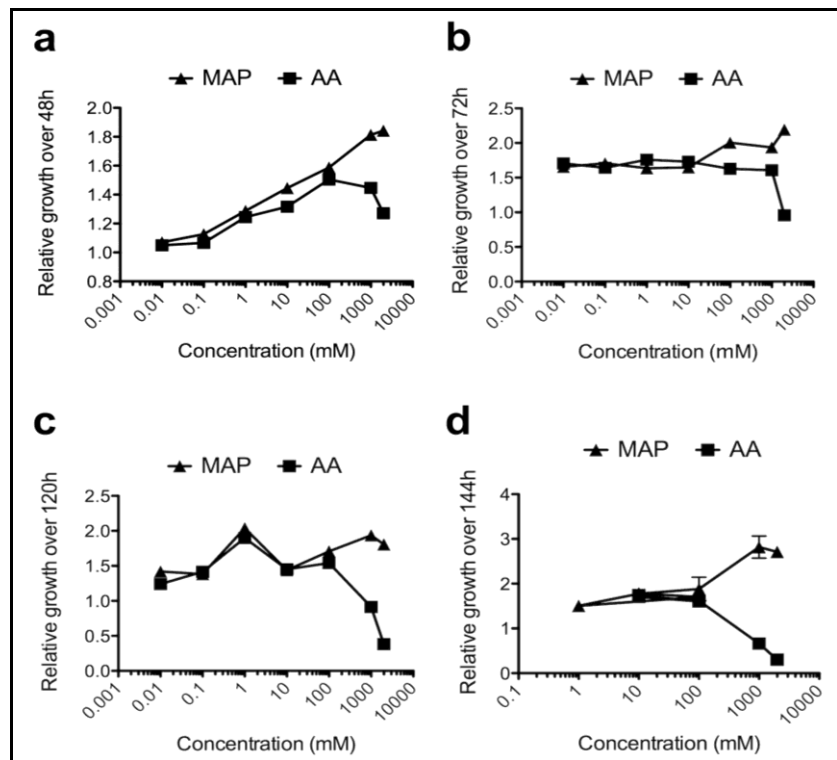


Figure 3. Fluorescence microscope images showing the confluence and morphology of tenocytes stained in green (actin filaments) and blue (DAPI, nucleus stain). Cells were exposed to 2 mM MAP (a), 2 mM AA (b), or no supplementation (c) for 7 days. Notable are clusters of rounded cells, visible as small rounded bundles of actin filaments in the culture supplemented with AA.

To avoid the toxic effect of acidified media, further work to measure collagen synthesis was done at a concentration up to 1 mM. To measure the effect of AA and MAP supplementation on collagen I and III expression, semi-quantitative PCR was performed. As can be seen in Fig.5, *COL1A1* (the alpha 1 chain of collagen I) was expressed by all groups, and there was a slight and statistically significant increase in its production by cells receiving 0.1 mM MAP. Whilst there was a small increase in expression by all groups receiving MAP, this was not statistically significant. *COL3A1* (the alpha 1 chain of collagen III) was also expressed by all groups with no statistically significant effect of neither AA nor MAP supplementation.

Slightly differing from gene expression, collagen accumulation, measured by quantifying hydroxyproline, was significantly influenced by the addition of 1 mM MAP. At all time points measured (days 7, 14 and 21; Fig.6), there was a large and significant increase of around 2-3 fold in total collagen in the plate. Moreover, in non-supplemented cultures, only minute quantities of hydroxyproline were

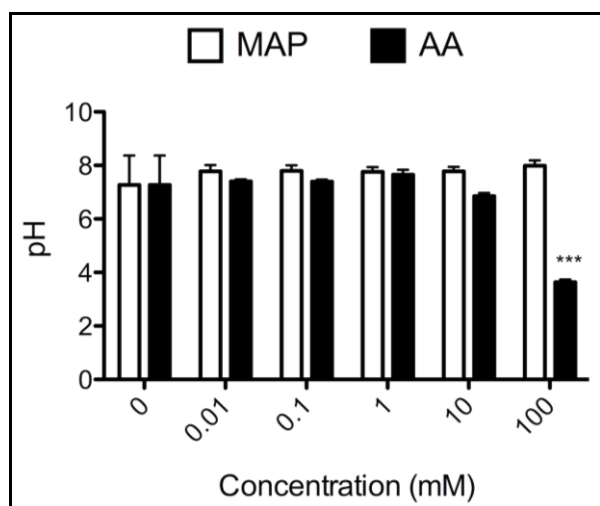


Figure 4. pH of complete cell growth media supplemented with AA and MAP.

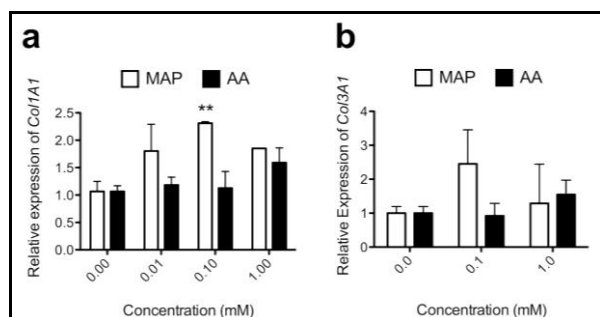


Figure 5. The expression of genes *COL1A1* (a) and *COL3A1* (b) by tendon-derived cells after 5 days in different ascorbic acid concentrations (0.01mM, 0.1mM, 1mM) or no supplementation (0mM). Expression was measured using semi-quantitative RT-PCR technique. GAPDH was used a housekeeper to normalize data (n=3).

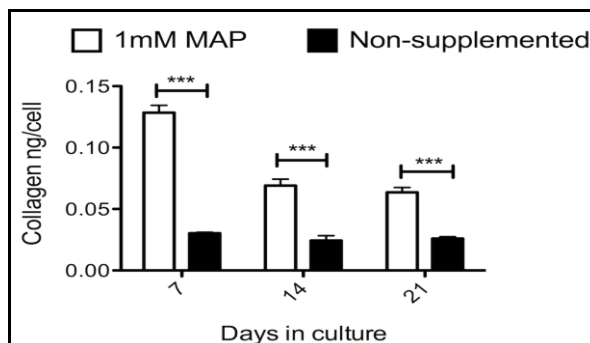


Figure 6. The accumulation of collagen in cultures supplemented with 1mM MAP or non-supplemented over 7, 14 and 21 days. Collagen content was measured by hydroxyproline assay and amounts are at ng/cell.

detected. This was visually confirmed by the presence of fully formed collagen fibrils, detected by MPM microscopy, in cultures of tendon-derived cells after 21 days supplementation of 1 mM MAP (Fig.7b), but no detectable SHG signal in non-supplemented cultures (Fig.7a). It should be noted that collagen content was lower after 14 and 21 days of supplementation compared to 7 days. This is most likely to be the result of the normalization to cell number, which nearly doubled in number between days 7 and 14.

Finally, further work to determine the localization of collagen revealed that whilst non-supplemented cultures did contain collagen I (stained green in Figs.8a&8b), it was confined to the cytosol of the tendon cells, and the staining is concentrated around the cell nuclei. In contrast, cells receiving 1 mM MAP appear to have secreted collagen I, as they are embedded in an extensive mesh of fibrils, which is localized both proximally and away from the cell nuclei.

DISCUSSION

Vitamin C has been proposed as a tendon culture supplement in the past, but no optimal dose or formulation has been indicated. Here, we investigated the effect of two vitamin C formulations, native AA and the modified derivative MAP in a very wide range of concentrations (0.001-2 M) on tendon-derived cells. Results showed a dose-dependent increase in cell growth rates in the presence of MAP. Similar results have been reported in previous studies of skin fibroblasts [21] and hyalocytes [22].

Moreover, we have found a differential response to AA and MAP. Whilst MAP appeared safe and effective even at very high concentrations (0.001-2 M), AA was strongly toxic to cells in concentrations above 10 mM, and negatively affected cell morphology at concentrations from 1 mM. Sommer *et al* [22], studying the effect of ascorbic acid on hyalocytes,

reported toxicity of AA at concentrations of 300 µg/ml or more (the equivalent of around 1.7 mM), but did not suggest any explanation for this toxicity. Another study has reported that depending on the growth media, ascorbic acid at concentrations above 1 mM could be toxic to various human cell lines because it generates hydrogen peroxide [23].

In this study, we confirmed strong toxicity at concentrations higher than 10 mM, which rendered the DMEM:F12 media acidic, but we also observed some toxicity when tenocytes were exposed to lower concentrations (2 mM ascorbic acid) in the form of altered, rounded tenocyte morphology.

More interestingly, we could not find any toxic response to MAP, even at concentrations as high as 100 mM and 1 M. Not only were these high concentrations non-toxic, but they appeared to have a positive effect on cell metabolism, used as a surrogate to indicate cell proliferation. Speculatively, The

positive effect of high MAP concentrations may be related to the increased osmolarity of the growth media, known to influence chondrocytes [24] and tenocytes *in vitro*. Alternatively, increased concentrations of magnesium ions may have an effect on various cell functions, as previously reported for many cell types.

Collagen production was also modulated by ascorbic acid supplementation. Supplementation with MAP resulted in a small but statistically significant increase in collagen I expression. Collagen accumulation, measured by hydroxyproline assay, was also shown to have increased by 2-3 folds in MAP-supplemented cultures. Imaging of collagen fibrils formed in the MAP-supplemented cultures further corroborated this. It is important to stress at this point that the media used to grow tendon cells (DMEM:F12) is formulated to contain 0.00863 mM ascorbic acid phosphate, but our findings demonstrated that this concentration was not sufficient to enable collagen I secretion.

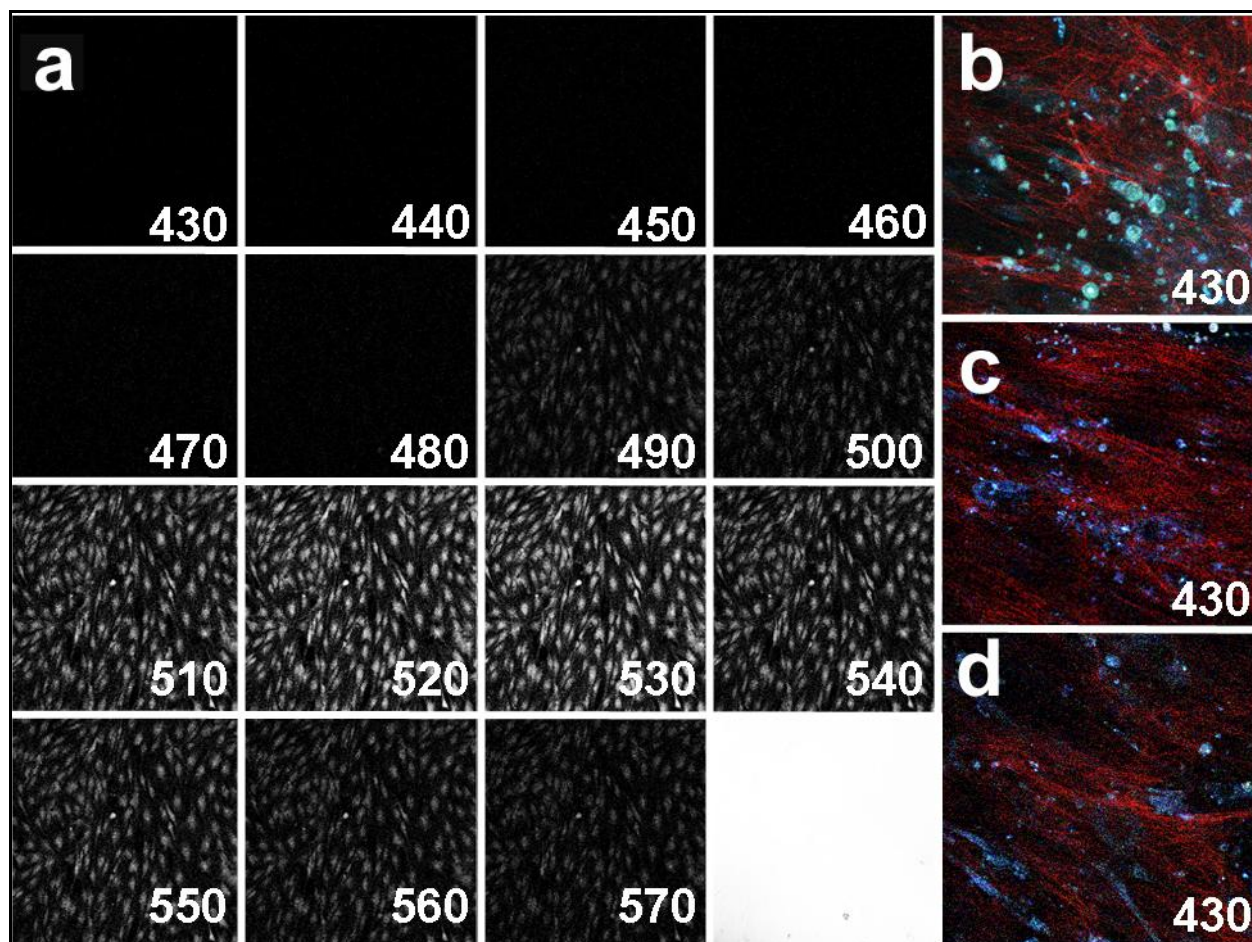


Figure 7. Spectral images acquired by MPM showing the presence of collagen in MAP supplemented cultures and its absence in non-supplemented cultures. Image (a) is showing a spectral scan between the wavelength of 430-570 nm of a culture with no vitamin supplementation. Cells can be seen at wavelength x-x due to auto-fluorescence (shown in white). Because the multiphoton laser was tuned to 860 nm, a second harmonic signal would be expected at 430 nm if collagen fibrils were present. Since there was no second harmonic signal visible at 430 nm (top left window), it was concluded that no significant collagen bundles were present. Images labeled (b), (c), and (d) are the second harmonic signal at 430 nm of cultures supplemented with 1 mM MAP for 21 days, showing clear collagen bundles (x40).

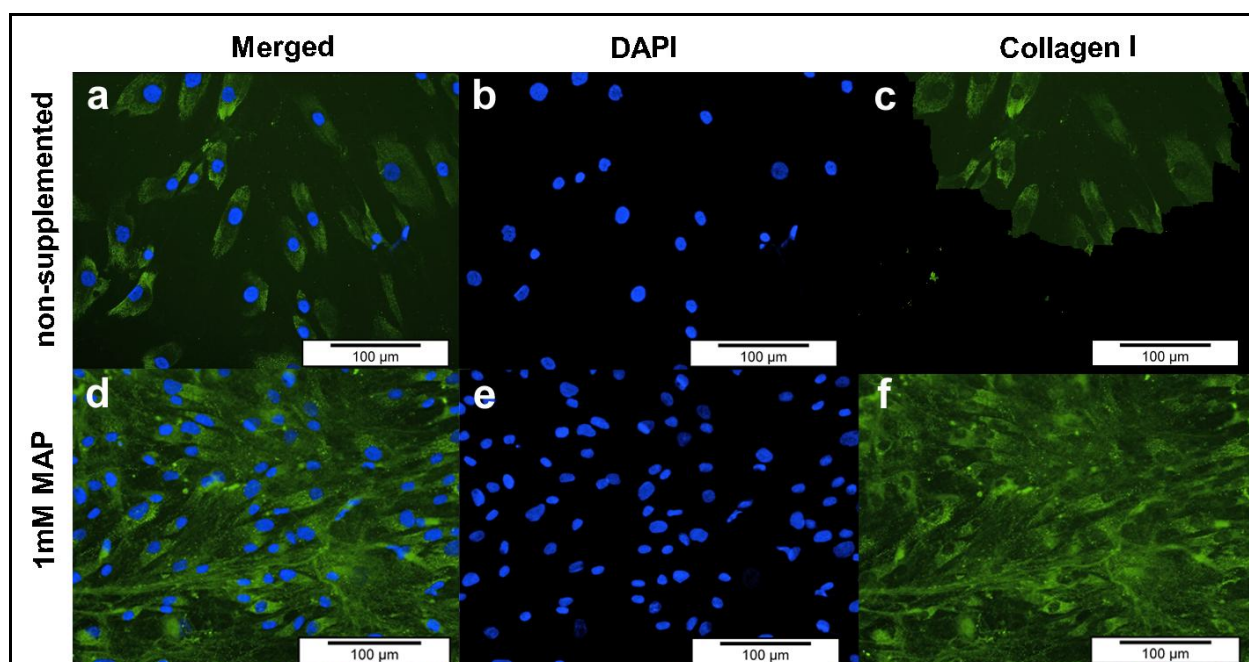


Figure 8. The presence and localization of collagen I in tendon cultures. Collagen is shown in green (c, f), nuclei in blue (b, e), and the merged images (a, d). Collagen is present in the non-supplemented cultures (a, b, c), but is limited to the cytoplasm at the proximity of the cell nuclei. In cultures supplemented with 1 mM MAP (d, e, f) the collagen is seen to form a denser, continuous fibrillar layer and is not concentrated exclusively around the nuclei.

Whilst there is no clear evidence for the exact role of ascorbic acid in collagen synthesis, there are many studies demonstrating its direct effect on its transcription, stability and accumulation [25, 26]. Studies on skin fibroblasts have shown that ascorbic acid increases the transcription rate and stability of type I collagen [27]. Moreover, ascorbate has been shown to act as a cofactor in the hydroxylation of proline to hydroxyproline and of lysine to hydroxylysine, both required to stabilize the collagen triple helix [28]. However, *in vitro* experimentation has shown only a minor effect of ascorbic acid on lysine hydroxylation or intracellular degradation of collagen [25]. It was clearly demonstrated by Graham *et al* [29] that ascorbate-deplete, intestinal smooth muscle cells and human dermal fibroblast secreted 75 and 95% less procollagen, respectively, than cells grown in ascorbate-supplemented media. Thus it has been suggested that the most critical role of ascorbic acid *in vitro* is related to collagen secretion rather than synthesis [29]. Our findings of a rather moderate change in collagen I expression, compared to a significant change in hydroxyproline content and the accumulation of collagen fibrils outside the cells, are in line with this hypothesis.

A previous study by Brink *et al* [30] has shown that although tendon-derived cells displayed intense collagen staining, the collagen appeared to be intracellular and localized around the cell nuclei. They hypothesized that this is likely due to the lack of

ascorbic acid in the culture medium, but did not go further to prove this [30]. Here, we demonstrated that high ascorbic acid supplementation was effective in inducing collagen secretion *in vitro*, and that cultures supplemented with 1 mM MAP demonstrated clear collagen fibril formation.

A number of studies have reported a contradictory effect of MAP as a cell culture supplement, and while work using human dermal fibroblasts showed good efficacy of this ascorbic acid formulation in stimulating collagen synthesis [21], studies on intestinal smooth muscle cells showed reduced efficacy of MAP compared to AA and no advantage to using the stable modification [29]. Such conflicting results indicate the importance of testing and reporting cell-specific and media-specific effects of vitamin supplementation, rather than deriving information from other cell types.

Finally, whilst the clinical implications of our *in vitro* study are not fully clear, they can provide a starting point to those wishing to incorporate ascorbic acid in tissue engineered scaffolds or use them as additives to other tendon treatments.

To summarize, our finding of the complete absence of extra-cellular collagen fibrils in human primary tenocyte cultures not receiving ascorbic acid, coupled with good levels of collagen accumulation and secretion in cells receiving ascorbic acid, provides a strong indication that it is a key factor for tenocyte function, and should be used as a standard supplement

in the *in vitro* culture of tendon-derived cells. Based on the evidence presented here, we propose a high dose (at least 1 mM) of MAP to be regularly added to tendon cell cultures, especially when collagen fibril formation is desired. However, it should be noted that under the standard *in vitro* culture conditions, similar doses of non-modified L-ascorbic acid are toxic to tenocytes.

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