UCDAVIS VETERINARY MEDICINE
Students Training in Advanced Research

Understanding the Tenogenic and Cytoprotective Roles of Vitamin C

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BACKGROUND:

Tendons function to transfer mechanical loads from muscle to bone [1]. Tendinopathy is an overarching term that describes a wide variety of tendon diseases and their respective severities [2]. Tendinopathy of the superficial digital flexor tendon can result in debilitating lameness for horses regardless of riding discipline [3,4]. Research continues to elucidate the complex mechanism of healing that occurs after disease or injury, however there are still no curative therapies for tendinopathies. Additionally, drugs utilized as an anti-inflammatory in tendon injuries, such as dexamethasone, have proved to be linked to a predisposition for rupture [5]. Recent studies have shown that dexamethasone causes an overall inhibition of cellular proliferation as well as collagen synthesis [6,7]. Novel therapies have suggested that Vitamin C supplementation is not only able to bolster collagen synthesis in the face of injury due to its role in collagen assembly but can also serve as a cytoprotective agent against glucocorticoid induced cellular apoptosis [6,7].

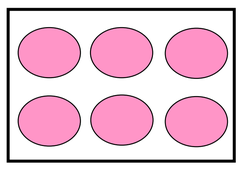
MAIN HYPOTHESIS:

We hypothesize that supplemental vitamin C will bolster collagen synthesis and act as a cytoprotective agent against negative anti-inflammatory glucocorticoid consequences in tendon proper (TP) and peritenon (PERI) stem/progenitor cells of the equine superficial digital flexor tendon (SDFT).

METHODS

Cell culture

Cells were harvested from SDFT of horses and grown to 95% confluence



Cells seeded in 6 well plates and treated with vitamin C in 3D constructs and in 2D for dexamethasone challenge

FOUR TREATMENTS:
0 µM
100µM
200µM
400µM
800µM
+
Each treatment was challenged with 10nM dexamethasone for 3 days in 2D for qPCR and biochemical assays

qPCR

RNA Isolation of Vitamin C treated cells in 3D
+
Vitamin C treated cells challenged with 10 nM treatment dose equivalent of dexamethasone in 2D

Reverse Transcription with High-Capacity Reverse Transcription Kit

TaqMan RT-qPCR

Marker	Gene
Tenogenesis	*SCX,MKX*
Matrix Assembly	*BGN, DCN, COL1A1, PLOD1, LARP6*
Vascular formation	*CSPG4*
Chondrification	*ACAN*
Cellular proliferation	*MKI67*
Apoptosis	*FOXO1, FOXO3A*

Hydroxyproline Assay

Dried Vitamin C treated 3D tendon constructs in a series of acid and boiling steps
↓
Generated L-hydroxyproline standards for standard curve analysis
↓
Plated in a clear 96- well plate and read in a spectrophotometer

Apoptosis Assay

Added 100 µl of Apo-ONE Caspase-3/7 Reagent to each well of a black 96-well plate seeded in 2D after 72 hour treatment with 10 nM of Dexamethasone
↓
Incubated for ~30min
↓
Measured fluorescence with a spectrophotometer

TENOGENIC EFFECTS OF VITAMIN C ON GENE EXPRESSION

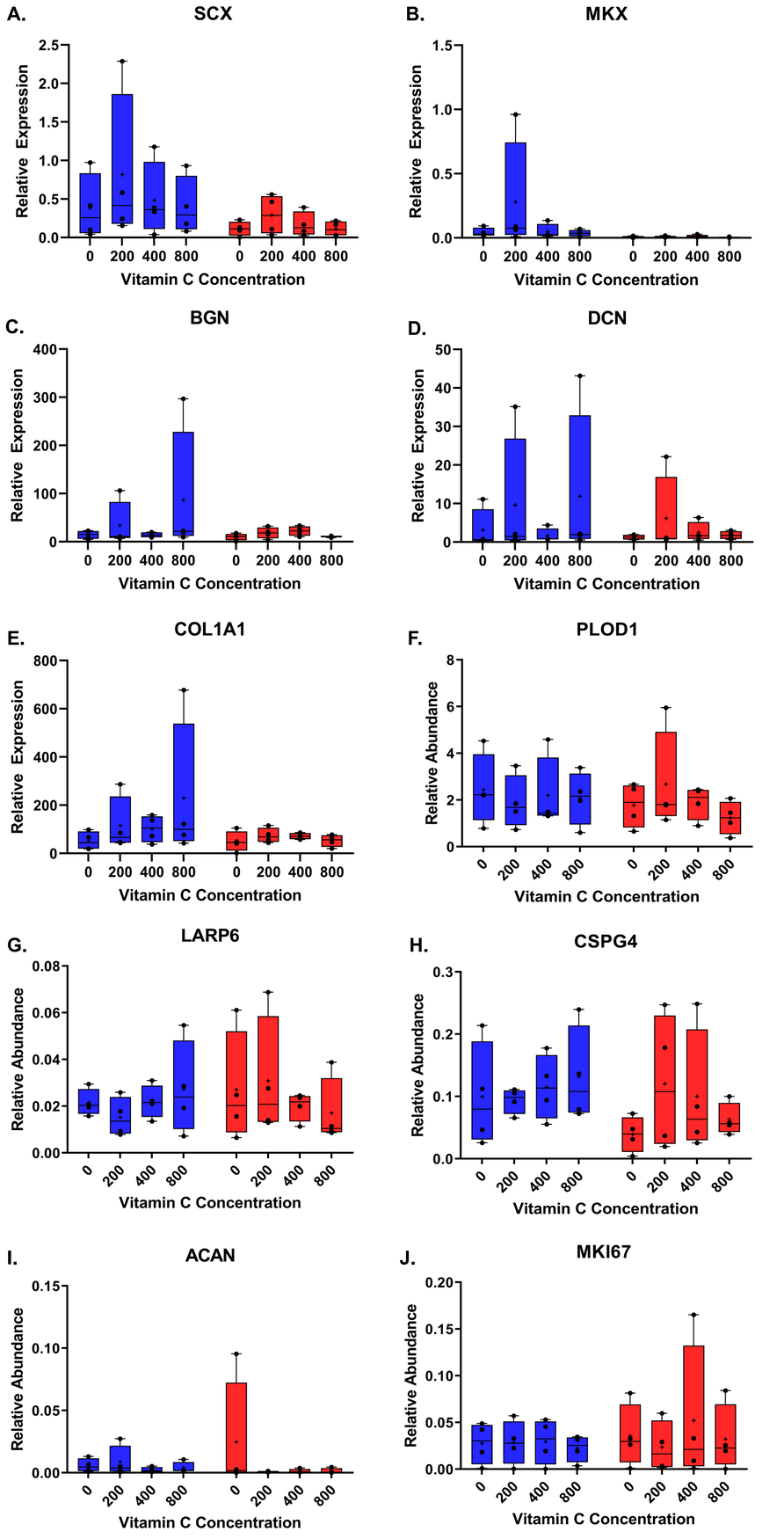


Figure 1: Vitamin C increased the relative expression of tenogenic markers in tendon constructs with TP and PERI-derived cell populations. (A,B): Tenogenic marker *SCX* trended upwards when supplemented with 200 nM in both cell types. *MKX* also increased expression when supplemented with 200 nM of vitamin C in TP-derived (BLUE) cells but remained unchanged in PERI-derived (RED) cells. **(C,D,E,F,G):** Matrix assembly markers BGN and DCN showed an increase at concentrations of 400 nM and 800nM in TP cells and no change in PERI cells. *COL1A1* increased in expression when supplemented with vitamin C in TP-derived cells whilst PERI-derived cells remained unchanged. *PLOD1* showed no change in expression in TP-derived cells but increased in PERI-derived cells supplemented with 200 nM. *LARP6* showed no change in expression in any cell type or treatment group. **(H):** Vascular marker *CSPG4* showed an overall increase in PERI cells compared to the control. **(I):** Chondrification marker *ACAN* showed an overall decrease in PERI-derived cells when supplemented with vitamin C while TP-derived cells remained unchanged. **(J):** Cellular proliferation marker (*MKI67*) showed an increase in expression when supplemented with 400 nM in PERI-derived cells while TP-derived cells remained unchanged. Statistical significance was analyzed via two-way ANOVA and Tukey's multiple comparison test; only trends were found.

HYDROXYPROLINE ASSAY

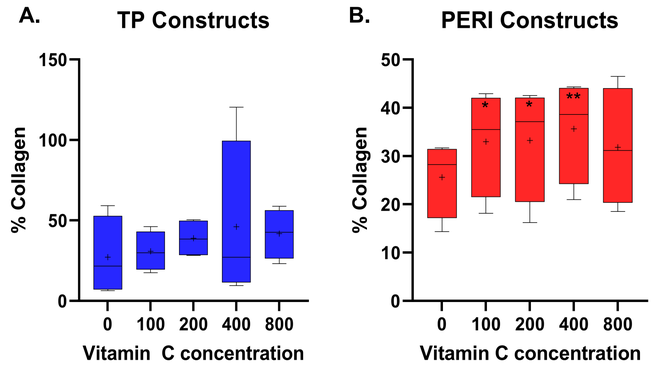
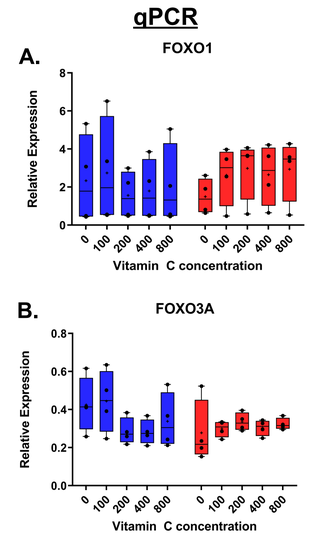


Figure 2: Vitamin C supplementation increases the overall collagen content in tendon constructs with TP and PERI cell populations. (A): Levels of collagen for constructs of TP-derived (BLUE) progenitor cells trended upward with increasing amounts of vitamin C. **(B):** Constructs of PERI-derived (RED) progenitor cells treated with vitamin C showed a significant ($p<0.05$) increase in collagen content relative to the control in 100 nM, 200 nM, and 400 nM ($p<0.01$). Statistical significance was found via a paired t-test utilizing GraphPad Prism.

APOPTOSIS

qPCR



Caspase 3/7 Assay

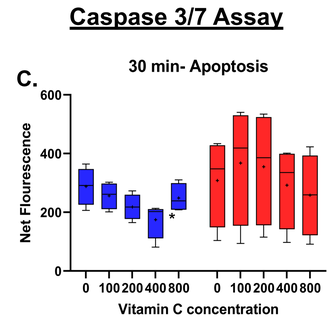


Figure 3: Vitamin C supplementation decreases overall apoptosis in TP and PERI cell populations treated with dexamethasone. (A): Vitamin C supplementation had no effect on the genetic expression of apoptotic marker *FOXO1* in both TP-derived (BLUE) and PERI-derived (RED) cells. **(B):** Apoptotic marker *FOXO3A* showed a relative decrease in expression mainly in TP-derived cells at 200 nM and 400 nM while expression for PERI-derived cells trended higher, but not with significance. **(C):** The Apo-ONE Caspase-3/7 assay showed the greatest decrease in apoptotic activity when dexamethasone-challenged TP-derived cells were supplemented with 400 nM of Vitamin C ($p<0.05$) while PERI-derived cells remained unchanged. Statistics were determined with a paired t-test utilizing GraphPad Prism.

CONCLUSION

In conclusion, vitamin C affected the genetic expression of TP and PERI derived 3D tendon constructs. More specifically, TP-derived cells seemed to increase tenogenic genes while PERI-derived cells increased both cellular proliferation and matrix assembly genes. Vitamin C also caused an overall decrease in apoptotic gene expression as well as apoptotic enzyme activity, mainly for TP cells. From these findings one can conclude that vitamin C not only caused an overall increase in tenogenic effects but also plays a cytoprotective role in dexamethasone challenged cells.

Literature Cited

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Acknowledgements

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