Effect of Hydrogen Peroxide Treatment on the Biomechanical and Biochemical Properties of Soft Tissue Allografts

Musculoskeletal Transplant Foundation Research and Development Department
125 May Street, Edison, NJ 08837

SUMMARY

Hydrogen peroxide (H_2O_2) is one of the most common chemicals used in the treatment of allograft bone and soft tissue for its potent disinfectant (cleaning) and anti-bacterial properties.[1] Some authors have studied the effects of exposure to a low concentration (3%) of H_2O_2 on cortical bone and have demonstrated a reduction in osteoinductivity after one hour.1 Since most tissue processors use similar concentrations and exposure times of H_2O_2 to treat soft-tissue allografts, it raises the question regarding the effect this treatment may have on the properties of soft-tissue allografts. The purpose of this study was to investigate the biomechanical and biochemical properties of soft tissue allografts when using H_2O_2 at concentrations and times of exposure that are similar to a commercially available tissue process.[2]

Bone patellar tendon bone (BPTB) allografts from three donors were used for the investigation. Both the control and treated samples were donor-matched. The grafts in the control group went through MTF's proprietary aseptic soft tissue processing method without the use of H_2O_2 while the grafts in the treated group were exposed to a cleaning and a disinfecting step of H_2O_2 treatment from a commercially available tissue process.[2] The biomechanical (tensile) properties as well as some biochemical properties (resistance to enzymatic degradation and native Collagen I levels) of the tendons were evaluated after treatment and compared to the control group.

The treatment of the tendons with H_2O_2 resulted in a reduction in biomechanical strength as well as alterations in the collagen composition. Deterioration of all the mechanical properties was detected after treatment with H_2O_2, with a significant difference in stiffness and ultimate stress. A

significant negative effect was also observed in the biochemical properties, where resistance to collagenase digestion was decreased in treated tissues and the levels of native, non denatured Collagen I were reduced compared to controls.

INTRODUCTION AND BACKGROUND

There are many different processing methods employed by tissue banks to eliminate objectionable microorganisms like bacteria and spores that may be present on some allograft tissues.[3] Most of these processes are proprietary and are designed to reduce the chance for infection and potential host rejection when implanted. Some of the treatment techniques used to clean, disinfect and sterilize allograft bone and soft tissues include exposure to the following: antibiotic solutions, boiling water, ethanol, alcohol, disinfectants such as H_2O_2, different levels of gamma radiation, acid wash, ethylene oxide, or a combination thereof.[4] The challenge is that different treatment methods can have deleterious effects on the biomechanical integrity and biochemistry of the allograft tissues.[1,5] In some cases, these can translate into less than desirable clinical outcomes.[6,7,8]

One of the most common treatment methods used for eliminating objectionable organisms on allograft tissues is exposure to varying levels of gamma radiation.[5] Exposure to levels of 20 kGy or higher have been shown to significantly compromise the mechanical properties of soft tissue grafts compared to non-irradiated controls both in the lab and in clinical settings.[5,6,7] When soft tissue allografts are exposed to radiation levels below 20 kGy the results have been mixed.[8,9] Shah et al. demonstrated clinical outcomes consistent with historic autograft controls (5.6% failure rate) when using

soft-tissue allografts treated with a dose of gamma radiation at 12-18 kGy for primary anterior cruciate ligament reconstruction (ACLR).[9] Gorschezky et. al. evaluated the use of soft-tissue allografts treated with a low dose of radiation (15 kGy) combined with a chemical treatment for primary ACLR.[8] The failure rate of the allograft group was statistically higher (44.7%) versus the autograft control group (5.9%) at six years.[8] This large disparity in outcomes between Shah and Gorchewsky raises the question regarding the effects of a chemical treatment process on the properties of soft tissue allografts.

In addition to varying levels of gamma, tissue processors also use chemical cleaning agents like H_2O_2 to treat allograft bone and soft tissue.[1,2,4] Hydrogen peroxide has oxidizing properties and is often used as an effective cleaning agent for anti-bacterial use.[1] Even brief exposure to H_2O_2 can change allograft bone and soft tissue from the tissue's natural color to a "white" or "bleached" appearance.[1,2] Besides the visual change, H_2O_2 can also change the inherent characteristics of the tissue.[1] DePaula et al. evaluated the effects of 3% H_2O_2 exposure over time to the osteoinductive capabilities of cortical bone.[1] The exposure of H_2O_2 to cortical bone demonstrated a statistically significant decrease in osteoinductivity over time.[1] If exposure to 3% H_2O_2 has a negative effect on the osteoinductive capabilities of cortical bone, then what effect does it have on the biomechanical and biochemical properties of soft tissue allografts?

MATERIALS AND METHODS

Sample Preparation

Hemi-BPTB's were processed from 3 human cadaveric donors (n=6 treated and n=6 controls; donor paired) with research consent, aged 50 to 54 years old. Specimens in the treated group were aseptically processed and then

exposed to H_2O_2 per the instructions of a published method for treating soft tissue allografts.[2] The grafts were subjected to one 30 minute soak in 3% H_2O_2 on an orbital shaker @ 75 RPM followed by a 120 minute soak in 6% H_2O_2 in a desiccator under vacuum followed by a series of water rinses. Control specimens were aseptically processed and subjected to an antibiotic cocktail solution followed by a series of water rinses without the use of H_2O_2.

Mechanical Testing

Prior to potting the samples for testing, the cross-sectional area of the tendon tissue was measured at 3 locations using a calibrated laser micrometer (LK-G87, Keyence, Woodcliff Lake, NJ) for the thickness of the gauge region and a calibrated digital caliper (Model 500-196-20, Mitutoyo, Aurora, IL) for the width. All samples were measured in the inferior, middle, and superior regions. After measuring the cross-sectional area, two perpendicular holes were drilled into the center of each bone block of the Hemi-BPTB samples. Two 3/32" stainless steel pins were positioned in the holes orthogonally to secure the sample in the potting fixture. The samples were subsequently potted into two stainless steel cups (25.4 mm in diameter and 25.4 mm in depth). Each bone block was positioned in the potting fixtures such that approximately 1-2 mm of bone was exposed above the rim of the potting fixture. The bone cement for potting was prepared by mixing AccurateSet copolymer powder and methyl methacrylate monomer (AccuSet, Hackensack, NJ). The bone cement was poured around the bone block in the potting fixture and care was taken to ensure the soft tissue did not come in contact with the bone cement. While the cement was curing, the sample was wrapped in saline-soaked gauze. The potting procedure was conducted with the inferior bone block first and then repeated with the superior bone block. The potted tissue samples were placed into the environmental fluid chamber

and positioned in the MTS tensile testing system (Minibionix 858, Eden Prairie, MN). Phosphate buffered saline (PBS) was poured into the fluid chamber and the tissue was allowed to rehydrate for 30 minutes *(Figure 1)*.

The samples were tested in an environmental chamber filled with PBS maintained at 37 ± 2°C using a heat lamp and a thermocouple to continuously monitor the temperature of the water bath. All testing was conducted using a servo-hydraulic MTS load frame equipped with calibrated LVDT and load cell. The samples were pre-tensioned to 89 Newtons (N) and help in displacement control for 25 minutes. The tension was adjusted back to 89 N at 5 minutes and 15 minutes following tissue relaxation. After 25 minutes, the samples were unloaded in displacement control to 30 N and held for 1.0 minute in force control before continuing with cyclic loading. The samples were then cyclically loaded in force control for 1000 cycles (1.0Hz) between 50 and 250 N. Cyclic creep was measured between cycle 3 and 1000, and stiffness was measured during cycle 10. Finally, the samples were ramped to failure at rate of 50 mm/min. The maximal load to failure was measured for each specimen and the location of the failure was noted.

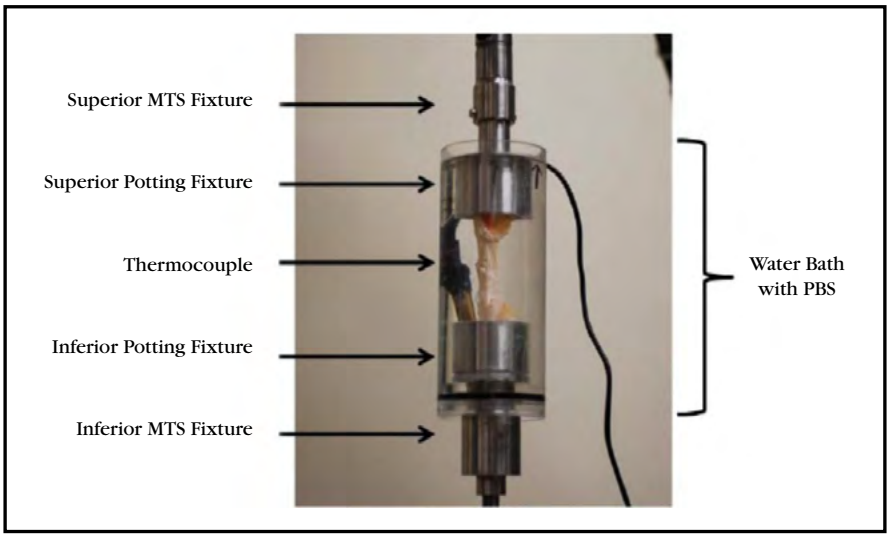

Figure 1: *Test setup*

Biochemical Testing

Collagenase Digestion

Enzyme degradation was measured by immersing 0.1g of wet tendon into a buffered solution containing 200 units/ml bacterial collagenase (C0130, Sigma-Aldrich, St. Louis, MO). Samples were incubated for about 4 hours with the enzymes and then the amount of each sample remaining was determined gravimetrically. If collagen in the tendons is denatured due to a harsh treatment, it would degrade more easily after exposure to bacterial collegenase.

Native Collagen I ELISA

Collagen I was extracted from tendons using a series of mechanical and enzymatic steps. The levels of native Collagen I in controls and treated samples were quantified using a commercially available Enzyme-Linked ImmunoSorbent Assay (ELISA) kit.

RESULTS & CONCLUSIONS

Visual and Tactile Assessment

After exposure to the different steps of H_2O_2 treatment, there was a noticeable change in the visual and tactile properties of the grafts.

Mechanical Testing

A general linear model was used to compare the treatment group to the control group. All statistical analysis was conducted using SAS 9.2 (Cary, NC). The data is summarized by treatment in Table 1. The general linear model showed a statistically significant treatment effect with a reduction in stiffness ($p = 0.022$, *Figure 3*) and ultimate stress ($p < 0.001$, *Figure 4*).

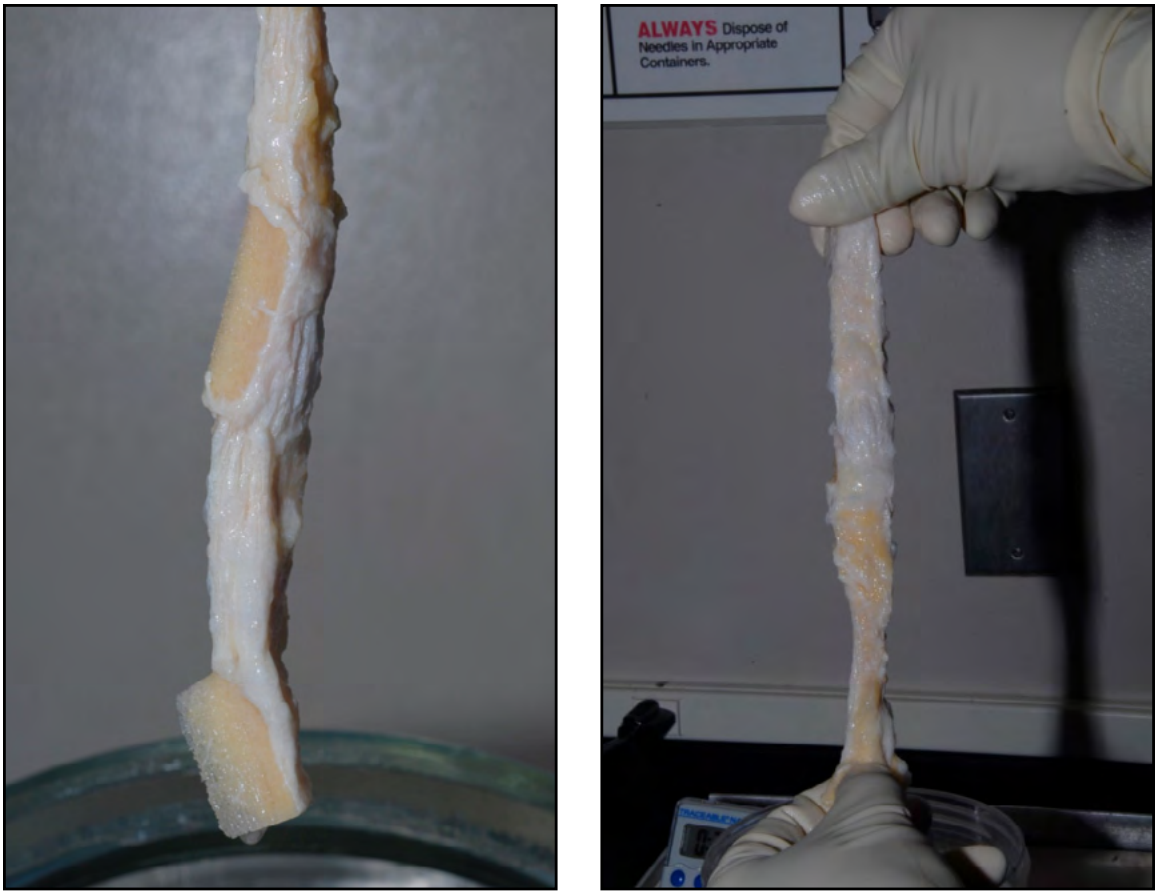

Figure 2: *Tendon and bone resembled a white (bleached) color with a swollen and "bubbly" appearance after H_2O_2 treatment*

Mechanical Testing

A general linear model was used to compare the treatment group to the control group. All statistical analysis was conducted using SAS 9.2 (Cary, NC). The data is summarized by treatment in Table 1. The general linear model showed a statistically significant treatment effect with a reduction in stiffness ($p = 0.022$, *Figure 3*) and ultimate stress ($p < 0.001$, *Figure 4*).

Table 1. Average (± St. Dev.) creep, stiffness, maximum load, and ultimate stress by treatment (treated and control)

Treatment	Creep [mm]	Stiffness [N/mm]$^{+}$	Maximum Load [N]	Ultimate Stress [MPa]$^{++}$
None (control)	0.38 ± 0.22	239.4 ± 5.7	1627 ± 344	22.6 ± 4.9
Treated	0.41 ± 0.28	198.3 ± 32.6	1354 ± 487	10.4 ± 4.3

$^{+}$ Statistically significant; p=0.022

$^{++}$ Statistically significant; p<0.001

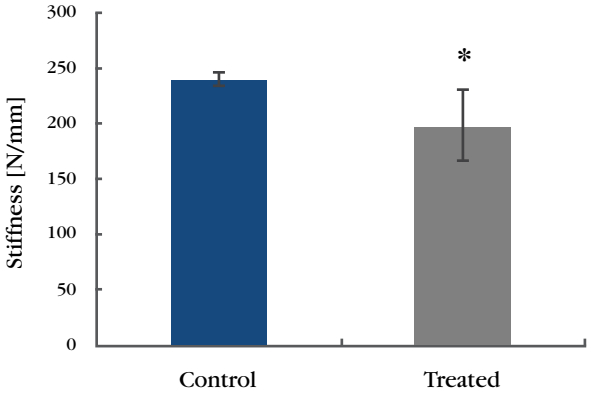

Figure 3: Stiffness by treatment condition. Error bars represent one standard deviation. Using the general linear model, the H_2O_2 treatment was found to cause a statistically significant decrease in stiffness (p = 0.022).

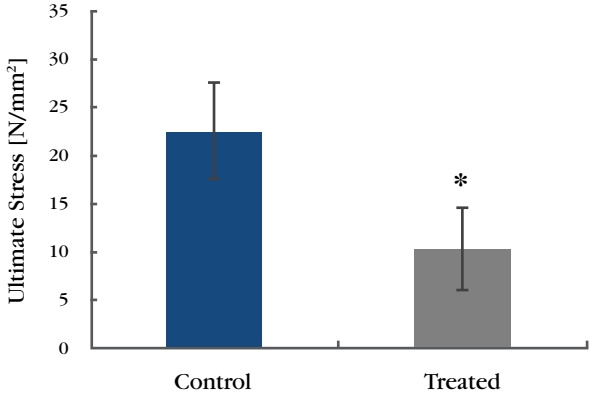

Figure 4: Ultimate Stress by treatment condition. Error bars represent one standard deviation. Using the general linear model, the H_2O_2 treatment was found to cause a statistically significant decrease in ultimate stress (p < 0.001).

Biochemical Testing

Enzymatic Degradation with Collagenase

After treatment of the tendons with H_2O_2, there was a significantly lower resistance to collagenase degradation, which indicates denaturation (breakdown) of the collagen molecules *(Figure 5)*

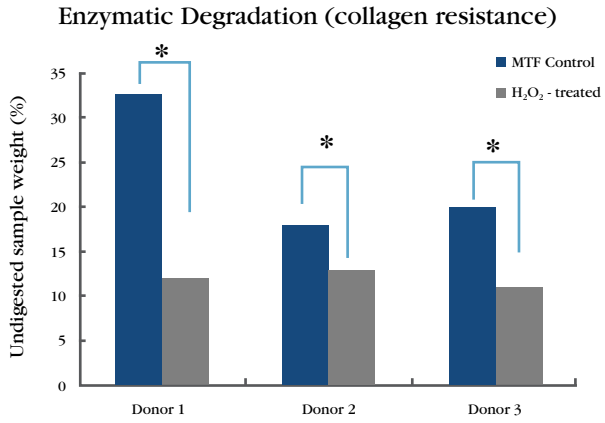

Figure 5: Enzymatic degradation by treatment condition. Using ANOVA Single Factor analysis, the effect of treatment was found to have a statistically significant lower resistance to enzymatic degradation of tendons after treatment with H_2O_2.

Native Collagen I ELISA

The levels of intact (native) Collagen I are also significantly reduced after treatment of the tendons with H_2O_2.

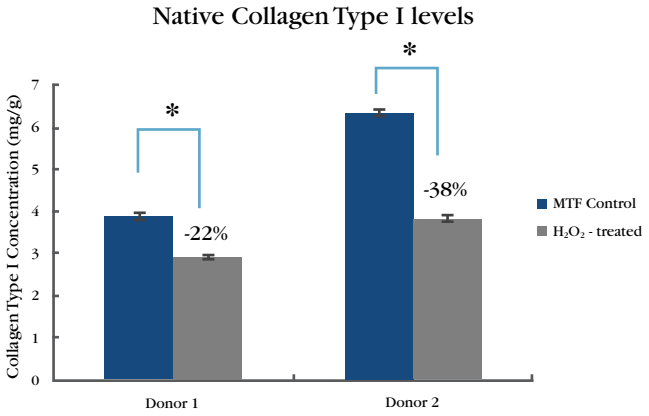

Figure 6: Native Collagen I assay. Using ANOVA Single Factor analysis, the effect of treatment was found to have a statistically significant reduction in the levels of native Collagen I after H_2O_2 treatment.

Conclusion

Treatment of tendons with H_2O_2 causes degradation in the matrix molecules and has a deleterious effect on the mechanical and biochemical properties of the tissue when compared to tendons processed without the use of H_2O_2.

REFERENCES:

1. Effects of hydrogen peroxide cleaning procedures on bone graft osteoinductivity and mechanical properties; *DePaula C.A., Truncale K.G., et. al., Cell and Tissue Banking; Vol 6:287-298; 2005.*
2. Process and apparatus treating implants comprising soft tissue: US 7,648,672 B2; *Milles R.C., et. al., January 2010.*
3. Current Safety Sterilization and Tissue Banking Issues for Soft Tissue Allografts; *Vangness T., Dellamaggiora R., Clin Sports Med; (28): 183-189; 2009*
4. Optimized process for producing tissue products: US 2003/0033044 A1; *Bilyeu D.,* February 2003.
5. The Biomechanical Effects of Low-Dose Irradiation on Bone-Patellar Tendon-Bone Allografts; *Curran A., Adams D., et al. Journal of Sports Medicine; Vol 32 No. 5; 2004*
6. Nonirradiated Versus Irradiated Achilles Allograft; *Rappe M., Horodyski M., et al. Journal of Sports Medicine; Vol 35 No. 10; 2007*
7. Anterior Cruciate Ligament Reconstruction with BPTB Autograft, Irradiated Versus Non-Irradiated allograft: a prospective randomized clinical study; *Sun K., Tian S., et al. Knee Surf Sports Traumatol Arthroscopy; 17: 464-474; 2009*
8. Clinical Comparison of the Tutoplast Allograft and Autologous Patellar Tendon (Bone-Patellar Tendon-Bone) for the Reconstruction of the Anterior Cruciate Ligament; *Gorschewsky O., Klakow A., et al. Journal of Sports Medicine; Vol 33 No.8; 2005*
9. Failure Rate of Achilles Tendon Allograft in Anterior Cruciate Ligament Reconstruction; *Shah A., McCulloch P., et al. Arthroscopy; Vol 25 No. 5; May 2010*

MTF Musculoskeletal Transplant Foundation
the better approach

125 May Street ■ Edison, NJ 08837 ■ 800-433-6576 ■ mtf.org