Improved Allograft Disinfection Technique Using GCIB

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Abstract

Background: Currently, soft tissue allografts are classified in two broad groups; aseptically harvested or end-sterilized by irradiation or chemicals. Surgeons perceive problems with both. Some surgeons see aseptically harvested tissues as "not clean enough" with potential for disease transmission. Others perceive the end-sterilized tissues to be potentially damaged by irradiation or chemicals.

Purpose/Objective: We have sought to process soft tissues by a technique called GCIB, which utilizes high energy ionized gas clusters comprised of large numbers of inert argon atoms to modify surfaces. This method results in a modified surface without any additional residues. We tested GCIB's effectiveness as a novel tissue disinfection method aimed to reduce or eliminate bio-burden, while preserving the inherent mechanical and biological characteristics of allograft tissues.

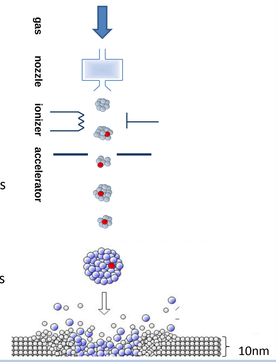
Materials/Methods: Cleanly harvested goat flexor tendons were placed in a mild cleansing solution consisting of PBS with 1% v/v Triton X-100, 2.5g/L Na deoxycholate, and 1% v/v penicillin/streptomycin overnight at 4°C. To assay for endogenous bacteria, flexor tendons were cut into 2 cm long sections and placed overnight in culture broth (n=3) without- (control group 1) or with- (control group 2) ampicillin (Amp, 50µg/ml). Additional tendon sections were inoculated with $1x10^6$ CFU Amp-resistant E. coli bacteria and frozen prior to control (test group I) or GCIB-treatment at a dose of $5x10^{14}$ Ar clusters/cm^2 (test group II, n=3). Following treatment, tendons were incubated overnight in 5 ml culture broth with Amp in a shaker-incubator at 37°C. The next day, 1ml of each culture was assayed for growth on a spectrophotometer at 595nm wavelength and absorbance was recorded (A595). 50µl of culture was then placed on pre-warmed LB-Agar-Amp plates in a dilution series and placed at 37°C overnight. The next day, individual plates were scanned and colonies were counted. The impact of GCIB treatment on biomechanical properties of goat tendons was evaluated by tensile testing on 18 matched pairs from three goats comparing fresh-frozen to GCIB-treated tendons. Tendons were loaded at a rate of 10 mm/min until failure using an 858 MiniBionix II MTS machine. Ultimate load and elongation at failure were measured directly and ultimate stress, ultimate strain and Young's modulus were calculated for each sample. To determine the effect of GCIB on cell attachment capacity of the tissue, fresh and GCIB-treated pig medial collateral ligaments (n=6) were decellularized and then their surface were seeded with porcine ligamentous fibroblasts at a concentration of 10^6 cells per ligament. The ligaments were incubated for 19 days in DMEM + 10% FBS. Ligaments were formalin fixed and slices were subjected to histological analysis to determine cellular on-growth. Statistical analysis was performed using the Paired t-Test.

Results: The use of the cleansing solution did not inhibit endogenous bacterial growth in the tendons of control group 1, which showed an A595 reading of 0.432 with over 500,000 colonies formed on the plates in dilutions; however, the use of ampicillin (control group 2) did eliminate virtually all bacteria from growing [A595 = 0.001 and 4 colonies on the plates ($p<0.01$)]. When bacteria are added exogenously to the tendons, the bacteria did grow well in ampicillin containing culture (test group I, A595 = 0.225, > 500,000 colonies). Exogenously added bacteria are completely inhibited by GCIB processing [test group II, A595 = 0.000, 0 colonies ($p<0.001$)]. There were no significant differences between fresh-frozen and GCIB-treated tendons as measured by ultimate load and elongation, as well as ultimate stress and strain or Young's modulus. Control ligaments revealed one cell layer in the cellular on-growth study; however, GCIB-treated ligaments displayed cells growing in a multi-layer fashion.

Conclusion: GCIB may be used to reduce or eliminate organisms in the superficial layers of soft tissue allografts, without compromising the inherent mechanical integrity or the ability of the tissue to support cell growth and adherence *in vitro*.

Introduction to GCIB

- Unique energetic ion bombardment technique.
- Clusters can be formed from a number of gases, Argon is typically used due to inert properties.
- Clusters that collide with surface have capability to modify material to about 10nm below the surface.
- Surface Treatment using Gas Cluster Ion Beams :
  - Changes the surface properties without changing the surface chemistry of a material
  - Very shallow penetration – at atomic levels
  - Extremely localized process that only impacts the surface



Materials and Methods

Bacterial studies: **Study 1**: *E. coli* DH5-α competent bacteria were transformed with pUC19 plasmid DNA to introduce an ampicillin-resistance gene in order to assay exogenously added bacteria. As described in Abstract, cleanly harvested goat flexor tendons were gently cleaned and cut into 2 cm long sections. Flexor tendon segments were placed in LB broth without- or with- ampicillin to determine background bacterial load. Flexor tendons were also inoculated with $1x10^6$ colony forming units (CFU) Amp-resistant *E. coli* and either left as controls or treated by GCIB at $5x10^{14}$ Ar clusters / cm^2 and then placed in LB broth with Amp overnight. Next day, aliquots of broth were measured on a spectrophotometer and absorbances at 595nm were recorded. Dilutions of the broth were also spread onto LB agar plates with Amp and grown overnight. Next day, colonies of bacteria were counted.

Study 2: Collagen sheets cut to 0.25" x 0.5" were used as surrogates for soft tissue allografts. *E. coli* (ATCC 8739) were grown according to manufactures instructions and dilution series were used to determine CFU of bacteria on LB agar plates. Collagen sheets were cut as described and 6 pieces were inoculated at $2.6x10^7$ CFU / sample for 15 minutes. All pieces were then frozen at -80°C for 30 minutes. The pieces were then lyophilized in a bench top lyophilizing unit for 1 hour. Three pieces were left as control, three additional pieces were treated on both sides using GCIB at $5x10^{14}$ dose. All the pieces of collagen were placed in 0.5ml LB broth for 1 hour at 37°C. 1µl of broth was then used for serial dilutions at 1:1,000 and 1:1,000,000 in LB broth and a subsequent 50µl were spread on individual LB Agar plates. Plates were incubated at 37°C over night. The next morning, individual colonies were counted to determine CFU / Sample.

Study 3: A validation of study 1 and 2 was performed at WuXi AppTec in Marietta, GA. Essentially similar to study 2, however a total of 9 pieces were used, 2 as in-house controls, 2 as shipping controls, and 5 as processed samples. Collagen pieces were inoculated at $2.7x10^7$ CFU / sample, 7 were shipped back to Exogenesis in MA and 2 were maintained at the facility in GA. 5 pieces were further processed as described above and all the pieces were sent back for analysis.

Tensile Strength studies: Goat flexor tendons were cleanly harvested, cut to 6cm segments, and frozen at -80°C. 18 matched pairs from three goats were used to compare fresh-frozen to GCIB-treated tendons. Treated tendons were lyophilized for 16 hours prior to GCIB treatment at $5x10^{14}$ Ar clusters/cm^2. Following treatment, all samples were analyzed at Clemson University and the Medical University of South Carolina. All tendon segments were rehydrated in saline for 1.5 hours at room temperature and clamped onto an 858 MiniBionix II MTS machine and were loaded at a rate of 10 mm/min until failure. Ultimate load and elongation at failure were measured directly and the initial cross sectional diameter and length were used to calculate ultimate stress, ultimate strain and Young's mdoulus.

Cell Attachment studies: Porcine anterior cruciate ligaments were cleanly harvested and ligamentous fibroblasts were grown out using an explant method. Medial colateral ligaments were decellularized using the method of Woods and Gratzer (1), cut to 1.5cm segments, and either left as controls or treated by GCIB as described. Porcine ligamentous fibroblasts were then seeded onto the surface of treated and un-treated MCL and allowed to attach and proliferate for 19 days. Ligaments were fixed in formalin and parafin embedded, sections were then H&E stained to visualize cells.

Statistics: Where needed, paired T-test were performed and $p<0.05$ was considered significant. Power analysis were also performed for the Tensile Strength studies.

(1) Woods T and Gratzer PF. Biomaterials 26 (2005) 7339–7349.

Results

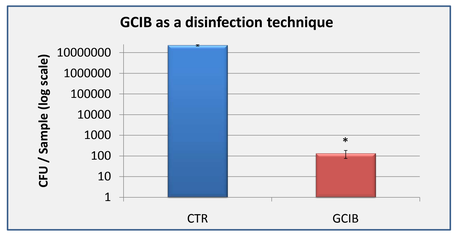
Bacterial Studies

Group #	Condition for Tendon	A_{595} Turbidity	Bacterial Colonies
Control 1	Fresh, incubated in LB	0.431	>50,000
Control 2	Fresh, incubated in LB+AMP	0.01	4 *
Test 1	Amp resistant *E. coli*, in LB+AMP	0.225	>50,000
Test 2	Amp res *E. coli* **+ GCIB** in LB+AMP	0	0 *

Table 1. Study 1 results show a significant reduction in background bacteria in control group 2 when tendons are placed in Amp containing broth (* $p<0.01$). When Amp resistant bacteria are added to tendons, they grow well in LB with Amp (Test 1), when similar tendons are treated by GCIB, there are no bacterial colonies that survive (Test 2, * $p<0.001$).

Inoculum Level	
Escherichia coli (ATCC 8739)	2.6×10^7 CFU / Sample
Sample	**Total Count – Recovered CFU / Sample**
Control 1	1.1×10^7
Control 2	1.3×10^7
Control 3	2.4×10^7
GCIB 4	18
GCIB 5	10
GCIB 6	23

Table 2. Study 2 results show a significant decrease in recovered CFU/sample in the GCIB treated collagen samples (4-6) as compared to controls (1-3, $p<0.02$).



Graph 1. Study 3 validation results from an outside vendor show a 5 log reduction in bacterial CFU/sample by GCIB treatment as compared to controls (* $p<0.0001$).

Questions or Further Information
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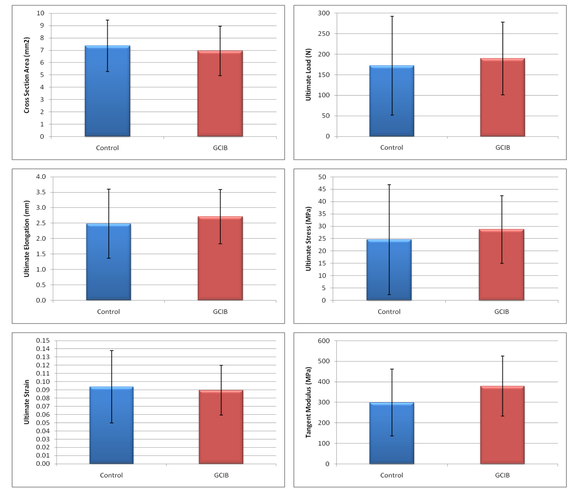
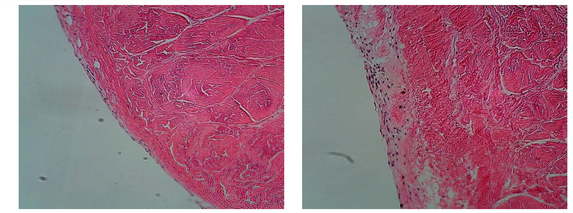
Results Continued

Tensile Strength Studies



No significant differences are found between fresh-frozen control goat flexor tendons to GCIB processed tendons in any of the mechanical testing properties. Cross section area, p=0.56; Ultimate load, p=0.64; Ultimate elongation, p=0.52; Ultimate stress, p=0.53; Ultimate strain, p=0.75; Tangent modulus, p=0.16.

Cell Attachment Studies



H&E staining reveals that GCIB treated MCL (right) do not inhibit cell attachment on the surface as compared with control ligaments (left).

Conclusions

GCIB is a novel method in which soft tissue allografts may be disinfected effectively without the use of harsh chemical washes or gamma-irradiation. The effect of GCIB does not negatively impact bio-mechanical properties or the ability of cells to attach and proliferate on the surface.