

Research Article

Comparison of Decontamination-Preservation Protocols for Delayed Surgical Re-implantation of Osteoarticular Fracture Fragments

James L Cook¹, Aaron M Stoker¹, Chantelle C Bozynski¹, Rown Parola¹, Gregory J Della Rocca¹, Tamara Gull², Julia AV Nuelle^{1*}

¹Thompson Laboratory for Regenerative Orthopaedics and Missouri Orthopaedic Institute, University of Missouri, Columbia, MO, USA

²Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO, USA

*Correspondence author: Julia AV Nuelle, MD, Thompson Laboratory for Regenerative Orthopaedics and Missouri Orthopaedic Institute, University of Missouri, Columbia, MO, USA; Email: julia.nuelle@health.missouri.edu

Abstract

Background: To avoid discarding contaminated, devascularized osteoarticular fragments required for joint reconstruction, fragments need to be decontaminated while preserving chondrocyte viability. We hypothesized that disinfection with povidone-iodine or chlorhexidine followed by preservation using the Missouri Osteochondral Preservation System (MOPS) would allow for effective decontamination while retaining essential chondrocyte viability in osteoarticular fracture fragments for up to 14 days of shelf-stable point-of-care storage.

Methods: With IACUC approval, purpose-bred hounds (n=16) were humanely euthanized for unrelated purposes and subjected to captive bolt trauma to create open distal humeral fractures. For each elbow (n=32), humerus, radius and ulna tissues were recovered such that 96 contaminated, devascularized osteoarticular fragments were randomly allocated to one treatment: Betadine (n=42): saline irrigation (1L), immersion in 10% povidone-iodine (20 min), saline irrigation; Chlorhexidine (n=42): saline irrigation, immersion in 0.002% chlorhexidine gluconate (20 min), saline irrigation; Injured Control (n=12): no decontamination treatment. After 7 or 14 days in MOPS, tissues were assessed by quantitative microbial culture and Viable Chondrocyte Density (VCD) measures.

Results: Captive bolt trauma consistently resulted in type 3 open articular fractures. Injured Control osteoarticular fragments produced high polymicrobial counts at days 7 and 14. Chlorhexidine treatment was effective for decontaminating fragments such that no CFUs for clinically relevant bacteria were produced, while Betadine treatment was not fully effective at decontamination. Chlorhexidine decontamination followed by MOPS preservation maintained VCD in osteoarticular tissues over the desired 70% mean for 14 days, whereas the Injured Control group was associated with significant loss of VCD (Day-7=59%, Day-14=13%), which was further

exacerbated by Betadine treatment (Day-7=29%, Day-14=6%).

Conclusion: Contaminated, devascularized osteoarticular fracture fragments can be effectively decontaminated while maintaining essential chondrocyte viability for 14 days after type 3 open articular fractures using a decontamination-preservation protocol that combines saline irrigation with 0.002% chlorhexidine immersion followed by shelf-stable point-of-care storage in MOPS.

Keywords: Articular Fracture; Osteoarticular; Joint Preservation; Decontamination; Osteochondral Autograft

Introduction

For open articular fractures, anatomic osteoarticular reconstruction is associated with the most optimal restoration of function such that retention of osteoarticular fracture fragments is desirable [1,2]. However, these fragments are often contaminated, devascularized and/or extruded, making immediate reimplantation rarely successful and potentially detrimental to joint health, such that these fragments are often discarded [3,4]. In these cases, the remaining treatment options include delayed arthroplasty,

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arthrodesis or amputation [4-10]. To avoid the need to discard osteoarticular fragments required for safe and effective anatomic osteoarticular reconstruction, the fragments need to be decontaminated while preserving chondrocyte viability and tissue integrity for delayed reimplantation as an osteochondral autograft.

Studies examining methods for retention and reimplantation of osteoarticular fracture fragments for joint reconstruction have not yet validated a safe and effective process for clinical use. In a rat model, Li, et al., successfully reimplanted large osteoarticular segments after sterilization using a combination of povidone-iodine scrub with autoclaving or antibiotic solution immersion to reconstruct open femur fractures [11]. While this method was highly effective for decontamination and preservation of articular surface morphology, chondrocyte viability and the related effects on joint health and function were not assessed [11]. Campbell et al., reported that immediate intraoperative treatment of potentially contaminated human femoral osteochondral allografts using 0.002% Chlorhexidine Gluconate (CHG) resulted in effective decontamination while preserving chondrocyte viability for up to 7 days [12]. However, this process has not been validated for decontamination and preservation of osteoarticular fracture fragments. As such, these methods need to be tested in a relevant preclinical open articular fracture model to develop and validate a protocol that optimizes the balance between decontamination and preservation of chondrocyte viability for point-of-care storage and reimplantation of contaminated, devascularized and/or extruded osteoarticular fragments. For translation to safe and effective clinical use, the goals for decontamination and preservation of osteoarticular fracture fragments were to achieve complete clearance of bacteria and maintain at least 70% of the Viable Chondrocyte Density (VCD) of healthy articular cartilage for a minimum of 7 days in storage [1-16].

Recent advances in shelf-stable Osteochondral Allograft (OCA) preservation have resulted in significant improvements in OCA transplantation outcomes [13-16]. We sought to further investigate the potential for similar methods to be coupled with validated tissue disinfection protocols for effective decontamination and point-of-care storage of osteoarticular fracture fragments such that delayed re-implantation for functional joint reconstruction could be considered (Fig. 1). Using a preclinical canine model, this study was designed to test the hypothesis that disinfection with povidone-iodine or chlorhexidine followed by storage using the Missouri Osteochondral Preservation System (MOPS) would allow for effective decontamination while retaining minimum essential viable chondrocyte density in osteoarticular fracture fragments for up to 14 days of shelf-stable point-of-care preservation for delayed re-implantation [17].

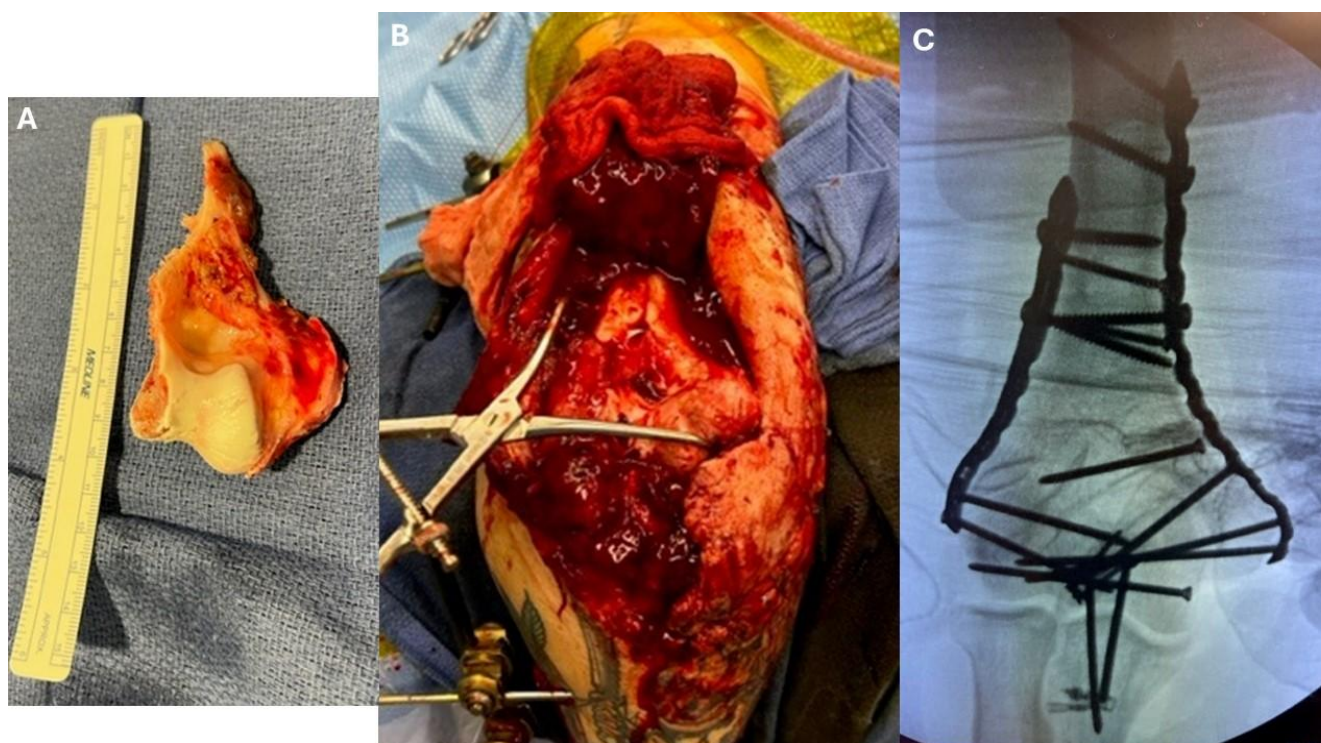


Figure 1: Clinical case example of type 3 open distal humeral fracture with contaminated, devascularized, extruded osteoarticular fracture fragment (A) with intraoperative gross (B) and fluoroscopic (C) images of delayed re-implantation.

Methods

With institutional Animal Care and Use Committee (ACUC) approval (#16680), skeletally mature purpose-bred research hounds (n=16) were humanely euthanized for purposes unrelated to the present study. Immediately following euthanasia, each dog was positioned in sternal recumbency with one forelimb extended and secured in a custom-made positioning apparatus. Fur was not clipped and no aseptic preparation or decontamination techniques were performed. Using a validated technique, a penetrating captive bolt stunner pistol (CASH Special, FRONTMATEC, Birmingham, United Kingdom) with a 1.25 grain cartridge was centered on the cranial (anterior) aspect of the distal humerus, immediately proximal to the elbow joint and with firm pressure against the secured elbow, the pistol was discharged, creating type 3 open distal humeral fractures (Fig. 2) [17]. After the fractures were induced and radiographs obtained, the skin and soft tissues were aseptically removed (Fig. 3). For each elbow (n=32), osteoarticular tissues were obtained from the distal humerus, proximal radius and proximal ulna such that 96 contaminated, devascularized, osteoarticular fracture fragments were randomly allocated to undergo treatment using one of the following protocols for comparison:

- Betadine (n=42): bulb irrigation using 1L of 0.9% saline, followed by immersion in 10% povidone-iodine solution for 20 min, followed by bulb irrigation using 1L of 0.9% saline
- Chlorhexidine (n=42): bulb irrigation using 1L of 0.9% saline, followed by immersion in 0.002% chlorhexidine gluconate solution immersion for 20 min, followed by bulb irrigation using 1L of 0.9% saline
- Injured Control (n=12): no decontamination treatment

Each fragment was then immediately placed in 200 ml of Missouri Osteochondral Preservation System (MOPS) solution in individual sterile closed containers, transported to the on-site laboratory and placed in a dedicated storage cabinet in standard room temperature and humidity conditions for 7 or 14 days.

Viable Chondrocyte Density

At days 7 (n=21) or 14 (n=21) of storage, osteoarticular tissues were collected and processed for assessment of viable chondrocyte density (VCD). VCD was assessed using a live-dead assay involving calcein AM (live stain) and ethidium homodimer (dead stain), as previously described [13,14]. Briefly, tissues were sagittally sectioned using a Buehler Isomet low speed saw (Lake Bluff, IL, USA) to produce representative 1mm-thick sections from each. Sections were placed in 6-well plates containing calcein AM (1µg/ml) solution and ethidium homodimer (1µM) in Phosphate Buffered Saline (PBS). The plates were incubated for 30 minutes at 37°C. After incubation, the stain was removed, the samples rinsed in PBS and tissues were mounted to the bottom of the plate using a 4% agarose solution. Fluorescent microscopy was used to obtain two images per section, which were analyzed to quantify tissue viability based on VCD. The VCD was determined by counting the number of viable cells in each image and dividing this by the area of the tissue in the image (# of viable cells/area of cartilage tissue mm²). The percentage of viable chondrocyte density (%VCD) for each fracture fragment was calculated based on the mean VCD of healthy day-0 samples using the formula: (fractured VCD/ control VCD) x 100 [17].

Microbial Cultures

At days 7 (n=21) or 14 (n=21) of storage, osteoarticular fracture fragments were also collected and processed for quantitative microbial culture. Each tissue intended for culture was placed into 18 ml thioglycollate broth and vortexed for 30 seconds, as effective aseptic grinding of bone specimens was not possible. One milliliter of the broth was immediately extracted for the 1:1 dilution and placed in an Eppendorf tube. Serial 1:10 dilutions were made from that subsample and plated within an hour. The plating process comprised 10ul of each dilution streaked onto tryptic soy agar with 5% sheep blood for aerobic culture and pre-reduced tryptic soy agar with 5% sheep blood for anaerobic culture. All dilutions were incubated for 72h under appropriate conditions before counting. Additional incubation in the 17 ml thioglycollate for 48h was performed and 10ul of thioglycollate was re-streaked at that time and incubated for another 72h. Organisms were identified via MALDI-TOF or 16S rRNA sequencing. Microorganism cultures were quantified using Colony-Forming Unit (CFU) counts per milliliter of broth. This method involved preparing a series of dilutions from the original sample to ensure countable colony growth on the Petri dish. Ten microliters of the sample were inoculated onto agar plates that were incubated under appropriate conditions to allow for the microorganisms' growth. Post incubation, the number of colonies formed on each plate was counted. The number of CFUs per milliliter (CFU/ml) was calculated using the formula: CFU/ml = (number of colonies x dilution factor) / volume inoculated. The CFU/ml data were reported as mean and range per specimen, providing both the typical value and variability in the microbial populations within the samples.

Statistical Analysis

All data were analyzed using statistical software R Studio, version 4.3.1 (Posit Software, Boston, MA, USA). Descriptive statistics for VCD, %VCD and CFU were calculated to report means, standard deviations, ranges and percentages. Differences in %VCD at each time point were compared among treatments using one-way ANOVA tests. Differences in %VCD over time in the Injured Control group were compared using one-way repeated measures ANOVA. Statistical significance was set at $p < 0.05$.



Figure 2: Representative cranial-caudal (anterior-posterior) radiographic views of type 3 open distal humeral fractures for creation of osteoarticular fracture fragments.



Figure 3: Representative images of contaminated, devascularized and extruded (by dissection) ulnar (U), radial (R) and humeral (H) osteoarticular fragments from type 3 open elbow fractures created in canine cadavers.

Results

The captive bolt penetrating trauma consistently resulted in type 3 open distal humeral articular fractures in each elbow of the canine cadavers, as previously described [17]. Injured Control osteoarticular fragments produced high polymicrobial counts with a mean of 19,818,000 CFU/specimen (range, 1,098,000 to 79,200,000 CFU/specimen) at day 7 and mean of 20,070,000 CFU/specimen (range, 7,740,000 to 50,400,000 CFU/specimen) at day 14. As such, tissues collected were representative of contaminated, devascularized, extruded osteoarticular fragments from type 3 open fractures (Fig. 3).

Decontamination Protocols

Chlorhexidine treatment was effective at decontamination of osteoarticular tissues based on quantitative microbial tissue cultures on days 7 and 14. No CFUs for clinically relevant bacterial species were produced at either time point. Betadine treatment was not fully effective at decontamination of osteoarticular tissues based on two (10%) osteoarticular tissues producing *Roseomonas* sp and one (5%) producing *Methylobacterium* sp CFUs at day 7 and six (29%) osteoarticular tissues producing *Methylobacterium* sp CFUs at day 14.

Viable Chondrocyte Density (Table 1, Fig. 4)

Trauma from captive bolt fracture creation was associated with a decrease in chondrocyte viability such that day-0 mean %VCD in Injured Control osteoarticular fragments was 91.7% of anatomically matched healthy (uninjured) day-0 tissues [17]. Mean %VCD further significantly decreased in Injured Control osteoarticular fragments assessed on days 7 ($p < 0.001$) and 14 ($p < 0.001$). On days 7 and 14, %VCD for the Chlorhexidine group was significantly ($p < 0.001$) higher than for the Betadine and Injured Control groups with the mean exceeding the minimum essential threshold of 70%. Interestingly, %VCD for the Betadine group was notably lower than for the Injured Control group, suggesting that 20-minute 10% povidone-iodine immersion was associated with additional cytotoxicity during storage of the osteoarticular fracture fragments.

	Day 0	Day 7	Day 14
Injured Control	91.7 $\pm$ 12.1	59.3 $\pm$ 26.2	12.7 $\pm$ 12.3
Betadine		29.2 $\pm$ 18.6	6.4 $\pm$ 7.4
Chlorhexidine		75.7 $\pm$ 24.4	70.1 $\pm$ 24.1

Table 1: Mean $\pm$ SD %-viable chondrocyte density in osteoarticular fracture fragments for injured control, betadine and chlorhexidine groups.

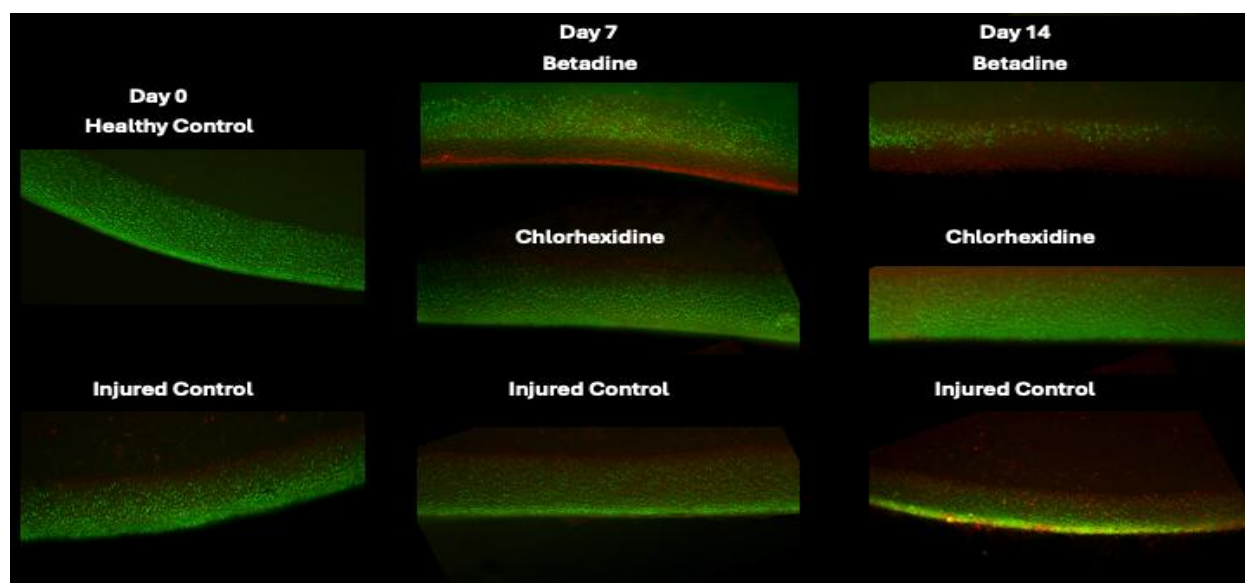


Figure 4: Representative images of distal humeral osteoarticular fracture fragments in Healthy Control and Injured Control groups at day-0 and Betadine, Chlorhexidine and Injured Control groups at days 7 and 14 illustrating chondrocyte viability in the respective tissues. Green dots represent viable cells, while red dots indicate dead cells. The live-dead assay, employing calcein and ethidium, was utilized for cell viability assessment.

Discussion

A protocol combining saline irrigation with 0.002% chlorhexidine gluconate immersion for 20 minutes followed by shelf-stable point-of-care storage in MOPS was effective for decontaminating extruded, devascularized osteoarticular fracture fragments while maintaining minimum essential chondrocyte viability for up to 14 days after type 3 open articular fractures of the elbow in a preclinical canine model. Importantly, saline-chlorhexidine decontamination followed by shelf-stable point-of-care MOPS preservation maintained VCD in the osteoarticular tissues over the desired 70% mean for the entire study period, whereas the trauma alone was associated with significant loss of VCD (Day-7=59%, Day-14=13%), which was further exacerbated by povidone-iodine treatment (Day-7=29%, Day-14=6%). Compared to the protocol combining saline irrigation with 10% povidone-iodine immersion, the chlorhexidine protocol was superior for decontamination and for preservation of cell viability, suggesting a chondroprotective effect associated with the combination of chlorhexidine decontamination and MOPS preservation. As chlorhexidine is available and used routinely in surgical settings, the results of the present study suggest that the saline-chlorhexidine-MOPS decontamination-preservation protocol has high potential for allowing for contaminated, devascularized and/or extruded osteoarticular fracture fragments to be safely and effectively recovered, treated, stored and reimplanted during subsequent joint reconstruction surgeries such that *in-vivo* preclinical animal model studies are warranted in order to effectively translate this method for functional joint preservation to clinical use.

Krueger, et al., validated the use of chlorhexidine solution to effectively reduce bacterial burden of grossly contaminated bone segments based on 20-minute immersion in 2% chlorhexidine removing >99% of a *Staphylococcus aureus* burden [18]. However, Bauer, et al., reported that decontamination with 2% chlorhexidine gluconate was associated with complete loss of cell viability in bone such that it was not recommended for applications that required preservation of viable cells [19]. Yazdi, et al., evaluated the effects of 10% povidone-iodine and 0.4% chlorhexidine 20-minute immersion on contaminated osteochondral tissues in a rabbit model; both solutions were effective at fully decontaminating these tissues but the effects on chondrocyte viability were not determined [20]. Campbell, et al., evaluated the effects of various concentrations of chlorhexidine on human chondrocyte viability after treatment of contaminated osteochondral allografts, reporting that pulse lavage using 0.002% chlorhexidine gluconate (1L) was effective for complete decontamination and was able to maintain chondrocyte viability at day-0 level for 7 days in tissue culture [12]. Higher concentrations of CHG were associated with significant chondrocyte viability loss within 24 hours of treatment [12]. The results of the present study provide further evidence for the use of 0.002% CHG (20-minute immersion) for osteoarticular tissue decontamination and use of MOPS for preserving minimum essential chondrocyte viability during shelf-stable point-of-care storage. Importantly, the decontamination-preservation protocol used in the present study did not require tissue culture and was effective in preserving VCD for 14 days after recovery and treatment of contaminated osteoarticular tissues. Seven days was selected for the minimum preservation period needed for clinical applicability based on typical time frames for definitive joint reconstruction surgeries following articular fractures initially managed with temporary stabilization; the capabilities for preserving the tissues for an additional 7 days verify clinical feasibility for use of this method in standard-of-care patient management and fracture treatment and entail important advantages for patient and wound optimization, timing and resource allocation.

Limitations to the present study include its *ex-vivo* design using a preclinical canine model, evaluation of only two decontamination protocols and the lack of assessment of decontamination-preservation effects on osteoarticular tissue material properties. The canine model was selected for this study based on its robust translational applicability in this area of research [13-17]. Specifically, the use of this *ex-vivo* model fully addresses ethical use of research animals while allowing for valid decontamination and viability preservation metrics in tissues that closely mimic the clinical scenario for patients. Only two decontamination protocols were evaluated to optimize the ethical use of research animals in conjunction with clinical relevance. Previous studies have included analyses of different titrations of each solutions studied such that the present study could avoid repeat testing of concentrations that were not effective for decontamination and/or preservation and instead focus on comparison of safe and effective protocols for specific clinically relevant application to decontamination and preservation of osteoarticular fracture fragments [11,12,18-20]. Similarly, osteoarticular tissue material properties were not assessed in the present study based on the robust peer-reviewed evidence for maintenance of these properties for much longer storage durations when VCD is maintained above 70% [13-15]. Specifically, OCA extracellular matrix composition, aggregate modulus and tissue permeability are well maintained through at least 56 days of storage in MOPS [14]. While these limitations prevent direct implementation of the decontamination-preservation protocol to generalized clinical use, the results of the study provide impetus for ethical *in vivo* studies using translational animal models to progress toward application to improve patient care.

The data from this preclinical animal model study verify that contaminated, devascularized, extruded osteoarticular fracture fragments can be effectively decontaminated while maintaining minimum essential chondrocyte viability for up to 14 days after type 3 open articular fractures using a decontamination-preservation protocol that combines saline irrigation with 0.002% chlorhexidine immersion followed by shelf-stable point-of-care storage in MOPS. These data provide the evidence for this decontamination-preservation protocol to be applied to clinical care by allowing for osteoarticular fracture fragments to not be discarded, but rather safely and effectively preserved during initial patient and wound management steps such that they can be reimplanted during subsequent joint reconstruction surgeries that result in functional joint and limb preservation ongoing studies in our laboratory are targeted on *in-vivo* preclinical assessment of functional outcomes associated with the use of this protocol towards validation of this protocol for broad clinical use.

Ethical Statement

Institutional Review Board approval was not needed to conduct this study. This study did receive approval from our institution's animal care and use committee (ACUC) (#16680).

Credit Authorship Contribution Statement

James L. Cook: Substantial contributions to research design, acquisition and interpretation of data, drafting the paper, revising the paper critically, approval of the submitted and final versions. Aaron M. Stoker: Substantial contributions to research design, acquisition and interpretation of data, revising the paper critically, approval of the submitted and final versions. Chantelle C. Bozynski: Substantial contributions to research design, acquisition and interpretation of data, revising the paper critically, approval of the submitted and final versions. Rown Parola: Substantial contributions to analysis and interpretation of data, drafting the paper and revising it critically, approval of the submitted and final versions. Gregory J. Della Rocca: Substantial contributions to research design, interpretation of data, revising the paper critically, approval of the submitted and final versions. Tamara Gull: Substantial contributions to research design, acquisition and interpretation of data, revising the paper critically, approval of the submitted and final versions. Julia A.V. Nuelle: Substantial contributions to research design, interpretation of data, revising the paper critically, approval of the submitted and final versions.

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Conflict of Interest

Co-authors James L. Cook and Aaron Stoker are patent holders in the MOPS system used in this research. They also receive IP royalties and research support from the Musculoskeletal Transplant Network (MTF Biologics), which has exclusive distribution rights to this preservation technology. Corresponding author Julia A.V. Nuelle receives research support from MTF. No other authors have disclosures in this study. The author group also reports the following disclosures:

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