Advanced Tissue Processing (ATP):

Development of a Cleaning Process for Allograft Bone

the better approach

MTF Musculoskeletal Transplant Foundation

Introduction

MTF is a non-profit organization founded in 1987 by academic orthopaedic surgeons dedicated to providing tissue of the highest quality and safety for transplantation. Everything we do at MTF begins with safety. MTF has distributed more than 3.5 million allografts since our inception and we have never experienced a case of viral disease transmission. MTF's exemplary safety record is directly attributed to our commitment to the donor families and to the tissue recipients we serve. This tremendous commitment provides our customers with the assurance that this gift of human tissue is safe and that it comes from a trustworthy source.

But we think beyond safety. While safety governs every decision we make, we know that quality also matters. Current techniques used by some tissue banks to clean, process and sterilize allografts have been shown to be detrimental to the quality of the tissue. These methods vary widely from bank to bank, because standards are open to interpretation. Allograft tissue of less-than-optimal quality may yield a graft that does not perform its intended function, leading to a less-than-optimal clinical outcome.

Preserving and protecting tissue integrity is integral to MTF's philosophy

MTF has developed and validated an improved tissue cleaning technology for processing bone tissue. This process provides safe, high-quality allograft bone and was developed through rigorous testing to ensure that the mechanical, biological or clinical performance of the tissue was not compromised.

Design Rationale

Today, most bone allografts are processed and cleaned before long term preservation and use.[1] Allografts are typically cleaned physically and chemically to provide an additional level of safety over and above donor screening, but it is critical not to jeopardize the integrity and quality of the graft with the use of these cleaning processes.

Commonly used chemical methods employ aqueous solutions of detergents or surfactants, hydrogen peroxide or other peroxides, organic solvents, acids and alcohol. Frequently, chemical methods are combined with mechanical methods to enhance the cleaning process. These processes must completely penetrate the bony matrix, remove endogenous materials such as blood, lipids, cells and bone marrow and reduce the level of microbiological and viral contamination. In addition, certain sterilization methods are often used in conjunction with processing and cleaning and are used for bioburden reduction or terminal sterilization.The most common terminal sterilization technique for bone is gamma irradiation.

When selecting the appropriate cleaning process, it is imperative to maintain the mechanical and biological integrity of the tissue. Strength is critical in cortical bone load-bearing applications, such as in spinal surgery and large joint reconstruction and, therefore should not be compromised during graft processing. Cortical bone allograft remodeling, a process termed "creeping substitution," occurs when allografts are incorporated into host bone. This is a process by which osteoclasts resorb the mineral content of the allograft thus exposing endogenous growth factors providing the capacity to form new bone. This process occurs until the allograft tissue is remodeled into the patient's own bone[2,3]. It is essential to maintain the biological activity of the endogenous growth factors thus providing the tissue with the proper biological balance for blood vessel formation and bony incorporation.

Several methods used to clean and sterilize allograft tissue have been shown to be detrimental to the quality of the tissue. While it is effective against bacteria, hydrogen peroxide is a common oxidizing agent with the potential to disrupt bone structural proteins, yielding a graft that may be compromised in either strength or biological activity. Typically, excessive exposure to hydrogen peroxide leads to an allograft with a pristine white appearance. This appearance may give the perception that the tissue is somehow "superior" due to its pure white and seemingly flawless look. However, caution should be used in these cases as the pure white appearance may be indicative of a prolonged exposure to hydrogen peroxide, which can diminish the biological activity of the endogenous growth factors.

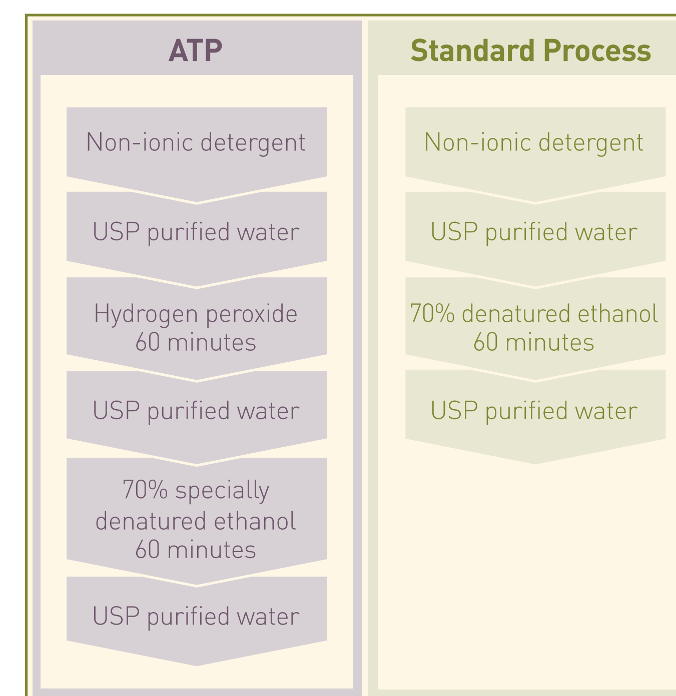
Terminal sterilization techniques, such as ethylene oxide and gamma irradiation, can have a negative impact on the natural structure and function of human bone. Ethylene oxide has been demonstrated to destroy nearly all of the osteoinductive potential of demineralized bone[4,5] making ethylene oxide an unpopular choice for allograft sterilization. Terminal gamma irradiation, at high doses, is known to reduce the mechanical strength of allograft bone through structural changes to collagen in both static[6] and fatigue testing[7] and has been shown to reduce its osteoinductive potential[5,8]. Finally, while heat treatment has been thought to provide some protection against certain viruses, high temperatures can also have a negative effect on bone. Temperatures above 60°C can degrade the beneficial osteoinductive factors present in bone.[9,10]

The design rationale for Advanced Tissue Processing (ATP) was established based on this published data. The process was designed to employ a series of chemical disinfectants that penetrate the tissue completely to remove endogenous materials, while minimizing exposure to hydrogen peroxide and heat.

Process Flow

The following flow diagrams represent both the ATP process and a standard cleaning process that was used as a control during design validation. Both processes consist of an initial antibiotic treatment.

The ATP process utilizes a series of detergents and disinfectants, followed by purified water washes. The primary solutions used: a non-ionic detergent, hydrogen peroxide and specially denatured alcohol, all of which were selected based on their proven ability to remove blood, lipids, cells and bone marrow. The process employs technologies to enhance the penetration of the detergents and disinfectants into the dense, lamellar structure of cortical bone for complete cleaning. In addition, the entire process is maintained at controlled temperatures to ensure that heat exposure is minimized to levels safe for the tissue.



Design Validation

1. Penetration of Tissue

The ATP process removes unwanted endogenous materials such as blood, lipids, bone marrow and cells within the cortical structure and medullary canal, as shown in *Figure 1* below.

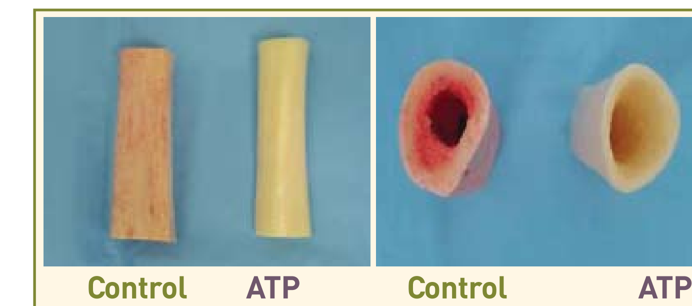


Figure 1: Cortical bone untreated (left) and treated (right) with the ATP process.

A dye penetration study was performed to confirm that the solutions in the ATP cleaning process penetrated the full lamellar structure of the cortical bone. *Figure 2* demonstrates complete penetration into the tissue at 20 minutes.

Figure 2: Dye penetration studies showing the full penetration of ATP disinfectants into the lamellar structure of cortical bone at 20 minutes in the image on the right.

2. Mechanical Integrity of Tissue

In order to assess the effects of the ATP process on the mechanical integrity of allograft, the following study was performed. Human femoral cortical bone was recovered from donors with research consent in accordance with the American Association of Tissue Banks (AATB) guidelines. Cortical bone was selected as the worst-case scenario, due to its high density and use in load-bearing applications.

All tissue was thawed in an antibiotic aqueous solution, debrided and cut to shape. Cylinders of 5.3mm x 5.3mm were cut from femoral mid-shafts in a direction corresponding to the loading axis of the bone. Samples were either packaged as frozen tissue (-70°C) or freeze-dried, to test the two methods currently used to preserve tissue forms. Control samples were cleaned and disinfected using the standard aseptic process, while test samples were cleaned using the ATP process with hydrogen peroxide treatment increased from 60 minutes to 300 minutes (5 hours) as a worst case. All samples were tested in compression which is the major loading pattern of bone *in vivo*.

The compression data show that there are no statistically significant differences in the maximum stress values between samples cleaned with the standard process and the ATP process (with an exaggerated hydrogen peroxide soak) in both frozen and freeze dried samples. See *Figure 3*.

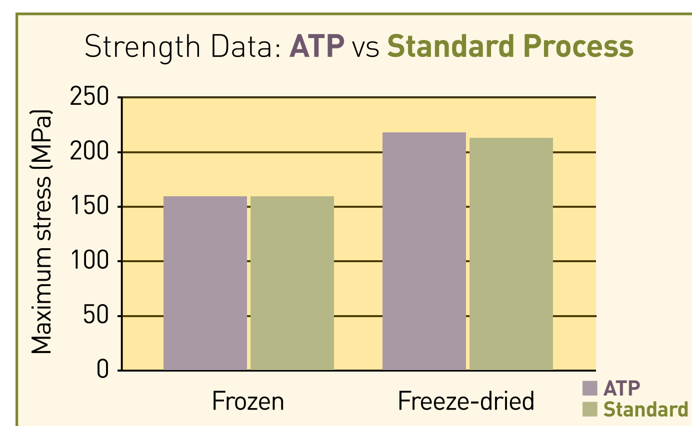


Figure 3: Maximum compressive strength (MPa) in specimens subjected to the standard and ATP cleaning process. N = 16.[11]

This data suggests that the ATP process does not compromise the structural integrity of cortical bone as demonstrated through compressive testing.[11]

3. Biological Integrity of Tissue

In order to determine the effects of hydrogen peroxide exposure on the biological integrity of cortical bone, the osteoinductivity of the bone was measured. As described earlier, during creeping substitution, the bone morphogenetic proteins (BMPs) and other endogenous growth factors are exposed within the bony matrix as a result of initial osteoclastic action. This provides a necessary and favorable environment for new bone formation. The capacity for new bone to form within a demineralized bone matrix (DBM) can be measured with a common animal model of osteoinduction.[12] Osteoinduction is defined as the ability to form new bone through the recruitment of host cells that ultimately form mineralized tissue.[13] In order to assess the biological activity of the allograft, osteoinductivity was measured through the use of this animal model.

Femoral cortical bone was cut into 5mm transverse sections and either cleaned with the standard aseptic cleaning process or with the ATP process with a 1, 3 or 5 hour hydrogen peroxide treatment. Once cleaned, bone was ground into a powder, demineralized with hydrochloric acid based on the Urist method[18] and mixed with a carrier to form a DBM. The specimens were implanted bilaterally into the hamstring muscles of athymic nude mice.[12] Animals were euthanized at 28 days post operative and histology was performed on the excised samples for evaluation. The scoring system used to assess the tissue for osteoinductivity scores is consistent with the industry standard and is based on the degree of new bone, bone cells, osteoid, calcified cartilage and marrow elements.[14] All scoring was done with the evaluator blinded.

A negative correlation was found between exposure time to hydrogen peroxide and osteoinductivity score, yielding a linear decrease with increasing time. The reduction in osteoinductivity reached statistical significance at the 5 hour time point when compared to the 0 hour control. Data is represented in *Figure 4* demonstrating the mean value of osteoinductivity score +/- standard deviation.

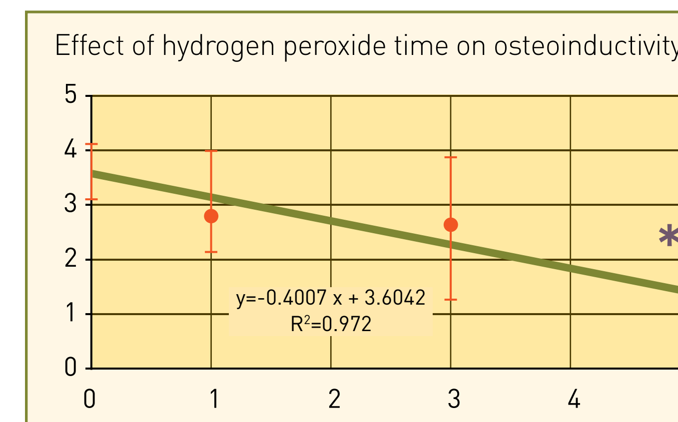


Figure 4: Sample sizes varied between groups due to the fact that a certain number of implants did not receive scoring becausethe amount of implant found was insufficient for scoring or there were artifacts in the slides. N = 8 (0 hours), N = 15 (1 hour), N = 12 (3 hour), N = 9 (5 hour).[11] **Statistically different from 0 hour treatment.*

The data clearly demonstrate a negative relationship between osteoinductivity and exposure to hydrogen peroxide, suggesting that there may also be a clinical impact at certain levels of exposure. The effects with a 1 hour exposure, however, were not statistically significant. It was for this reason that the hydrogen peroxide step of the ATP process was limited to 1 hour. To further confirm that 1 hour exposure maintains the quality of the tissue, an experimental canine model of allograft healing was initiated to ensure that the *in vivo* performance was not compromised.

4. *In Vivo* Performance

An ulnar diaphyseal canine defect model was utilized to examine cortical bone graft healing. Bilateral intercalary ulnar allografts or autografts were implanted into a 2.5cm defect in the right and left forelimbs of skeletally mature male coonhounds. Bone grafts were stabilized using dynamic compression plates with 2.7mm cortical bone screws. Thirteen animals were randomized to receive two allografts, one autograft and one allograft or two autografts. The allografts were cleaned by either the standard processing technique or the ATP processing technique and were frozen in sterile packaging prior to implantation.

Animals were euthanized at 90 days post operative and each specimen was subjected to high-resolution radiographic imaging, histologic and histomorphometric analysis. Blinded observers scored the radiographic images using a modified scoring system to evaluate graft integrity and quality of new bone formation at the graft-host interface.[15] Similarly, blinded observers scored the histology slides using a modified scoring system,[16] which was further developed for this canine model and evaluated bony bridging at the proximal and distal interface. Finally, histomorphometric analysis was performed using a digital image analysis system which quantified the total bone area at the graft-host interface.

Grafts processed with ATP showed normal bony healing and were statistically equivalent to those cleaned with the standard process. *Figures 5 and 6* demonstrate equivalent union at the host-graft junctions between the two processing techniques by radiographic and histologic evaluation.

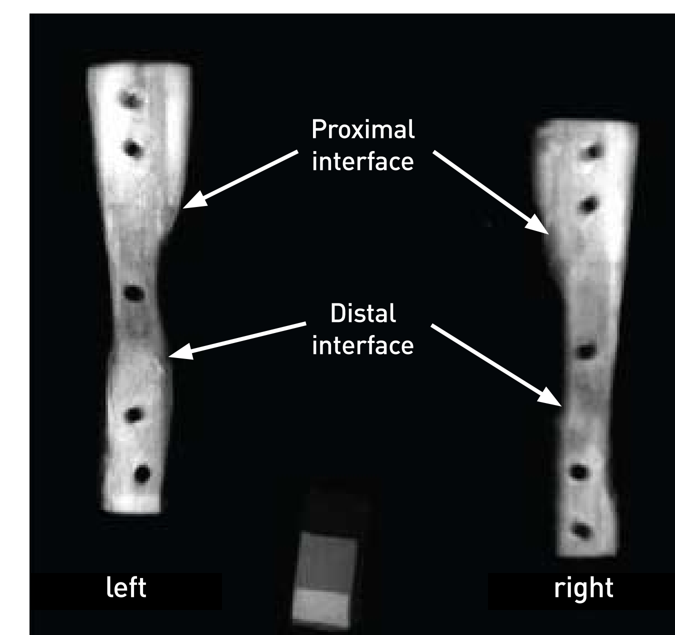


Figure 5: High resolution anteroposterior radiographs show grafts processed with the standard cleaning process (left) and the ATP process (right).

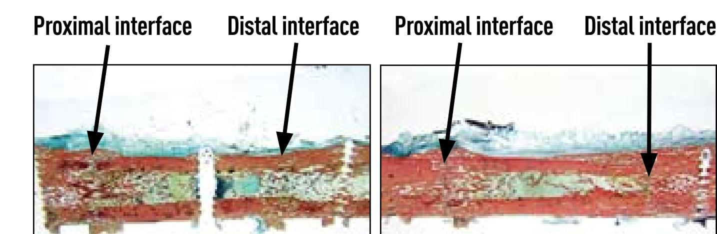


Figure 6: Histology images represent grafts processed with the standard cleaning process (left) and the ATP process (right). Both host-graft junctions appear bridged by newly formed woven bone undergoing remodeling.

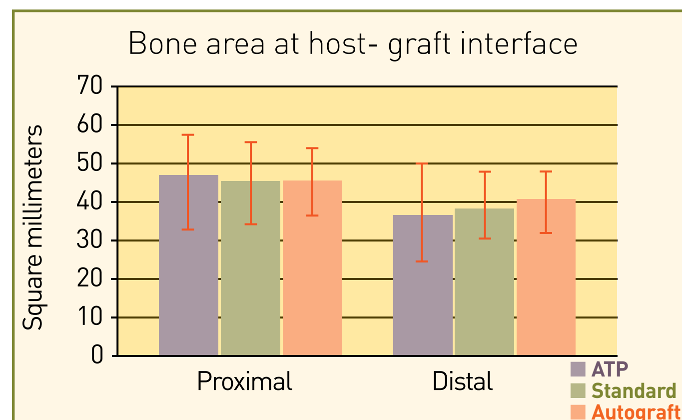


Figure 7: Total bone area as determined through histomorphometric analysis at both the proximal and distal host-graft interface.[17]

The data suggest that the ATP process, with a one hour hydrogen peroxide exposure, does not adversely affect the healing properties of cortical bone allograft in a canine model.[17]

Comparison of ATP to Two Proprietary Processing Methods

Tissue treated with the ATP process has been compared to allograft tissue subjected to other processing and cleaning methods from two other tissue banks: Regeneration Technologies Inc. Biocleanse® and LifeNet Allowash.® All tissue in this study was prepared into DBM based upon the Urist method.[18] Biological activity was assessed in an athymic mouse model through associated ostoinductivity,[12] and scored based on industry standard.[14] A positive and negative control were utilized. The ATP-treated tissue demonstrated significantly higher osteoinductivity scores when compared to tissue processed at other tissue banks,[19] as seen in *Figure 8*.

These results suggest that the higher osteoinductivity of the tissue prepared from ATP-treated bone compared to the tissue prepared from Biocleanse® and Allowash®-treated bone may result in improved incorporation of allografts treated with the ATP process.[19]

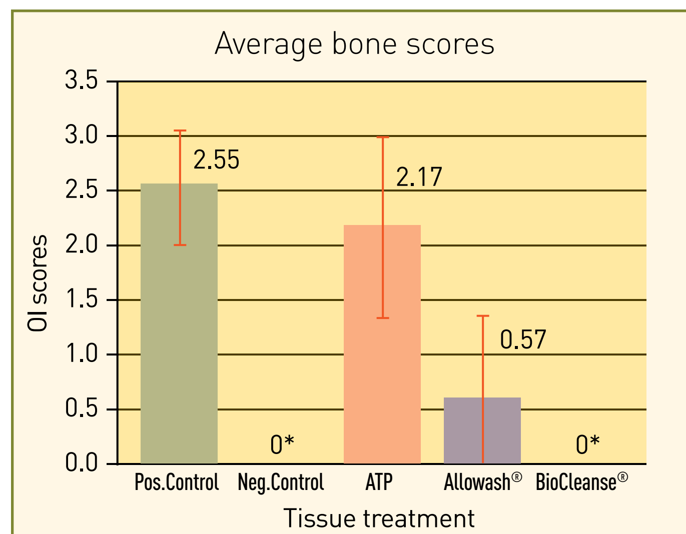


*Figure 8: Osteoinduction scores for tissue processed through various techniques. *Statistically different to ATP and control.*[19]

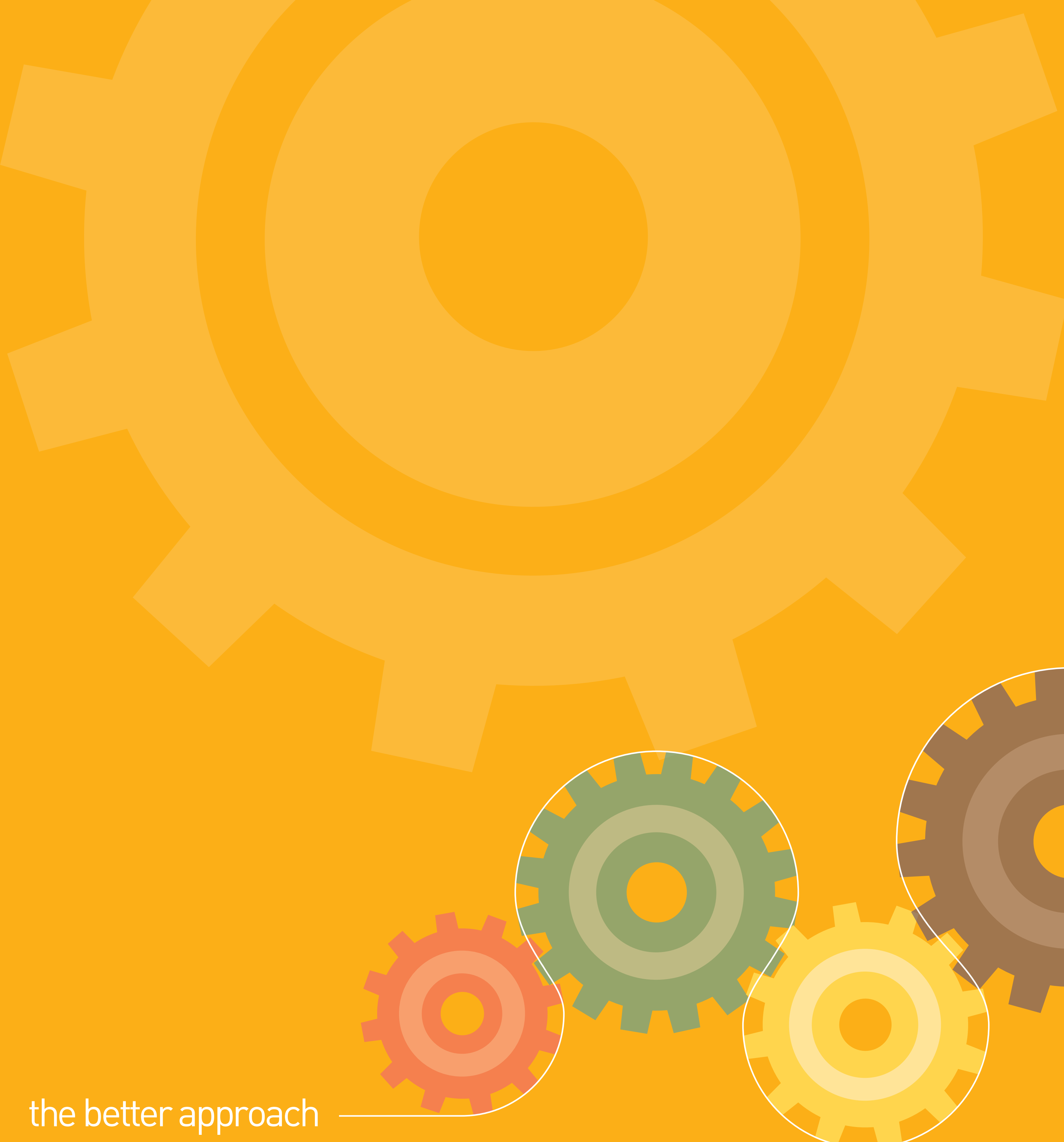
Summary

At MTF, we are driven by our strong commitment to safety. It is because of this commitment that we continue to maintain an exemplary safety record, providing our customers with allograft tissue from a source they can trust. As part of our philosophy, we believe that providing safe tissue is not enough—we also must not compromise the inherent biomechanical and biological properties of bone. In this vein, MTF has developed and validated a tissue cleaning and disinfecting process to further ensure the safety of our allografts without adversely affecting their mechanical or biological performance. The studies described here demonstrate that the ATP process has no harmful effects on the mechanical strength, natural biological properties or *in vivo* performance of the allografts.

The ATP process employed by MTF, yields a safe, effective allograft designed and validated to maintain the natural function of allograft bone.

References:

1. **Tomford WW and Mankin HJ:** Bone banking: update on methods and materials. *Orthop Clin North Am 30:* 565-570, 1999.
2. **Kalfas I:** Principles of bone healing. *Neurosurg Focus* 10: 1-4, 2001.
3. **Khan SN, Cammisa FP, Sandhu HS, Diwan AD, Girardi FP, Lane JM.:** The biology of bone grafting. *J Am Acad Orthop Surg*
4. **Aspenberg P, Johnsson E, Thorngren KG:** Dose-dependent reduction of bone inductive properties by ethylene oxide. *J Bone Joint Surg Br. 72:* 1036-1037, 1990.
5. **Munting E, Wilmart JF, Wijne A, Hennebert P, Delloye C:** Effect of sterilization on osteoinduction. Comparison of five methods in demineralized rat bone. *Acta Orthop Scand. 59:* 34-38,1988.
6. **Currey JD, Foreman J, Laketic I, Mitchell J, Pegg DE, Reilly GC:** Effects of ionizing radiation on the mechanical properties of human bone. *J Orthop Res.*15: 111-117, 1997.
7. **Mitchell EJ, Stawarz AM, Kayacan R, Kayacen R, Rimnac CM:** The effect of gamma radiation sterilization on the fatigue crack propagation resistance of human cortical bone. *Bone Joint Surg.* 86-A: 2648-2657, 2004
8. **Ijiri S, Yamamuro T, Nakamura T, Kotani S, Notoya K:** Effect of sterilization on bone morphogenetic protein. *J Orthop Res.* 12: 628-636, 1994.
9. **Hallfeldt KK, Stutzle H, Puhlmann M, Kessler S, Schweiberer L:** Sterilization of partially demineralized bone matrix: the effects of different sterilization techniques on osteogenetic properties. *J Surg Res.* 59: 614-620, 1995.
10. **Ito T, Sakano S, Sato K, Sugiura H, Iwata H, Murata Y, Seo H:** Sensitivity of osteoinductive activity of demineralized and defatted rat femur to temperature and duration of heating. *Clin Orthop.* 316: 267-275, 1995.
11. **DePaula CA, Truncale KG, Gertzman AA, Sunwoo MH, Dunn MG:** Effects of hydrogen peroxide cleaning procedures on bone graft osteoinductivity and mechanical properties. *Cell and Tissue Banking.* 6:287-298, 2005.
12. **Schwartz Z, Somers A, Mellonig JT, Carnes DL, Dean DD, Cochran DL, Boyan BD:** Ability of commercial demineralized freeze dried bone allograft to induce new bone formation is dependent on donor age but not gender. *J. Periodontal Surg.* 69: 470-478, 1998.
13. **Wolfe MW, Salkeld SL, Cook SD:** Bone morphogenetic proteins in the treatment of non-unions and bone defects: historical perspective and current knowledge. *Univ Penn Orthop J.* 12: 1-6, 1999.
14. **Draft Standard:** Standard Guide for the Assessment of Bone Inductive Materials, ASTM F04.4 Division, Draft by Barbara Boyan, University of Texas Health Science Center at San Antonio, downloaded from ASTM website 5-8-2000.
15. **Stevenson S, Li XQ, Martin B:** The fate of cancellous and cortical bone after transplantation of fresh and frozen tissue-antigen-matched and mismatched osteochondral allografts in dogs. *J Bone Joint Surg Am.* 73: 1143-1156, 1991.
16. **Bos GD, Goldberg VM, Powell AE, Heiple KG, Zika JM:** The effect of histocompatibility matching on canine frozen bone allografts. *J Bone Joint Surg Am.* 65: 89-96, 1983.
17. **Beebe KS, Benevenia J, Tuy BE, DePaula CA, Harten RD, Enneking WF:** Effects of a new allograft processing procedure on graft healing in a canine model: A preliminary study. *Clin Orthop Relat Res.* 467: 273-280, 2009.
18. **Urist M:** Bone formation by autoinduction. *Science.* 150: 893-899, 1965
19. **Dunn M, Sunwoo M, Reynoso W, Chnari E, Cartmell J, Gertzman A:** Effect of allograft bone processing on structural cortical grafts: A comparison of three proprietary processing methods. *Proceedings of the NASS 23rd Annual Meeting / The Spine Journal* 8 138S: P78, 2008.



MTF Musculoskeletal Transplant Foundation

125 May Street ▪ Edison, NJ 08837 ▪ 732-661-0202 ▪ mtf.org

BioCleanse® is a registered trademark of Regeneration Technologies Inc.,
Allowash® is a registered trademark of LifeNet

©2009 Musculoskeletal Transplant Foundation 5/09 10M 0002 RJ