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A new sterilization technique of bovine pericardial biomaterial using microwave radiation

Abstract

Bioprosthetic valves created from chemically treated natural tissues such as bovine pericardial biomaterial are used as heart valve scaffolds. Methods currently available for sterilization of biomaterial for transplantation include the application of gamma radiation and chemical sterilants. These techniques, however, can be problematic because they can be expensive and lead to a reduction in tissue integrity. Therefore, improved techniques are needed that are cost effective and do not disrupt the physical properties, functionality, and lifespan of the valvular leaflets. This study examined a novel technique using nonthermalmicrowave radiation that could lead to the inactivation of bacteria in bovine pericardial biomaterial without compromising valve durability. Two common pathogenic species of bacteria, Escherichia coli and Staphylococcus aureus, were used as test microorganisms. Optimized microwave parameters were used to determine whether inactivation of pathogenic bacteria from bovine pericardium could be achieved. In addition, the effect of microwave sterilization on tissue integrity was examined. The mechanical properties (assessed using dynamic mechanical analysis) and tensile strength testing (using a Universal Tensile Tester) as well as thermal analysis (using thermogravimetric analysis and differential scanning calorimetry) indicated that microwave sterilization did not compromise the functionality of bovine pericardial biomaterial. Scanning electron microscopy imaging and cytotoxicity testing also confirmed that the structure and biocompatibility of transplant biomaterial remained unaltered after the sterilization process. Results from the application of this newmicrowave (MW) sterilization technique to bovine pericardium showed that nearcomplete inactivation of the contaminant bacteria was achieved. It is concluded that nonthermal inactivation of pathogenic bacteria from bovine pericardial biomaterial could be achieved using microwave radiation.

Disciplines

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A New Sterilization Technique of Bovine Pericardial Biomaterial Using Microwave Radiation

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Bioprosthetic valves created from chemically treated natural tissues such as bovine pericardial biomaterial are used as heart valve scaffolds. Methods currently available for sterilization of biomaterial for transplantation include the application of gamma radiation and chemical sterilants. These techniques, however, can be problematic because they can be expensive and lead to a reduction in tissue integrity. Therefore, improved techniques are needed that are cost effective and do not disrupt the physical properties, functionality, and lifespan of the valvular leaflets. This study examined a novel technique using nonthermal microwave radiation that could lead to the inactivation of bacteria in bovine pericardial biomaterial without compromising valve durability. Two common pathogenic species of bacteria, *Escherichia coli* and *Staphylococcus aureus*, were used as test microorganisms. Optimized microwave parameters were used to determine whether inactivation of pathogenic bacteria from bovine pericardium could be achieved. In addition, the effect of microwave sterilization on tissue integrity was examined. The mechanical properties (assessed using dynamic mechanical analysis) and tensile strength testing (using a Universal Tensile Tester) as well as thermal analysis (using thermogravimetric analysis and differential scanning calorimetry) indicated that microwave sterilization did not compromise the functionality of bovine pericardial biomaterial. Scanning electron microscopy imaging and cytotoxicity testing also confirmed that the structure and biocompatibility of transplant biomaterial remained unaltered after the sterilization process. Results from the application of this new microwave (MW) sterilization technique to bovine pericardium showed that near-complete inactivation of the contaminant bacteria was achieved. It is concluded that nonthermal inactivation of pathogenic bacteria from bovine pericardial biomaterial could be achieved using microwave radiation.

Introduction

Bovine pericardial biomaterial remains one of the most common biomaterials used for the production of prosthetic heart valves.[1,2] To reduce the antigenicity and immunogenic properties of these biomaterials and to prolong the structural and mechanical integrity, the tissue used to make bioprostheses needs to be initially fixed with glutaraldehyde.[2] After this chemical fixation, further sterilization of the bioprostheses is required before they can be used in clinical applications.[3]

One of the main causes of implant-associated infections is the adhesion of bacteria on the surface of biomaterials.[4] Implant-related infections are commonly caused by bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*.[5] Other pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Proteus vulgaris* have also been shown to cause infections in implants.[5] In those instances where it is found that implanted prosthetic valves have been infected by *S. aureus*, surgical intervention is required to replace the valves due to the virulency of the bacteria and the high mortality rates associated with the infection.[6]

Currently available methods for sterilizing bovine pericardial biomaterial include the application of dry heat, pressurized steam, gamma irradiation, and the use of liquid and gaseous chemicals.[3] Because it has been clearly shown that the proteins of biological tissue denature at relatively low temperatures, dry heat and pressurized steam cannot be employed to sterilize pericardium.[3] Gamma irradiation has been shown to cleave the peptide bonds of collagenous biomaterial, thereby damaging tissue integrity by reducing its tensile strength and accelerating its degradation rate.[3] Ethylene oxide is a commonly used gaseous sterilant; however, in certain instances, the presence of residual ethylene oxide is considered to be detrimental, even in small quantities.[3] Further, transplants containing biological tissue have been sterilized

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by immersion in antibiotic mixtures, but such processes are very expensive and do not destroy certain bacterial spores and viruses.[7]

Accordingly, improved methods of sterilization of bovine pericardial biomaterial are needed that do not disrupt the physical properties, functionality, and lifespan of the valvular leaflets while being a cost-effective process. Microwave disinfection technology is regarded as a promising alternative method for eliminating unwanted bacteria. The application of microwave radiation to control bacterial proliferation is particularly appealing for the biomedical industry due to its effectiveness and low cost. Much research has led to the conclusion that microwave radiation extends tissue preservation by reducing microbial levels.[8–17] Microwave treatment is known to inactivate many microorganisms, such as *Burkholderia cepacia*, *Clostridium perfringens*, *E. coli*, *Photobacterium leiognathii*, *Streptococcus faecalis*, *S. aureus*, *Salmonella*, and *Listeria* spp.[10–13,15,17–23] Bacterial and mould spores have also been reported to be sensitive to microwave radiation.[24]

Such findings provide important implications for the acquittal of pathogenic bacteria from transplant material.[28–31] The use of microwave radiation to sterilize transplant tissue could be used to deliver an effective sterilization technique for bovine pericardial biomaterial. In this context, the aim of the present study was to evaluate the effectiveness of MW radiation with regard to the inactivation of contaminant bacteria in a commonly used biomaterial—bovine pericardium. Two common pathogenic species of bacteria, *E. coli* and *S. aureus*, were used as model microorganisms. To preserve the structure and functionality of bovine pericardial biomaterial, it was proposed that the application of the electromagnetic sterilization technique would take place at temperatures below the thermal degradation point of the biomaterial and bacteria involved, hence ensuring a nonthermal sterilization mechanism. The first stage of the study aimed at examining the mechanical properties (viscoelastic properties and tensile strength) and thermal degradation (thermogravimetric analysis and differential scanning calorimetry) of bovine pericardial biomaterial after the MW sterilization process. The second stage was designed to use previously optimized microwave parameters[25] to identify the point at which optimum bacterial inactivation could be achieved without compromising the structure and functionality of the bovine pericardial biomaterial.

Materials and Methods

Bovine pericardium collection and preparation

The bovine pericardial biomaterial was collected immediately after slaughter from an abattoir. Membranes of bovine pericardium were mechanically cleaned and washed in 0.85% (w/v) NaCl phosphate buffered saline (PBS). After this, one portion of membrane was subjected to 70% ethanol solution for 12 h and then transferred to flasks containing PBS until further use and was regarded as a control sample of original tissue or untreated sample; another portion was fixed in 0.5% glutazaldehyde (GA) (Merck, Darmstadt, Germany) for 12 h and then transferred to flasks containing PBS until further use and MW treatment.

Cell culture and reagents

The mouse fibroblast NIH 3T3 cell line was obtained from American Type Culture Collection (ATCC). Cells were grown at 37°C in a 5% (v/v) CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum up to 4 days.

Cytotoxicity assay

The assay was performed in compliance with the USP and/or applicable ISO guidelines (ISO 10993–5; USP 87). Samples of pericardium were cut into pieces of 1×1 cm and were placed into an empty well of a six-well culture plate. Mouse 3T3 fibroblast cells were then seeded into the well at a density of 50,000 cells per mL. The plate was incubated at 37°C with 5% CO_2 up to 4 days. After the incubation, fibroblast cells were stained with Lilie Mayer hematoxylin and observed directly to examine growth and morphology, using Microscope System Olympus IX81 (20×, UIS objectives). The system was equipped with a transmitted light differential interference contract attachment and a CCD camera (Cool view FDI, Photonic Science).

Bacterial strains and cultivation procedures

E. coli ATCC 15034 and *S. aureus* CIP 103594^T were used as test strains in all experiments. Bacterial strains were obtained from the ATCC (Manassas, VA) and the Culture Collection of the Pasteur Institute (France). Pure cultures were stored at −80°C in nutrient broth (Waltham, MA, Oxoid) supplemented with 20% (v/v) of glycerol. Both strains were routinely cultivated on nutrient agar (NA; Oxoid).

Bacterial suspensions were freshly prepared for each independent experiment from cultures of *E. coli* and *S. aureus* that were grown overnight at 37°C, yielding the stationary phase cells as described in previous work.[31] Briefly, the cell density was first adjusted to OD_{600} 1 to yield 0.8×10^8 bacterial colony forming units (cfu) per mL in PBS (10 mM, pH 7.4). Each initial suspension was subjected to five serial dilution steps (dilution factor 10^5) to obtain the recovery of 300 cfu from 50 μL of bacterial suspension spread per plate. These suspensions optimized by the plate technique were further used in all experiments as controls.

Sample preparation for microwave sterilization

A sample size of 10.0×10.0×0.5 mm (length×width×thickness) was considered suitable for these experiments. At 12 h before microwave sterilization the samples were submerged in 70% ethanol solution to eliminate possible contaminants. One hour before treatment the samples were washed with PBS to cleanse out the ethanol. The samples were kept in this state until further use. For the inoculation procedure, it was determined that 30 μL of the optimized bacterial suspension would be adequate to coat the test sample. Each sample was placed onto a micro Petri dish (35 mm; Esser, Germany, Griener) and inoculated with one of the bacterial test strains. After MW treatment, each sample was removed from the micro Petri dish and washed in 500 μL of PBS to remove bacteria. For each sample, five 50 μL aliquots taken from the washed solution were spread onto NA plates to determine recovery of viable bacterial cells. The remainder of the bacterial suspension was used for scanning electron microscopy (SEM) analysis to determine whether any differences in cell morphology were visible after microwave sterilization. Controls for this experiment were set up by inoculating the samples with 30 μL of bacterial suspension, washing it in 500 μL of PBS, and spreading ten 50 μL aliquots onto NA plates.

Microwave apparatus

The microwave apparatus that was used in the present study had the option of a variable frequency ranging from 1 to 18 GHz (Vari-Wave Model LT 1500; Lambda Technologies, Cincinnati, OH). The LT1500 is a computer-controlled variable frequency processing cavity for delivering excellent levels of control and uniformity of energy distribution into a multimode microwave cavity. Both the amplitude and frequency of the microwave could be varied allowing a significant expansion of the parameter space within which a process could be optimized. A data-logging option allowed processing data capture from the embedded computer system over a standard RS-232-C serial interface. A cavity characterization option was also available that allowed evaluation of the performance of a material in the cavity to assist in determining the optimum processing conditions as described in our previous design.[25]

Treatment of inoculated sample with microwave radiation

To separate the thermal and nonthermal effects of microwave radiation, it was attempted to control and record the maximal temperature reached during exposure. Previous studies indicated that during microwave sterilization of bacteria performed at 50°C, around 30% reduction in bacterial numbers was achieved as a result of the heating effect alone and not radiation.[25] Therefore, for the threshold treatment temperature, 45°C was preferred, as it is the temperature at which the target pathogens were determined to be unaffected by heat. The experiments were prepared using the highest available frequency (lowest wavelength) of 18 GHz. The incident power output was adjusted so that the cavity and material therein absorbed a measured amount of energy, to prevent the temperature of the sample exceeding 45°C. In this design, an output of 16 W incident power was used. To ensure uniformity of exposure, each sample was placed on a Teflon pedestal in the same position, which had been determined by electric field modeling using CST Microwave Studio 3D electromagnetic simulation software.[25] This analysis allowed for the microwave power distribution at that particular position to be uniform, thereby eliminating hot spot effects.[17] Further, because the wavelength of microwaves in the cavity was much greater than the dimensions of the sample (10×10×0.5 mm), the possibility of noneven heating due to a nonuniform field distribution was negligible.

A second complexity in microwave processing is that within an anisotropic heterogeneous system (comprised of different substances or different phases) microwave field energy is converted to heat by dissimilar amounts in different parts of the system. This inhomogeneous energy dissipation allows some parts of a sample to be heated more quickly than others and may therefore lead to temperature variations within the sample. The energy absorbed as a result of microwave irradiation is primarily due to the existence of permanent dipole (polar) molecules that tend to re-orientate/vibrate under the influence of a microwave electric field. The loss mechanism based on the polarization in the alternating electric field of the microwaves causes molecular friction, which produces heat. This effect occurs much more strongly in liquids than it does in solids.

Polar liquids such as PBS are heated when their component molecules are forced to rotate within the field and therefore lose energy as a result of collisions. The amount of power (P, in W/m^3) that is absorbed by dielectric is given by:

$$P = 2\pi F \varepsilon_0 \varepsilon'' |E|^2$$

where $\varepsilon_0 = 8.854 \times 10^{-12}$ (F m^{-1}), F is the frequency (Hz), E is the electric field amplitude (V/m), and ε'' is the imaginary part of complex permittivity:

$$\varepsilon = \varepsilon_0(\varepsilon' - j\varepsilon'')$$

where ε' is the dielectric constant, a property of a dielectric that determines the electric energy stored, while ε'' is the dielectric loss, a measure of the energy transferred from the internal electric field to the material being irradiated.

In the present study, bovine pericardium samples contaminated with bacteria were immersed in PBS. This can be expressed in terms of the ratios of the temperature rise rate:

$$\left[\frac{\Delta T}{\Delta t}\right]_{saline} \div \left[\frac{\Delta T}{\Delta t}\right]_{pericardium} \div \left[\frac{\Delta T}{\Delta t}\right]_{bacteria} = \frac{\varepsilon''_{saline}}{\varepsilon''_{pericardium}} \times \frac{C_{pericardium}}{C_{saline}} \times \frac{\rho_{pericardium}}{\rho_{saline}}$$

and

$$\left[\frac{\Delta T}{\Delta t}\right]_{saline} \div \left[\frac{\Delta T}{\Delta t}\right]_{bacteria} = \frac{\varepsilon''_{saline}}{\varepsilon''_{bacteria}} \times \frac{C_{bacteria}}{C_{saline}} \times \frac{\rho_{bacteria}}{\rho_{saline}}$$

where $\Delta T/\Delta t$ is the rate of temperature rise; C_p, C_s, and C_b are the specific heat capacity (kJ/kg K) for the pericardium, saline, and bacteria, respectively; and ρ_p, ρ_s, and ρ_b are their corresponding densities (kg/m^3). According to previously published dielectric constant data,[26,27] the PBS would absorb microwave radiation much more rapidly than the pericardium

Table 1. Decontamination Rates of *E. coli* and *S. aureus* After Multiple Exposures of Bovine Pericardium to MW Radiation at 18 GHz and 16 W

	E. coli		S. aureus	
	Cfu/30µL (average)	*Decontamination rate (%)*	*Cfu/30µL (average)*	*Decontamination rate (%)*
Control	301 ± 129	0	268 ± 62	0
3 Exposures				
Trial 1	3.2 ± 3	98.9	14 ± 11	94.8
Trial 2	6.4 ± 2	97.9	12 ± 12	95.5
Average	4.8	98.4	13	95.2

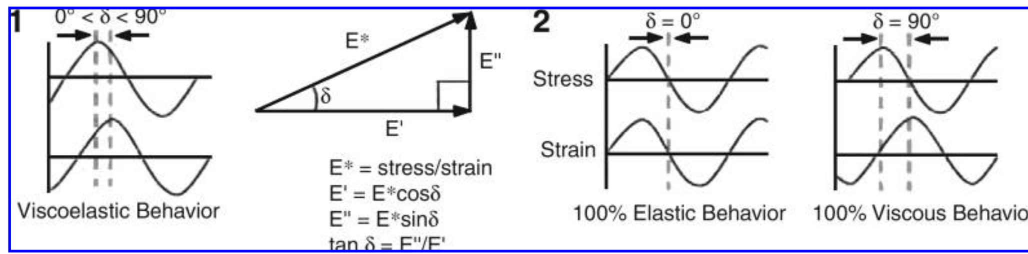

FIG. 1. Theoretical evaluation of the measurement of viscoelastic behavior of pericardial tissue.

and bacteria. Consequently, it would be expected that the internal temperature of the bacteria could not have risen higher than its surrounding pericardium or PBS, suggesting that nonthermal mechanisms of MW inactivation were in action.

Our previous study[25] showed that when using MW settings of 18 GHz and 16 W, three repeated exposures (with cooling in between exposures) allowed for the nonthermal effects of the MW radiation to be considerably manifested, leading to a significantly higher bacterial mortality rate than that obtained when using a single exposure. Therefore, the same methodology was adopted in the present study, and samples were exposed to three repeated exposures to MW radiation. Each sample was allowed to cool to 23°C between exposures to ensure that samples had the opportunity to receive the longest MW exposure possible without the core temperature exceeding the threshold of 45°C. Internal temperature control was achieved through the attachment of fiber optic probes to the sample within the microwave apparatus. All other environmental factors were kept constant during the study.

Mechanical properties of bovine pericardium

The viscoelastic properties of the pericardium tissue were determined using the Dynamic Mechanical Analyser (DMA 2980; TA Instruments, New Castle, DE) and analyzed using the Universal Analysis 2000 (TA Instruments). Further mechanical tests were completed using the Universal Tensile Tester (UTT) (Zwick Z010) and recorded using the Test Expert 8.1 (Zwick, Kennesaw, GA).

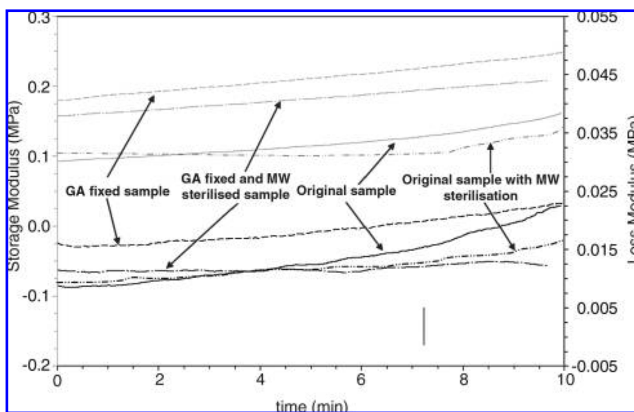

FIG. 2. Mechanical properties of bovine pericardium analyzed using dynamic mechanical analysis. Samples include original tissue, GA-fixed tissue, and GA-fixed tissue followed by MW sterilization. Top half of the streaks refers to the storage modulus, and the bottom half refers to the loss modulus.

Samples studied included the original bovine pericardium that had been stored in PBS before use, original pericardium that had been exposed to MW sterilization, pericardium that had been fixed with 0.6% GA, and fixed pericardium that was further subjected to MW sterilization.

Tests of dynamic mechanical analysis were run in the tensile oscillation mode using the Tension Film clamp. In this mode the samples were placed in tension between a fixed and moveable clamp. According to manufacturer's specifications, the bovine pericardial biomaterial was cut into rectangular pieces approximately 20.0×5.0×0.5 mm (length×width×thickness). An electromechanical motor attached to one arm drove the sample to selected amplitude (15 μm). An optical encoder coupled with a driven arm measured the sample response, strain, and frequency, as a function of the applied stress, and it provided feedback control to the motor. The sample was positioned in a temperature-controlled chamber and was measured at ambient temperature (23°C). The "fixed-frequency" mode of dynamic mechanical analysis operation was used throughout the experiment. In this mode the sample was allowed to oscillate at a fixed frequency (1 Hz or 60 oscillations/min). The resulting deformation strain was recorded as a function of time (for 10 min).

Tensile tests performed using the UTT was used to determine whether a difference existed in the maximal tensile strength of each sample (to the point of tearing). For this mechanical analysis, the bovine pericardial samples were cut into rectangular pieces approximately 50.0×5.0×0.5 mm (length×width×thickness) and kept in PBS until use.

Thermal analysis of bovine pericardium

Thermal analysis of the bovine pericardial biomaterial was performed using a Thermogravimetric Analyser (SDT 2960; TA Instruments) and Differential Scanning Calorimetry (DSC) (DSC 2920; TA Instruments). All data were recorded using the Universal Analysis 2000 (TA Instruments).

Thermogravimetric analysis was used to determine the thermal degradation of the pericardium by measuring changes in the mass of each sample as a function of temperature. This study allowed the identification of any changes in the thermal properties brought about by application of the MW sterilization technique.

DSC was used to identify the temperature associated with the thermal transitions in the tissue. This analysis provided quantitative information about possible changes in heat capacity of the pericardial tissue caused by MW sterilization.

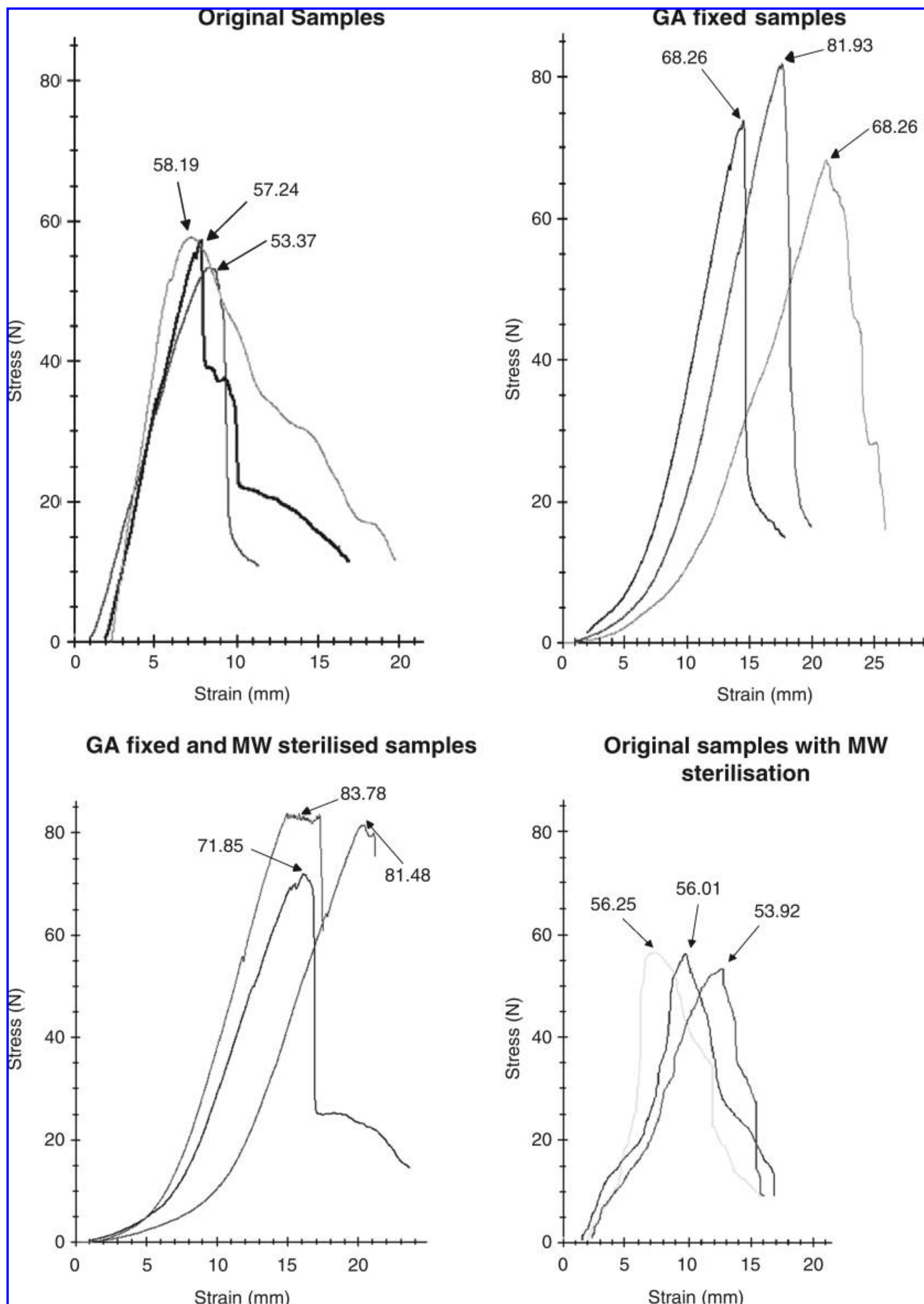

FIG. 3. Maximal tensile strength analysis of bovine pericardium using the UTT. Graph 1, original tissue; Graph 2, GA-fixed tissue; Graph 3, GA-fixed tissue followed by MW sterilization.

SEM

An FeSEM–ZEISS SUPRA 40VP was used to obtain high-resolution images of the bacterial cells and pericardium tissue. Primary beam energies of 3 to 15 kV were used, which allowed features on the sample surface or within a few microns of the surface to be observed, respectively. The samples were freeze-dried, and sputter-coated with gold before imaging.

Statistical analysis

Statistical data processing was performed using SPSS 15.0 (SPSS, Chicago, IL). Single independent groups *T*-test were

performed to compare consistency of sterilization rates across bacterial test strains and to determine whether a difference in tensile strength existed between the original bovine pericardium, original tissue followed by MW sterilization, GA-fixed tissue, and fixed tissue that was further subjected to MW sterilization.

Results

Microwave treatment

Repeated-exposure microwave treatment was used to study the nonthermal effects of the MW radiation at 18 GHz and 16 W. Each sample was subjected to three exposures of MW radiation, and then allowed to cool to room temperature (23°C) between exposures. The results of this experiment (Table 1) showed that three repeated exposures were able to sterilize 97.7% of pericardium contaminated with *E. coli* and 96% of the pericardium contaminated with *S. aureus.* A statistical analysis of the data highlighted that there were no significant differences in sterilization rates between the two bacterial test strains [$t(2) = 0.35$, $p > 0.05$].

Mechanical properties testing

Dynamic mechanical analysis is a technique used to measure the mechanical properties of a wide range of materials that behave both like an elastic solid and a viscous fluid. Under dynamic loading, viscoelastic materials such as polymers exhibit an intermediate phase difference. The complex modulus, E^*, can be calculated as the ratio of stress and strain (Fig. 1). From E^* and the measurement of phase shift, δ, the storage modulus, E^{I}, and loss modulus, E^{II}, can be obtained. E^{I} is the elastic component and related to the sample's stiffness. E^{II} is the viscous component and is related to the sample's ability to dissipate mechanical energy through molecular motion. The tangent of phase difference, or tan δ, is another common parameter that provides information on the relationship between the elastic and inelastic components. All of these parameters were calculated using the Dynamic Mechanical Analyser software, as a function of time, temperature, frequency, or amplitude (stress or strain).

Mechanical durability tests were conducted to determine whether the pericardium tissue would display altered mechanical functionality when subjected to MW sterilization after glutaraldehyde fixation. The durability of the pericardial tissue was tested for the original samples, the original sample followed by MW sterilization, GA-fixed samples, and GA-fixed samples followed by MW sterilization.

The original sterilized sample was included in the analysis to affirm that any change in mechanical properties due to MW radiation would not be shielded by the GA fixation. The results showed that the mechanical functionality of bovine pericardium was unaltered after MW sterilization of the original and fixed tissue compared to that of the unsterilized tissue samples. As can be seen from the data presented in Figure 2, the original (unfixed) tissue sample demonstrated a storage modulus that was only slightly lower than that of the fixed and MW-sterilized samples. This indicated that GA fixation had slightly altered the mechanical properties of the pericardium, causing the tissue to become more rigid. Results from the maximal tensile strength analysis using the UTT indicated no difference between the unfixed samples ($M = 56.27$, $SD = 2.55$) and the unfixed samples that had been subjected to the MW sterilization process ($M = 55.39$, $SD = 1.47$). A difference was found between the unfixed tissue compared to the fixed tissue ($M = 72.820$, $SD = 7.89$) and MW-sterilized tissue ($M = 79.04$, $SD = 6.33$) (Fig. 3). A statistical analysis of the data showed that the GA-fixed sample and the fixed followed by MW-sterilized samples had significantly higher tensile strengths than the original samples [$t(4) = 3.46$ and $p < 0.05$, and $t(4) = 5.78$ and $p < 0.05$, respectively]. Comparison of the data pertaining to the original and original followed by MW-sterilized tissue as well as comparison of the fixed and MW-sterilized tissue did not reveal a significant difference in tensile strength.

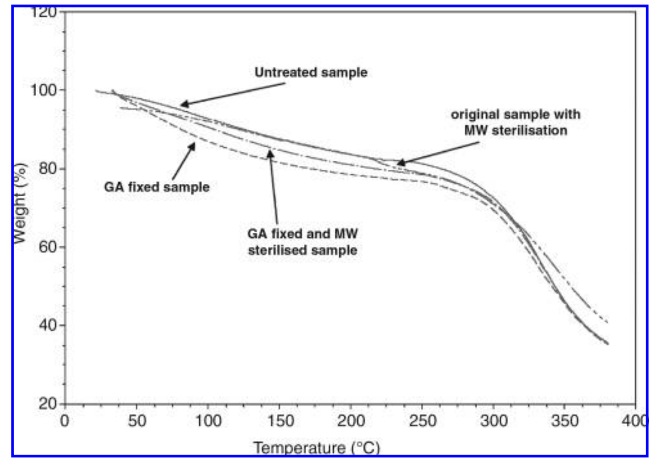

FIG. 4. Thermal analysis of bovine pericardium using Thermogravimetric Analyser.

Thermal analysis

Thermogravimetric analysis revealed no differences in the thermal degradation of the pericardial tissue of the MW-sterilized, GA-fixed only; original followed by MW sterilization; and control samples. These results indicated that the MW sterilization technique did not alter the thermal properties of the bovine pericardial biomaterial (Fig. 4). DSC analysis confirmed that all four samples were similarly stable. A large endothermic peak observed in all samples was related to evaporation of water at around 90–120°C (Fig. 5).

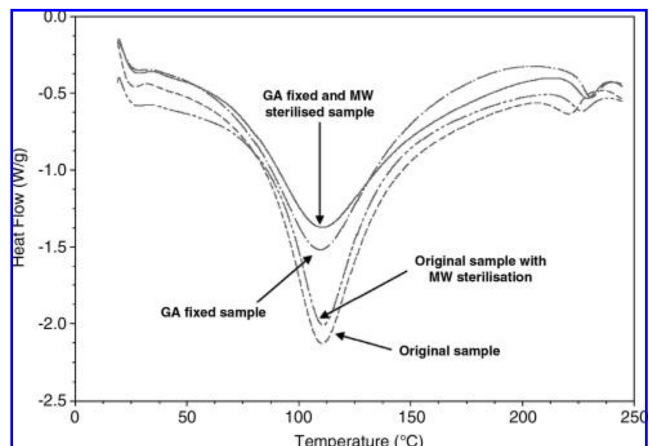

FIG. 5. Thermal analysis of bovine pericardium using DSC.

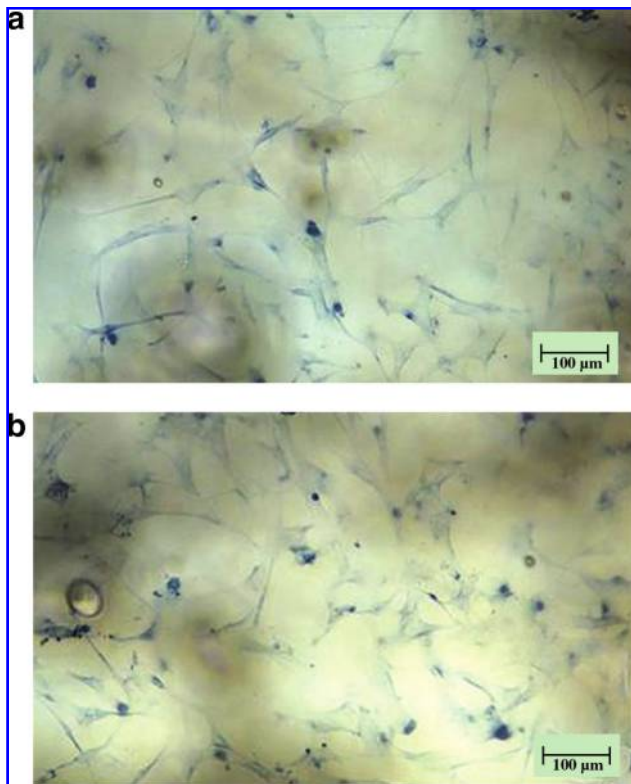

FIG. 6. *In vitro* mouse 3T3 fibroblast cell coverage of (**a**) GA-fixed pericardium tissue and (**b**) GA-fixed pericardium tissue followed by MW sterilization. Color images available online at www.liebertonline.com/ten.

Biocompatibility testing

In vitro cytotoxicity of GA-fixed pericardial tissue followed by MW sterilization was assessed visually as validity of this method was proven for mouse fibroblast cells that visual cytotoxicity scoring is predictive of the percentage of viable cells.[28] The visual assessment revealed (Fig. 6) that cells formed a confluent monolayer of well-defined cell-to-cell contact; cell morphology and cell density were unaltered; no cell lysis was observed. Thus, a score of five was assigned as indicative of a nontoxic reaction.

SEM analysis

Bovine pericardial biomaterial samples that had been subjected to GA fixation as well as GA fixation followed by MW sterilization were further compared using SEM to identify whether any changes in morphology had occurred. High-resolution images of the bovine pericardium are shown in Figure 7. An untreated control is included for comparison. It follows from the images presented in Figure 4 that SEM structural comparison of pericardial tissue did not reveal any visible differences for the GA-fixed tissue, GA-fixed tissue followed by MW sterilization, or original tissue used as a control. Most likely the change in properties is not reflected in the SEM visual appearance of the tissue, as even after treatment with glutaraldehyde, bovine pericardial biomaterial remains suitable for transplantation.

SEM analysis of bacterial cells treated with MW radiation revealed no visible morphological changes or any mechanical disruptions of bacterial cells for both test strains compared to the untreated controls (Fig. 8). This finding suggested that the bactericidal effects of MW radiation were not reflected in the morphological appearance of the cells, rather due to various types of molecular transformations and fatal metabolic changes that might have occurred.[29,30]

Discussion

The aim of the present study was to examine a novel sterilization technique using MW radiation that could lead to the inactivation of bacteria in bovine pericardial biomaterial

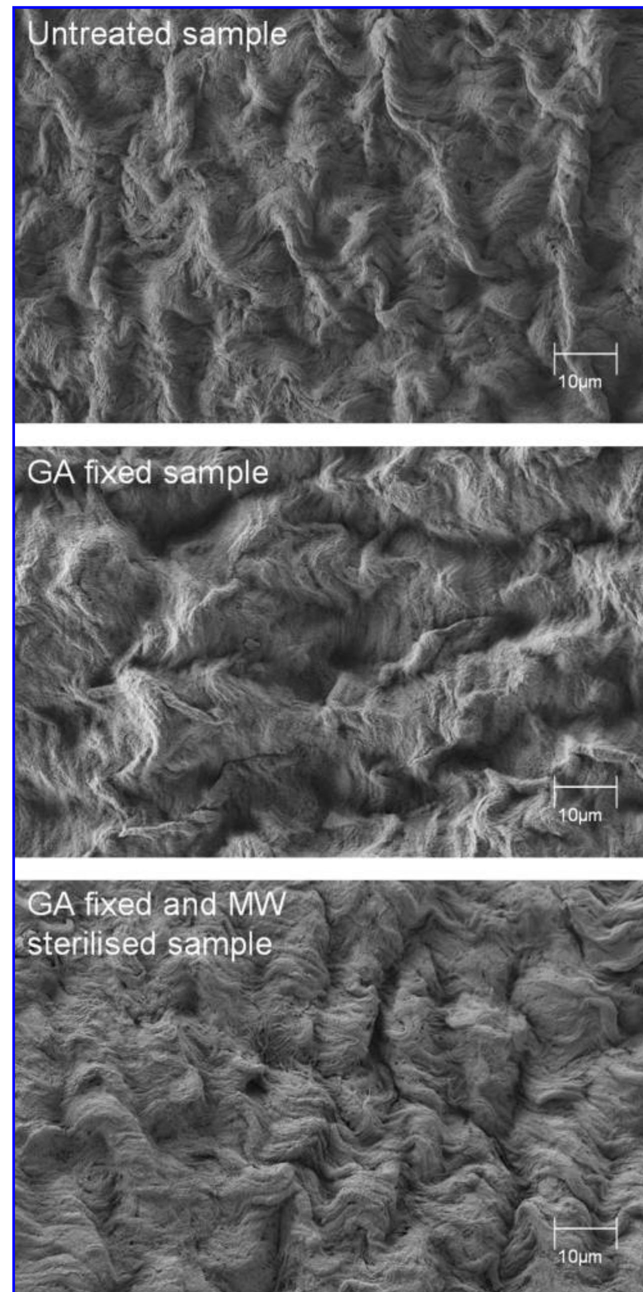

FIG. 7. Typical SEM images of the bovine pericardium. Samples include original tissue, GA-fixed tissue, and GA-fixed tissue followed by MW sterilization. The magnification used was ×1000.

used as transplant biomaterial. The data obtained in the present study suggested that using optimized settings, nonthermal MW radiation was up to 97% effective in decontaminating pericardium of bacteria with no adverse effects on biocompatibility or on the mechanical and thermal properties of the transplant tissue. Comparable levels of bacterial decontamination are currently achieved using gamma radiation.[28] The International Atomic Energy Agency recommends a gamma radiation value of 25 kGy to comply with sterility assurance level of 10^{-6} in transplant tissue.[28] In practice, however, this dose of radiation is not sufficient to guarantee such a level of sterilization because even under comparable conditions, resistance to radiation exposure varies widely among different bacteria.[28] To guarantee sterility assurance level of 10^{-6}, gamma radiation doses as high as 50 kGy are required, which have been found to evoke many physical and chemical changes in tissue grafts that lead to a reduction in their biological properties.[28] Therefore, using gamma radiation to sterilize tissue grafts necessitates a compromise in sterilization to maintain tissue integrity. Although tissue integrity was maintained throughout the present study, further work is needed to optimize the sterilization rates to reach the sterility assurance standard.

While near-complete bacterial inactivation using nonthermal MW radiation was achieved in the present study, the mechanisms of action were not determined and are generally not understood. The results reported here are novel for these MW settings; however, our results are in accordance with the conceptual approach of a number of other studies that have also demonstrated a perturbation in bacterial cellular activity (as well as cell death) using nonthermal MW mechanisms.[11,16,29–31] Because these other investigations have employed a wide range of microwave energy absorption or intensities, a direct comparison of the results of these studies to those of the current study is difficult, if not impossible, due to the lack of common experimental conditions.

An assortment of studies has suggested different directions for cellular damage that occurs as a result of microwave radiation.[10,30,31] The limitation of many of these studies is that the treatment of the bacterial cells was conducted at lethal or close to lethal temperatures (50°C to 60°C). Such temperatures could cause the denaturation of enzymes, proteins, and nucleic acids, as well as the disruption of membranes,[13] causing the basis of cell damage (nonthermal or thermal) to become hard to distinguish.

The effect of MW radiation on cell metabolism has also been previously studied. Dreyfuss and Chipley[10] indicated that microwave radiation affects the metabolic activity of bacteria in a manner that could not be explained by thermal effects alone. These conclusions were made based on increases in the specific activities of several key enzyme systems after sublethal microwave treatment. Samarketu *et al*.[31] suggested that at the molecular level, microwaves (at 9.575 GHz) induce different biological effects in a nonthermal method by changing the

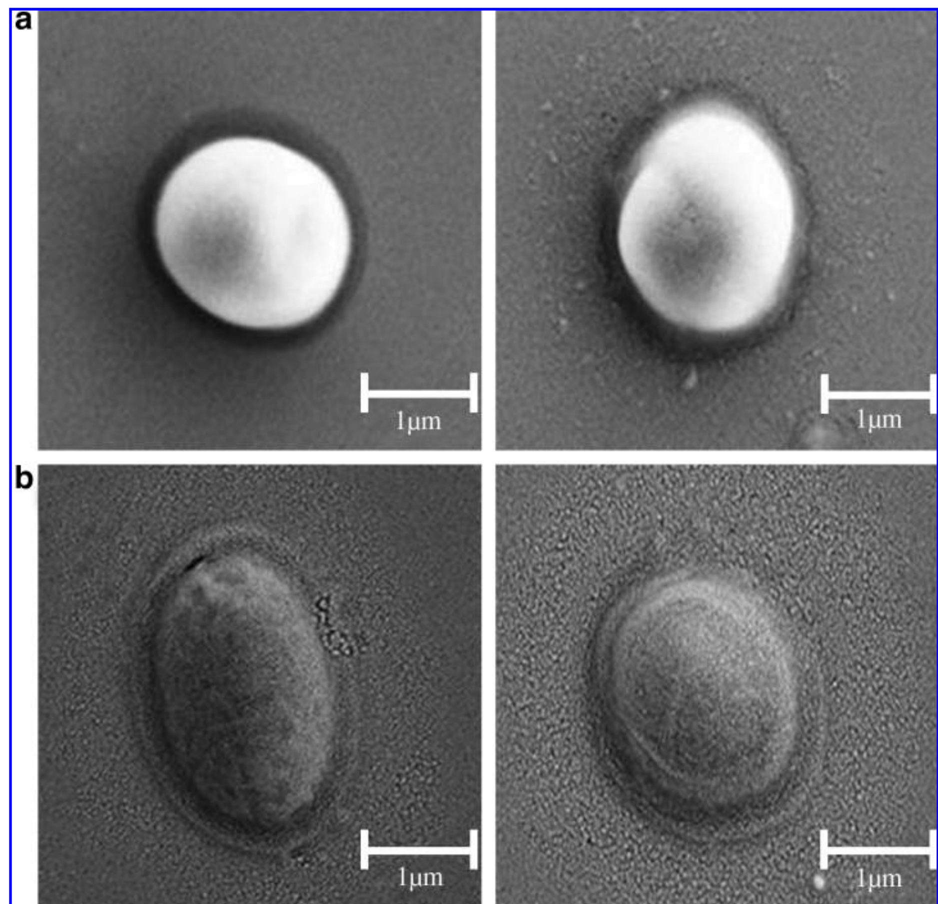

FIG. 8. Typical SEM images of *S. aureus* and *E. coli* cells: control (left) and MW-treated cells (right). (**a**) *S. aureus*; (**b**) *E. coli*. The effect of MW radiation did not result in mechanical disruptions of bacterial cells.

structures by differentially partitioning the ions, altering the rate and/or direction of biochemical reactions in *Cyanobacterium*. Following on from the literature mentioned above, the present study has provided evidence suggesting that nonthermal bacterial inactivation can be achieved by the application of MW radiation. While near-complete inactivation of bacteria was evident, however, the reason for the observed reduction in bacterial numbers was not determined. Future research is needed to determine the specificity of the damage that occurs to bacterial cells, and the current MW parameters need to be further fine-tuned to achieve complete biomaterial sterilization.

In conclusion, this study has demonstrated that the inactivation of pathogenic bacteria in bovine pericardial biomaterial can be achieved by the application of nonthermal microwave radiation. In particular, this study is the first in its kind to demonstrate that the mechanical and thermal properties of bovine pericardium are not affected by nonthermal microwave radiation at the frequency of 18 GHz. This study also demonstrated that the application of MW radiation at a frequency of 18 GHz, power of 16 W, and with three repeated exposures, up to 97% of pathogenic bacteria could be eliminated from bovine pericardial biomaterial.

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Disclosure Statement

No competing financial interests exist.

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