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ORIGINAL ARTICLE

Sterilization using electrolyzed water highly retains the biological properties in tissue-engineered porcine liver scaffold

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Purpose: *The aims of this study were to investigate the effects of sterilization with peracetic acid (PAA) and ethanol on the biological activity of porcine liver scaffolds and to develop a new technique for sterilization using slightly acidic electrolyzed water (SAEW).*

Methods: *Decellularization of liver slices was performed using 0.1% sodium-dodecyl-sulfate, then evaluated by histological and polymerase chain reaction analyses. Decellularized slices were treated with either PAA or ethanol or SAEW, and then DNA content was quantified. We determined sterilization efficiency by culturing scaffolds in culture medium and on blood agar. We next analyzed the glycosaminoglycan and collagen contents of the scaffolds. Finally, we tested the cytotoxicity of the scaffolds as well as the effects of sterilization on host cell attachment and proliferation.*

Results: *Complete cell and antigenic epitopes removal emphasized the decellularization efficiency. PAA and SAEW treatments achieved the highest efficiency of sterilization compared to that of the ethanol treated scaffolds, and were able to remove a considerable fraction of DNA from decellularized livers. The retained glycosaminoglycan content decreased in all treatments in the following order: SAEW, ethanol, and PAA. Ethanol caused a significant loss in collagen content compared to the other groups. A cytotoxicity evaluation revealed that all scaffolds were nontoxic. SAEW-treated scaffolds supported cell attachment and proliferation at a significantly higher rate than other groups.*

Conclusions: *These data suggest that SAEW is highly efficient for sterilizing scaffolds and allowed the scaffolds to retain their bioactivity in addition to its high efficiency for cell remnant removal.*

Keywords: *Decellularization, Sterilization, Scaffold, Electrolyzed water*

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INTRODUCTION

The incidence of hepatitis B virus-related end-stage liver disease and hepatocellular carcinoma is extremely high in Asia, particularly in eastern and southeastern areas (1). About 30 million people in the United States have liver disorders, with approximately 27,000 deaths reported per year due to liver disease (2). Liver transplantation is the

gold standard for treating patients with severe hepatic failure. However, the shortage of donors limits its application and has led to a dramatic increase in the number of patients wait listed for liver transplantation (3).

Many studies have reported that scaffolds mimic the biological properties of the native extracellular matrix (ECM) (4). Indeed, scaffolds have been prepared and applied in the fields of tissue engineering and regenerative medicine. Scaffolds are composed of a sophisticated assembly of proteins, glycosaminoglycans (GAGs), and growth factors. A scaffold represents the ideal microenvironment and can be seeded with cells to facilitate their migration, proliferation, and differentiation (5).

Animal-originating scaffolds must undergo treatment to minimize or eliminate the host immune response and to avoid zoonotic disease transmission after *in vivo* implantation in recipients (6, 7). Many agents have been used for sterilization; however, each has disadvantages that may adversely affect ECM properties, rendering it unfit for transplantation (8-10). Preserving GAGs, collagen, and growth factors in their natural state is highly desirable because of their critical role in the maintenance of scaffold bioactivity (11, 12). Many researchers have attempted to determine the relationship between the DNA content of scaffolds and the host immune response (13). They suggested that xenogenic DNA within scaffold materials plays a role in the inflammatory and tissue remodeling responses after implantation; therefore, DNA must be effectively removed from tissues and organs (14). Primary porcine hepatocytes provide a valuable tool for different research strategies and clinical applications. However, the utility of cultured hepatocytes is hindered by many difficulties in obtaining populations of primary cells, which cannot be expanded *in vitro* because of a limited life span. Even the availability of porcine hepatocytes cell lines is still limited (15). Fibroblast is commonly used for biocompatibility evaluation of different extracellular matrices obtained from tissues such as liver, urinary bladder (8), heart (16), intestine (7) and pericardium (17). For these reasons, in this study we used porcine fibroblasts to check the effect of sterilization on the behavior of cells in terms of attachment, growth, and proliferation.

The present study reports on the effects of two agents commonly used for scaffold sterilization and their effect on the biological properties of porcine liver ECM. The treatments included 0.1% peracetic acid and 70% ethanol. We also established a new technique to sterilize scaffolds using slightly acidic electrolyzed water (SAEW). SAEW, which contains free chlorine and hypochlorous acid, is effective for disinfecting surgical equipment and treating wounds. SAEW is easily generated by electrolysis of a solution containing hydrochloric acid and sodium chloride in a chamber without a membrane (18, 19). Therefore, we investigated the effect of sterilization on the structure and biological properties of scaffolds.

MATERIALS AND METHODS

Liver harvest and decellularization

All procedures were approved by our Institutional Animal Care and Use Committee (Kangwon National University, Korea). Porcine livers were collected from mixed breed adult pigs weighing 40 kg to 50 kg directly after slaughtering and evisceration. Harvested liver lobes were separated and trimmed into several thin slices of size 2.5 × 1.5 × 0.2:0.3 cm with a weight of 5 g per slice. The slices were washed twice in phosphate buffered solution (PBS) containing heparin (Chungwae Pharma Co., Seoul, Korea) in a concentration of 500 IU/l for 1 h using a shaker at a speed of 120 rpm at 4°C. The slices were decellularized using 0.1% sodium dodecyl sulfate (SDS, Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 72 h on a shaker. The decellularization solutions were changed every 8 h. Finally, all slices were rinsed three times in PBS for 2 h each to flush the residual SDS from the ECM.

Sterilization treatment

Scaffolds were treated with either 70% ethanol solution (Daejung Chemicals and Metals, Seoul, Korea), 0.1% peracetic acid solution (Sigma-Aldrich, St Louis, MO, USA), or SAEW. Scaffolds in the control group were treated with PBS. Sterilization was performed using these agents for 2 h as suggested by other studies. The sterilizing agents were changed every 15 min, followed by eight washes with PBS for 15 min each time (8, 14, 20).

Preparation of SAEW

SAEW (pH, 6.2-6.4), with an oxidation reduction potential (ORP) of 780-797 mV and available chlorine concentration of 24 mg/l, was produced by electrolysis of 6% HCl and 2 M NaCl in a chamber without a membrane at 12 A

using an electrolysis device as described by Cao et al (18). SAEW was placed into tightly sealed sterile bottles and directly used after preparation.

Morphological evaluation

Native and decellularized livers were fixed, paraffin embedded, and sectioned according to standard protocols. Then, samples were cut into 4 µm sections and stained with hematoxylin and eosin (H&E) to evaluate the efficiency of our decellularization protocol in preserving the tissue architecture and removing the cellular components. To confirm the removal of nuclear materials, sections were also stained by DAPI staining (Vector Laboratories, Burlingame, CA, USA).

DNA extraction and polymerase chain reaction (PCR) analysis

DNA was extracted from lyophilized samples of native, treated, and untreated decellularized livers using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Five milligrams of lyophilized samples was digested with proteinase K buffer at 56°C on a hot plate for 24 h. The digest was treated with AE buffer and ethanol. DNA was eluted using AE buffer and centrifuged at 6000 rpm for 4 min. The concentration of extracted DNA from each sample was measured with a NanoDrop spectrophotometer ND-1000 (PeqLab, Erlangen, Germany). Equal amounts of extracted DNA were separated on a 1% agarose gel containing 0.5% ethidium bromide and visualized with ultraviolet transillumination (G:BOX F3, Syngene, Cambridge, UK) using a reference 100-base pair DNA ladder (GeneRuler, Fermentas, Burlington, ON, Canada) to determine residual DNA fragment size. To validate the effectiveness of the decellularization procedure in elimination of the porcine DNA sequences encoding immunogenic and pathogenic antigens, 50 ng of genomic DNA extracted from decellularized livers was used for PCR analysis by TProfessional standard 96 gradient machine (Biometra, Goettingen, Germany). The sequences of primers used are summarized in Table I. Thermal cycler conditions for amplification were the following: 94°C for 3 min, followed by 34 cycles of 94°C for 30 s, annealing temperature for 30 s and 72°C for 45 s, and a final extension at 72°C for 10 min. PCR products were analyzed on 1% agarose gel stained with ethidium bromide.

TABLE I - PRIMERS USED FOR PCR ANALYSIS

Primer	Importance	Primer sequence	Annealing temperature	Product size (bp)
1,3 α gal	Responsible for hyperacute rejection after xenotransplantation (1)	F: 5'-GCTCCACCTGGCAGTCATAG-3' R: 5'-GTCCTGGAGGATTCCCTTGA-3'	54.95	361
SLA-2	Plays a role in acute rejection (2)	F: 5'-GTCACCTTGAGGTGCTGGG-3' R: 5'-TGGCAGGTGTAGCTCTGCTC-3'	55.04	185
SLA-DRA	Plays a role in acute rejection (2)	F: 5'-CGAGAAGAGGTGGCAAGACA-3' R: 5'-GTCCTGGAGGATTCCCTTGA-3'	54.5	220
pvWF	Responsible for thrombosis problems after xenotransplantation (3)	F: 5'-GCC CCT TTG CAG GAG AAG AT-3' R: 5'-ATA CAG CCC TTT GCT GGC AT-3'	60.03	375
PERV	Possible risk infection after xenotransplantation (4)	F: 5'-CTACCCCGAGATTGAGGAGC-3' R: 5'-GGGGGATGGTTAGTTTTCCA-3'	54.9	317
β-actin	cell remnant marker (5)	F: 5'-TCC CTG GAG AAG AGC TAC G-3' R: 5'-TGT TGG CGT AGA GGT CCT TC-3'	60.5	280

1, 3 gal = alpha 1, 3 galactosyltransferase; SLA-2 = swine leukocyte antigen 2 = SLA-DRA swine leukocyte antigen DR alpha; PERV = porcine endogenous retrovirus-gag; pvWF = porcine von Willebrand factor; ß-actin = beta actin; F = forward; R = reverse.

(1) Puga Yung GL, Li Y, Borsig L, et al. Complete absence of the alphaGal xenoantigen and isoglobotrihexosylceramide in alpha 1,3 galactosyltransferase knock-out pigs. Xenotransplantation. 2012;19(3):196-206.
(2) Park KM, Park SM, Yang SR, Hong SH, Woo HM. Preparation of immunogen-reduced and biocompatible extracellular matrices from porcine liver. J Biosci Bioeng. 2013;115(2):207-215.
(3) Lin CC, Cooper DKC, Dorling A. Coagulation dysregulation as a barrier to xenotransplantation in the primate. Transpl Immunol. 2009;21(2):75-80.
(4) Ota T, Taketani S, Iwai S, et al. Novel Method of Decellularization of Porcine Valves Using Polyethylene Glycol and Gamma Irradiation. Ann Thorac Surg. 2007;83(4):1501-1507.
(5) Petersen TH, Calle EA, Zhao L, et al. Tissue-engineered lungs for *in vivo* implantation. Science. 2010;329(5991):538-541.

Sterility testing

Sterility efficiency was evaluated by incubating the sterilized scaffolds in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37°C and on Columbia blood agar (Fluka Chemie, Zurich, Switzerland) for 96 h as described by Shearer et al (21). Samples were checked periodically for signs of infection, as indicated by any discoloration or turbidity of the media or growth on Columbia blood agar. Native liver, untreated, and PBS-treated scaffolds were used as positive controls, whereas medium without scaffolds was used as the negative control.

GAG quantification

GAG content in native liver and in untreated and treated scaffolds was determined using a dimethyl methylene blue dye-binding assay kit (Blyscan, Biocolor, Carrickfergus County Antrim, UK), according to the manufacturer's instructions. In brief, 5 mg of lyophilized sample was homogenized and solubilized. A 100 µL aliquot from each sample lysate was added to 1 mL of dimethyl methylene blue and agitated on a shaker at 25°C for 30 min. Solutions were then centrifuged at 10,000 × g for 10 min to collect the GAG-dye complex, and the supernatants were discarded. The remaining pellet was suspended in 1 mL of the provided dissociation reagent, and absorbance was read at 655 nm.

Collagen quantification

Total collagen levels in native liver, and decellularized and sterilized scaffolds were determined using a Sircol collagen dye-binding assay kit (Biocolor, Carrickfergus, County Antrim, UK), according to the manufacturer's instructions. Briefly, 5 mg of lyophilized sample was homogenized, and total acid pepsin-soluble collagen was obtained after incubation in 0.5 M acetic acid containing 0.1 mg/ml pepsin. A 100 µL aliquot of acid neutralizing reagent was added to the acid-pepsin extract, followed by cold isolation and concentration reagent. The tubes were centrifuged after an overnight incubation at 4°C. Then, 1 mL Sircol dye reagent was added to the pellet and incubated at 25°C for 30 min. After centrifugation, the pellet was washed with acid-salt wash reagent and suspended in 1 mL of alkaline reagent, and absorbance was read at a wavelength of 540 nm.

Cell attachment assay (in vitro direct contact assay)

Small discs of liver scaffolds that had been treated with different sterilizing agents were placed into a 96-well plate and evaluated for their ability to support the attachment and viability of porcine fibroblasts (passage 4). Fibroblasts were harvested from tissue culture dishes, counted, and resuspended in complete DMEM containing 10% FBS. Aliquots of 100 µL containing 5×10^4 cells were added to each scaffold. The plate was incubated at 37°C in 5% CO_2 for 3 h to allow the fibroblasts to attach. The scaffold substrates were transferred to another plate after 3 h, so the cells that had attached to the scaffold would remain undisturbed. Then, the plate was incubated at 37°C in 5% CO_2 for 12 h. After 12 h, 10 µL of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) was added to each well followed by a 4 h incubation at 37°C in 5% CO_2. Then, the media were removed and 200 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. After 10 min incubation, 100 µL solutions from respective substrate wells were pipetted into another 96-well plate. Thereafter, the absorbance was measured using a spectrophotometer with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain the sample signal. Here, the results represent the percentage of seeded cells that attached to the scaffold substrate after 3 h incubation and remained metabolically active.

We used DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St Louis, MO, USA)-labeled porcine fibroblasts in the same manner for 16 h to confirm the results obtained from the *in vitro* direct contact assay. Then, the scaffolds were checked by phase-contrast fluorescence inverted microscopy (Olympus, Tokyo, Japan) to count the number of attached cells. DAPI-labeled cells were counted in two fields per well using Image J software (National Institutes of Health, Bethesda, MD, USA).

In vitro cytotoxicity assessment using the extraction method

This test aimed to exclude any cytotoxic effect that might result from compounds leached from the scaffolds (22, 23). Serum-free DMEM cell culture medium was incubated overnight with each scaffold at a concentration of 0.2 g/ml media at 37°C in a shaker at 70 rpm/min. Additional DMEM without scaffolds and minced, powdered, latex

gloves were similarly processed to provide unconditioned negative and positive controls, respectively. Then, the scaffolds were removed and the medium was filtered through a 0.4 µm filter. Porcine skin fibroblasts were harvested, counted, and resuspended in complete DMEM. A total of 0.1×10^4 cells were added to each well in 100 µL media. Samples were allowed to incubate at 37°C in 5% CO_2 for 24 h. The medium was aspirated, followed by the addition of conditioned or control medium after adding 10% FBS and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). The cell response against the extraction fluid was evaluated after incubating the plate for 24 h and 48 h. The culture medium was changed after 24 h. At the end of the incubation, 10 µL of MTT solution was added to each well followed by a 4 h incubation. The medium was aspirated, and 200 µL DMSO was added to each well to dissolve the formazan. Then the absorbance was recorded at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Ki-67 cell proliferation staining

The objective of this test is to evaluate the ability of scaffolds to release soluble factors that stimulate cell proliferation (10, 12). Conditioned and control media were prepared as described for the previous cytotoxicity test. Porcine fibroblasts were cultured at a concentration of 0.5×10^4 on gelatin-coated 4-well plates for 24 h. Then, the medium was switched to conditioned or control medium. The medium was changed every 24 h to offer the fibroblasts a continuous supply of soluble factors. The proliferation potential of the soluble factors that eluted from the scaffolds was checked after incubation for 24 h and 48 h. Cells were immunostained using anti-Ki-67 antibody (Abcam, Cambridge, UK). Texas Red goat anti-rabbit IgG (Invitrogen) was used as a secondary antibody. Nuclei were counterstained using a mounting medium containing DAPI. The percentage of Ki-67 positive cells was calculated in three fields as per the ratio of average positive Ki-67 cells relative to 250 DAPI-stained cells.

Statistical analysis

Results are reported as mean ± standard deviation. A one-way analysis of variance followed by Tukey's test was performed to determine significance differences in DNA, GAG, and collagen contents between treated and untreated decellularized liver samples. Tukey's test was also used to analyze the results of cytotoxicity and Ki-67 assays. The results of the cell attachment and *in vitro* cytotoxicity assays are expressed as the percentage of the corresponding negative controls conducted within the same experiments. A two-tailed Student's *t*-test was performed to assess differences between the control and different groups for the cell attachment assay. A p value <0.05 was considered statistically significant.

RESULTS

Characterization of decellularized liver matrix

The slices were decellularized by agitating them with 0.1% SDS for 72 h at 4°C. As shown in (Figs. 1A and B), translucent white-colored slices, which retained the same gross shape of liver slices before decellularization, were generated. Histological evaluation showed hexagonal-shaped lobules which were separated by connective tissue in addition to lack of cytoplasmic and nuclear staining in decellularized liver matrices (Figs. 1C and D). A lack of DAPI staining in scaffolds confirmed the absence of cells whereas abundant nuclei were observed in native livers (Figs. 1E and F).
PCR analysis of DNA extracted from decellularized liver samples after SDS treatment revealed that our decellularization protocol was efficient in removing of the antigenic and pathogenic epitopes (Fig. 1G) that include alpha 1,3 galactosyltransferase (1,3 α gal), swine leukocyte antigen 2 (SLA-2), swine leukocyte antigen DR alpha (SLA-DRA), porcine endogenous retrovirus-gag (PERV), porcine von Willebrand, and porcine beta actins (ß-actin). Therefore, these results suggested that the scaffolds generated were immunogen-reduced and can be applied for potential xenotransplantation.

SAEW removed a considerable fraction of immunogens from decellularized tissues

DNA is a cell remnant indicator; hence, we quantified the amount of DNA to evaluate the efficacy of the sterilizing agents for removing these remnants. The amount of DNA in decellularized matrices was 42.5 ± 3.21 ng/mg dry weight ECM. The statistical analysis indicated that the DNA concentration in scaffolds treated with PAA or SAEW was significantly lower (2.9 ± 1.20 ng/mg dry weight ECM and 16.85 ± 2.84 ng/mg dry weight ECM, respectively)

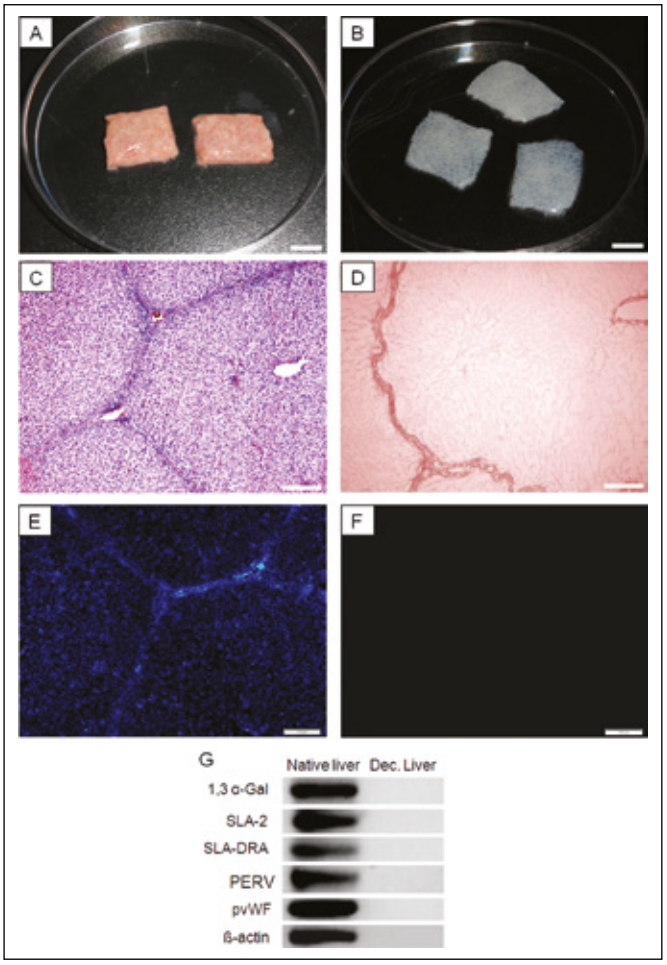

Fig. 1 - *Gross appearance of the porcine liver slices subjected to decellularization by agitation.* ***(A)*** *Before decellularization, and* ***(B)*** *after decellularization (Scale bar = 10 mm). H&E staining of decellularized porcine liver* ***(D)*** *showed no cytoplasmic or nuclear staining compared to native liver* ***(C)*** *as well as preserving of the honeycomb-shaped connective tissue that outlines the removed hepatocytes (Scale bar = 100 µm). DAPI staining revealed absence of the nuclear materials in liver matrices* ***(F)****, whereas nuclear materials were evident in native liver* ***(E)****.* ***(G)*** *PCR analysis of decellularized liver samples showed that 1.3 α gal, SLA-2, SLA-DRA, pvWF, PERV, and ß-actin antigens were completely eliminated after decellularization.*

compared to scaffolds treated with ethanol (25.7 ± 3.63 ng/mg dry weight ECM) or PBS (32.11 ± 2.88 ng/mg dry weight ECM) (Fig. 2A).

In other words, PAA revealed the highest efficiency for removing cell remnants and removed approximately 93.13 ± 2.82% of the DNA content from decellularized liver. SAEW was also efficient for removing DNA content (60.29 ± 6.68%) from decellularized liver. No significant difference was observed between the DNA content in scaffolds treated with PBS or ethanol.

The fragment size of the isolated DNA was assessed using agarose gel electrophoresis. Both decellularized and sterilized scaffolds showed no DNA band, whereas the native liver showed a large band at >1000 bp (Fig. 2B).

SAEW is a highly efficient sterilizer of liver scaffolds

Sterilization efficacy was evaluated qualitatively by the absence of signs of infection after a 96 h culture period in DMEM supplemented with 10% FBS and on Columbia blood agar medium. As shown in (Tab. I), neither discoloration of DMEM nor growth on blood agar was observed in treated scaffolds after 48 h incubation. After 60 h incubation, scaffolds treated with ethanol showed slight growth on blood agar, which increased with time, and slight discoloration of the DMEM occurred after 72 h of incubation. No growth on blood agar or discoloration of DMEM was observed in scaffolds treated with PAA or SAEW after 96 h of incubation.

SAEW retains GAGs in the scaffolds

A significant decrease in GAG content was observed in scaffolds treated with PAA (43 ± 0.80%) compared to that of native liver and 56 ± 1.04% compared to that of decellularized liver (Fig. 2C). The amount of GAGs retained in scaffolds treated with PBS was 68.7 ± 0.320% compared to that in native liver, whereas it was 89.8 ± 0.418% compared to that in decellularized liver. Scaffolds treated with SAEW showed significantly higher GAG content (53.3 ± 0.83% and 69.6 ± 0.58%) compared that of native and decellularized liver and scaffolds treated with ethanol, which contained 49.1 ± 1.47% and 64.16 ± 1.92% of the native and decellularized liver, respectively.

SAEW had no adverse effects on collagen

The amount of collagen in the liver ECM after decellularization and sterilization was estimated and compared to that in native liver. The results indicated no differences between scaffolds treated with PBS, SAEW, PAA, or decellularized scaffolds where the amounts were 111.61 ± 2.44%, 111.14 ± 2.19%, 110.37 ± 3.59%, and 113.11 ± 2.97% compared to those in native liver (Fig. 2D). In contrast, scaffolds treated with ethanol exhibited a significant loss in collagen, and the amount of collagen was 87.54 ± 4.07% compared to that in native liver.

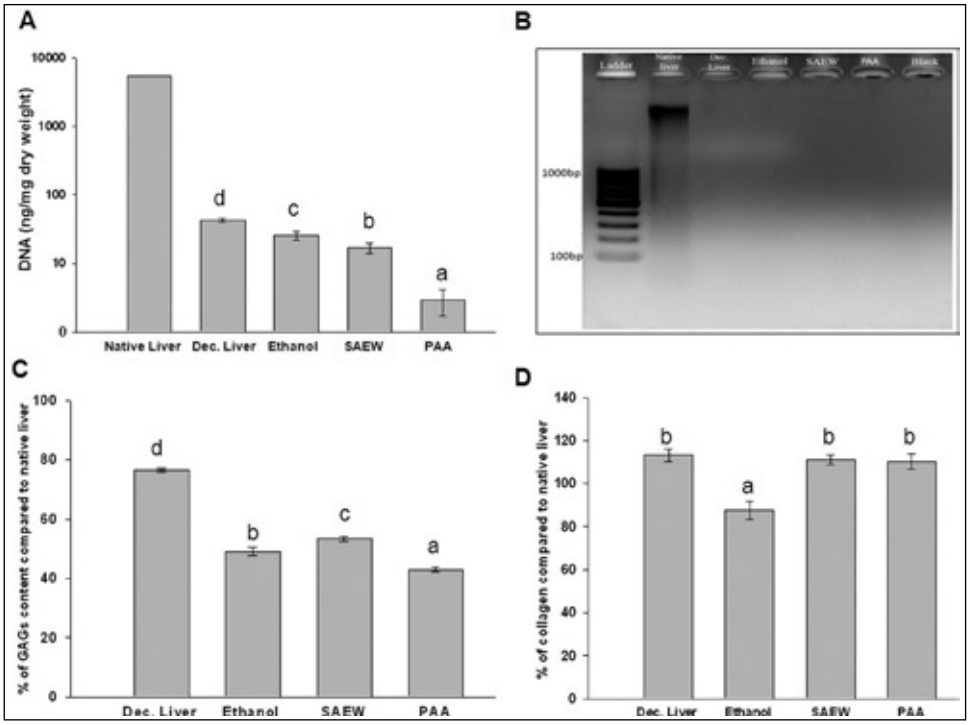

Fig. 2 - ***(A)*** *DNA quantification of untreated and treated decellularized liver scaffolds compared to native liver. All scaffolds after sterilization showed significantly lower DNA content than those of untreated liver matrix.* ***(B)*** *Native liver showed a large amount of DNA on gel electrophoresis, whereas untreated and treated scaffolds did not.* ***(C)*** *Glycosaminoglycans (GAGs) in untreated and treated liver scaffolds. All scaffolds had significantly reduced GAG content after sterilization.* ***(D)*** *Collagen content was only significantly reduced in scaffolds treated with ethanol and no significance difference was observed between scaffolds treated with slightly acidic electrolyzed water (SAEW), peracetic acid (PAA), or untreated. Bars with the same letter are not significantly different. Error bars: ± standard deviation; n = 8, p<0.05.*

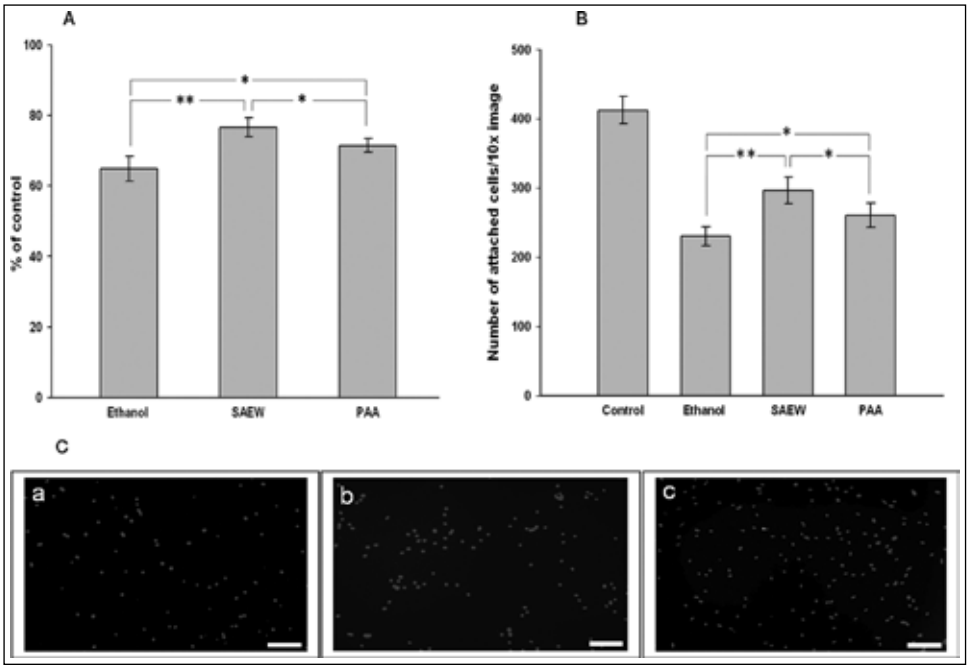

Fig. 3 - ***(A)*** *MTT cell attachment assay revealed the ability of slightly acidic electrolyzed water (SAEW)-treated scaffolds to support the attachment of seeded porcine fibroblasts at a significantly higher percentage than in other groups.* ***(B)*** *Cell attachment assay using DAPI-labeled porcine fibroblasts confirmed the ability of cells to adhere to SAEW-treated scaffolds in a higher number than that of other scaffolds. (Error bars: ± standard deviation; n = 8, *p<0.05, **p<0.01).* ***(C)*** *Attached DAPI-stained fibroblasts on scaffolds treated by ethanol* ***(a)****, peracetic acid (PAA)* ***(b)*** *and slightly acidic electrolyzed water (SAEW)* ***(c)****. Scale bar = 50 µm.*

SAEW-treated scaffolds support cell attachment

The effect of sterilization on host cell attachment was evaluated to determine whether the scaffolds were biologically active. Compared with fibroblasts exposed to media alone, 64.86 ± 3.48% of seeded fibroblasts were attached to scaffolds treated with ethanol during the 3 h incubation period and remained viable for 16 h (Fig. 3A). Scaffolds treated with PAA and SAEW supported the attachment and viability

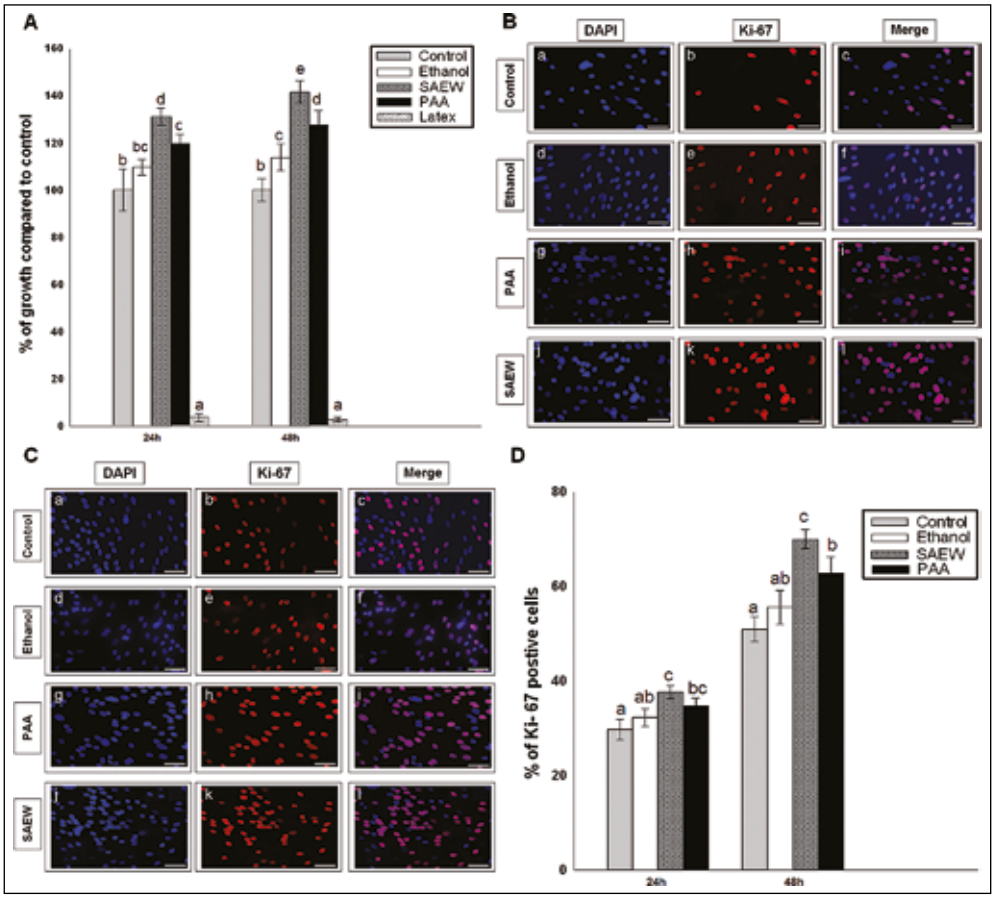

Fig. 4 - ***(A)*** *Cytotoxicity assay by extract method. This assay was performed over 48 h and demonstrated that all extracts prepared from scaffolds were biocompatible, nontoxic, and did not inhibit growth of porcine fibroblasts. Results were analyzed relative to the negative control. Cells were unable to proliferate in the positive control.* ***(B, C)*** *Immunofluorescence staining for the Ki-67 nuclear antigen in porcine fibroblasts after 24 h and 48 h of incubation, respectively. DAPI was used as a counterstain for the same field* ***(a, d, g, j)****. Ki67-staining* ***(red) (b, e, h, k)****. Merge images of DAPI and Ki-67* ***(c, f, i, l)****. Scale bar = 100 µm.* ***(D)****. Culturing of fibroblasts using conditioned media prepared from peracetic acid (PAA) or slightly acidic electrolyzed water (SAEW) showed a significantly higher percentage of proliferating cells, whereas no significant difference was observed between conditioned media prepared from ethanol-treated scaffolds and the control group. Bars with the same letter are not significantly different, p<0.05. Error bars: ± standard deviation; n = 6 for each group.*

of 71.50 ± 1.95% and 76.65 ± 2.66% of the seeded fibroblasts, respectively.

An evaluation of the repopulated DAPI-labeled fibroblasts using phase-contrast fluorescence inverted microscopy confirmed the ability of SAEW-treated scaffolds to support cell adherence, and the number of cells was higher than scaffolds treated with PAA or ethanol (Figs. 3B and C).

Scaffolds treated with SAEW, ethanol, or PAA have no cytotoxic or growth inhibitory effects on cells

The cytotoxic effect was distinct in the positive control, and cells were unable to proliferate, whereas cells grew and proliferated in the negative control (Fig. 4A). In contrast to the expected results, the extract prepared from scaffolds not only showed no growth inhibiting effect after 24 h or 48 h, but cells also grew and proliferated at a significantly higher rate. Scaffolds treated with SAEW showed the highest proliferation rate of 131.29 ± 5.49% and 141.46 ± 4.78% compared to that of the negative control after 24 h and 48 h incubations, respectively. Scaffolds treated with PAA demonstrated a high proliferation rate of 119.18 ± 5.6 and 127.43 ± 6.88% compared to that of scaffolds treated with ethanol, which showed proliferation rates of 109.22 ± 5.58% and 113.95 ± 6.67% compared to that of the control after 24 h and 48 h of incubation.

SAEW-treated scaffolds release soluble factors that stimulate cell proliferation

The percentage of Ki-67 expressing cells was significantly higher in fibroblasts cultured in conditioned media prepared from SAEW-treated scaffolds for 24 h (37.6 ± 1.45%) or 48 h (70.2 ± 1.33%) (Figs. 4B-D). No significant difference was observed between conditioned media prepared from the ethanol- and PAA-treated scaffolds. Additionally, the ethanol-treated scaffolds were not different from the control group.

DISCUSSION

Tissue-engineered liver bio-scaffolds must be sterilized to meet clinical application requirements (6). However, no direct comparison of different sterilization protocols for natural decellularized scaffolds has been reported. Therefore, our goal was to determine the ideal sterilization technique that has minimal damage to the structural components of scaffolds and can minimize the DNA content of scaffolds.

Presence of xenogeneic cells within a biologic scaffold is already known to play a critical role in induction of recipients' immune system. Therefore, it is necessary to remove the intracellular components and antigenic epitopes for generation of animal-originated scaffolds for further human use. Previous studies concerning porcine heart valves and liver matrices have shown that successful decellularization should be accompanied by removal of antigenic and pathogenic epitopes (24, 25). Similar to these studies, our decellularization protocol was efficient in removing of α gal and SLA epitopes that were involved in hyperacute and acute rejection. Matrices were also depleted from PERV that has been identified as a possible infection risk after xenotransplantation (24). Interestingly, porcine vWF was also eliminated in our study. According to previous studies, vWF was considered responsible for xenograft dysfunction and rejection because of its capability to bind xenoreactive antibodies and aggregate primate platelets (26). Subsequently, generation of tissues or organs that lack this gene will aid in reduction of the coagulation problems associated with xenotransplantation.

These small-sized scaffolds need to be sterilized for further diverse applications such as stem cell differentiation and migration and *in vitro* drug screening studies. Ethanol has a strong bactericidal effect against Gram-positive, Gram-negative, and acid-fast bacteria as well as lipophilic viruses. Ethanol has been classified as a disinfectant instead of a sterilizing agent because of its inability to destroy hydrophilic viruses or bacterial spores at concentrations of 60% to 80% (27). This may be why growth occurred on blood agar and the DMEM discolored in scaffolds treated with 70% ethanol.

The results of our study demonstrate that PAA was highly efficient at removing the cellular material from the thin ECM and was able to sterilize it. The ECM retained 56% of the GAG content in decellularized liver following PAA treatment. These results are consistent with previous studies which reported the oxidative destructive effects of PAA on glycosaminoglycans (7). The proliferation assay revealed that PAA treatment preserved the function of many soluble factors that are resident in the ECM. Moreover, PAA did not appear to have any adverse effect on collagen and the subsequent mechanical behavior of the scaffolds.

Electrolyzed water has strong antimicrobial and antifungal activity (28). Researchers have also demonstrated that it has antiviral activity on blood borne pathogenic viruses including hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. It is unclear whether pH, chlorine compounds, ORP, or a combination of these factors are responsible for the disinfecting effect of SAEW (29). Park et al suggested that the low pH of electrolyzed water leads to reduced bacterial growth and makes the bacterial cells more sensitive to active chlorine by increasing the influx of hypochlorus acid (HOCl) (30). Other researchers have suggested that this activity is due to its high ORP (31, 32). We developed a new sterilization method using SAEW after optimizing the duration required for sterilization. We checked the efficiency of sterilization after 30 min, 60 min, 90 min, and 120 min and changed the SAEW every 15 min, as many researchers have reported that electrolyzed water can lose its activity with time (30, 33). Treatment of the scaffolds for 90 min revealed high efficiency sterilization; however, we used 120 min treatment for further experiments to ensure sterilization efficiency. Scaffolds sterilized with SAEW retained 69.6% of their GAG content in decellularized liver and the SAEW appeared not to have a significant effect on collagen of decellularized liver. Scaffolds treated with SAEW were able to support the attachment of 76.64% of the seeded fibroblasts and retained the important soluble factors needed for host cell proliferation. To our knowledge, this is the first report to suggest the sterilization of scaffolds by SAEW.

The ECM contains different mixtures of GAGs that bind to growth factors and cytokines protecting them from denaturation (34). These growth factors and cytokines such as basic fibroblast growth factor aid in cell attachment, migration, proliferation, and differentiation. GAGs promote water retention due to their high concentration of negative charges and hydrophilicity, and contribute to the gel properties of the ECM (35, 36). Hence, retention of GAGs in decellularized materials was considered important for maintaining biological activity. We suggest that the higher amount of GAGs in SAEW-treated scaffolds resulted in the higher attachment percentage. Although the amount

of GAGs retained in the ethanol-treated scaffolds was significantly higher than that of the PAA-treated scaffolds, the percentage of cell attachment was higher in the PAA-treated scaffolds. A reasonable explanation is the loss of collagen as observed in our study or changes that occurred in the surface topology as suggested by other studies after treatment with ethanol (6, 21).

Concern about the cytocompatability of sterilized liver scaffolds is critical to exclude the presence of any toxic reagents within scaffolds which can affect the interactions of the scaffold with the transplanted cells (12). An unexpected finding is that extracts prepared from scaffolds demonstrated no cytotoxic effect on fibroblasts and facilitated proliferation. This may have resulted from the release of soluble factors from scaffolds into the conditioned medium. These factors were not studied in our work. To confirm the ability of the extract to stimulate proliferation, parallel experiments were performed using Ki-67 and suggested that the extracts prepared from PAA and SAEW-treated scaffolds induced fibroblast proliferation. The proliferation rate of fibroblasts on scaffolds treated with ethanol was significantly lower than that in the other groups. The direct effect of ethanol on growth-stimulated cell proliferation has been studied and it has been demonstrated that ethanol targets growth factors that regulate cell proliferation and survival (37, 38). We established a new test based on the iodoform reaction to exclude the presence of residual ethanol within the scaffolds in the cell proliferation assay (unpublished data). In this test, ethanol reacts with iodine solution in the presence of sodium hydroxide solution to form a yellow solid, tri-iodomethane, called iodoform (39). This experiment showed the absence of ethanol residues on conditioned media prepared from ethanol-treated scaffolds. Subsequently, the only other possibility was the ability of ethanol to react with these factors and change or modulate their activity. This explanation is consistent with previous studies that suggested retention of ECM components does not necessarily mean that they are retained in a form that can interact with cells and induce cell activities (7). Based on a literature review about the similarities between fibroblasts and hepatocytes concerning attachment and spreading, we expect similar behavior for hepatocytes on sterilized scaffolds (40, 41).

We quantified the collagen content within scaffolds due to its role of preserving ECM strength and cell adhesion. Our results demonstrated that the collagen content in decellularized liver was higher than the content in native liver, suggesting the removal of cellular proteins from decellularized tissue (42). Ethanol appeared to have adverse effects on collagen, which we suspect affected cell attachment.

Xenogenic DNA within scaffolds materials has been suggested to be responsible for the adverse immune and tissue remodeling responses in recipients. Previous studies suggested that the amount of retained DNA in scaffolds should be less than 50 ng/mg dry weight ECM to avoid adverse host responses (14, 18). In the present study, DNA quantification revealed the ability of PAA and SAEW to remove most of the DNA and cell remnants. This loss of DNA and cell remnants suggests a decrease in immunogenicity of the scaffold materials and subsequently facilitates scaffold remodeling after *in vivo* implantation.

The results presented in this paper support that decellularized matrices provide seeded cells with a network of biological fibers facilitating cell attachment in addition to bioactive molecules that promote cell attachment, growth, and proliferation. These characteristics, which most synthetic scaffolds lack, makes these biological scaffolds preferable for tissue engineering and drug screening purposes compared to synthetic scaffolds. Future studies would be directed for clinical application of sterilized whole porcine liver scaffolds for development of a transplantable bioengineered liver for further transplantation in humans.

We conclude that ethanol, which is commonly used to sterilize naturally derived scaffolds, causes structural damage to scaffolds in addition to reducing cell attachment and proliferation. More importantly, our findings indicate the inability of ethanol to completely sterilize the scaffolds. Furthermore, PAA can be used to sterilize scaffolds for transplantation purposes and facilitate cell attachment and proliferation. SAEW showed excellent cytocompatability and the highest capacity to retain the important components of ECM. Therefore, we suggest that SAEW should be used to sterilize scaffolds for tissue engineering purposes.

ABBREVIATIONS

SAEW	=	slightly acidic electrolyzed water
ECM	=	extracellular matrix
PAA	=	peracetic acid
PBS	=	phosphate buffered saline
GAGs	=	glycosaminoglycans
ORP	=	oxidation reduction potential

H&E = hematoxylin and eosin
DAPI = 4',6-diamidino-2-phenylindole
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DMSO = dimethyl sulfoxide

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REFERENCES

1. de Villa V, Lo CM. Liver transplantation for hepatocellular carcinoma in Asia. Oncologist. 2007;12(11):1321-1331.
2. Uygun BE, Soto-Gutierrez A, Yagi H, et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med. 2010;16(7):814-820.
3. Punch JD, Hayes DH, LaPorte FB, McBride V, Seely MS. Organ donation and utilization in the United States, 1996-2005. Am J Transplant. 2007;7(5 Pt 2):1327-1338.
4. Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol. 2005;23(1):47-55.
5. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. FASEB J. 2006;20(1):9-22.
6. Reing JE, Brown BN, Daly KA, et al. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. Biomaterials. 2010;31(33):8626-8633.
7. Hodde J, Janis A, Hiles M. Effects of sterilization on an extracellular matrix scaffold: part II. Bioactivity and matrix interaction. J Mater Sci Mater Med. 2007;18(4):545-550.
8. Brown B, Lindberg K, Reing J, Stolz DB, Badylak SF. The basement membrane component of biologic scaffolds derived from extracellular matrix. Tissue Eng. 2006;12(3):519-526.
9. Siritientong T, Srichana T, Aramwit P. The effect of sterilization methods on the physical properties of silk sericin scaffolds. AAPS PharmSciTech. 2011;12(2):771-781.
10. Rosario DJ, Reilly GC, Ali Salah E, Glover M, Bullock AJ, Macneil S. Decellularization and sterilization of porcine urinary bladder matrix for tissue engineering in the lower urinary tract. Regen Med. 2008;3(2):145-156.
11. Hoganson DM, Owens GE, O'Doherty EM, et al. Preserved extracellular matrix components and retained biological activity in decellularized porcine mesothelium. Biomaterials. 2010;31(27):6934-6940.
12. Ning LJ, Zhang Y, Chen XH, et al. Preparation and characterization of decellularized tendon slices for tendon tissue engineering. J Biomed Mater Res A. 2012;100A(6):1448-1456.
13. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011;32(12):3233-3243.
14. Keane TJ, Londono R, Turner NJ, Badylak SF. Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials. 2012;33(6):1771-1781.
15. Meng FY, Chen ZS, Han M, et al. Porcine hepatocyte isolation and reversible immortalization mediated by retroviral transfer and site-specific recombination. World J Gastroenterol. 2010;16(13):1660-1664.
16. Eitan Y, Sarig U, Dahan N, Machluf M. Acellular cardiac extracellular matrix as a scaffold for tissue engineering: *in vitro* cell support, remodeling, and biocompatibility. Tissue Eng Part C Methods. 2010;16(4):671-683.
17. Mirsadraee S, Wilcox HE, Watterson KG, et al. Biocompatibility of acellular human pericardium. J Surg Res. 2007;143(2):407-414.
18. Cao W, Zhu ZW, Shi ZX, Wang CY, Li BM. Efficiency of slightly acidic electrolyzed water for inactivation of Salmonella enteritidis and its contaminated shell eggs. Int J Food Microbiol. 2009;130(2):88-93.
19. Tanaka N, Fujisawa T, Daimon T, Fujiwara K, Yamamoto M, Abe T. The effect of electrolyzed strong acid aqueous

solution on hemodialysis equipment. Artif Organs. 1999; 23(12):1055-1062.
20. Brown BN, Barnes CA, Kasick RT, et al. Surface characterization of extracellular matrix scaffolds. Biomaterials. 2010;31(3):428-437.
21. Shearer H, Ellis MJ, Perera SP, Chaudhuri JB. Effects of common sterilization methods on the structure and properties of poly (D, L lactic-co-glycolic acid) scaffolds. Tissue Eng. 2006;12(10):2717-2727.
22. Salgado AJ, Coutinho OP, Reis RL. Novel starch-based scaffolds for bone tissue engineering: cytotoxicity, cell culture, and protein expression. Tissue Eng. 2004;10(3-4):465-474.
23. Pariente JL, Kim BS, Atala A. *In vitro* biocompatibility assessment of naturally derived and synthetic biomaterials using normal human urothelial cells. J Biomed Mater Res. 2001;55(1):33-39.
24. Ota T, Taketani S, Iwai S, et al. Novel method of decellularization of porcine valves using polyethylene glycol and gamma irradiation. Ann Thorac Surg. 2007;83(4):1501-1507.
25. Park KM, Park SM, Yang SR, Hong SH, Woo HM. Preparation of immunogen-reduced and biocompatible extracellular matrices from porcine liver. J Biosci Bioeng. 2013; 115(2):207-215.
26. Lin CC, Cooper DKC, Dorling A. Coagulation dysregulation as a barrier to xenotransplantation in the primate. Transpl Immunol. 2009;21(2):75-80.
27. Holy CE, Cheng C, Davies JE, Shoichet MS. Optimizing the sterilization of PLGA scaffolds for use in tissue engineering. Biomaterials. 2001;22(1):25-31.
28. Al-Haq MI, Sugiyama J, Isobe S. Applications of Electrolyzed Water in Agriculture & Food Industries. Food Sci Technol Res. 2005;11(2):135-150.
29. Huang Y-R, Hung Y-C, Hsu S-Y, Huang Y-W, Hwang D-F. Application of electrolyzed water in the food industry. Food Contr. 2008;19(4):329-345.
30. Park H, Hung Y-C, Chung D. Effects of chlorine and pH on efficacy of electrolyzed water for inactivating Escherichia coli O157:H7 and Listeria monocytogenes. Int J Food Microbiol. 2004;91(1):13-18.
31. Kim C, Hung YC, Brackett RE. Roles of oxidation-reduction potential in electrolyzed oxidizing and chemically modified water for the inactivation of food-related pathogens. J Food Prot. 2000;63(1):19-24.
32. Liao LB, Chen WM, Xiao XM. The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. J Food Eng. 2007;78(4):1326-1332.
33. Len SV, Hung YC, Chung D, Anderson JL, Erickson MC, Morita K. Effects of storage conditions and pH on chlorine loss in electrolyzed oxidizing (EO) water. J Agric Food Chem. 2002;50(1):209-212.
34. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell. 1991;64(4):841-848.
35. Pike DB, Cai S, Pomraning KR, et al. Heparin-regulated release of growth factors *in vitro* and angiogenic response *in vivo* to implanted hyaluronan hydrogels containing VEGF and bFGF. Biomaterials. 2006;27(30):5242-5251.
36. Elia R, Fuegy PW, VanDelden A, Firpo MA, Prestwich GD, Peattie RA. Stimulation of *in vivo* angiogenesis by in situ crosslinked, dual growth factor-loaded, glycosaminoglycan hydrogels. Biomaterials. 2010;31(17):4630-4638.
37. Hicks SD, Miller MW. Effects of ethanol on transforming growth factor B1-dependent and -independent mechanisms of neural stem cell apoptosis. Exp Neurol. 2011;229(2): 372-380.
38. Miller MW, Luo J. Effects of ethanol and basic fibroblast growth factor on the transforming growth factor beta1 regulated proliferation of cortical astrocytes and C6 astrocytoma cells. Alcohol Clin Exp Res. 2002;26(5):671-676.
39. Dean JA, Lange NA. Handbook of chemistry. New York, NY: McGraw-Hill; 1999.
40. Saltzman WM, Parsons-Wingerter P, Leong KW, Lin S. Fibroblast and hepatocyte behavior on synthetic polymer surfaces. J Biomed Mater Res. 1991;25(6):741-759.
41. Rovensky YA, Domnina LV, Ivanova OY, Vasiliev JM. Locomotory behaviour of epitheliocytes and fibroblasts on metallic grids. J Cell Sci. 1999;112(Pt 8):1273-1282.
42. Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. Hepatology. 2011;53(2):604-617.