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(54) **Title**: STERILIZED, ACELLULAR EXTRACELLULAR MATRIX COMPOSITIONS AND METHODS OF MAKING THEREOF

(57) **Abstract**: Methods for sterilizing and decellularizing extracellular matrix materials are disclosed. Extracellular matrix compositions produced using the disclosed methods are also disclosed.

STERILIZED, ACELLULAR EXTRACELLULAR MATRIX COMPOSITIONS AND METHODS OF MAKING THEREOF

Cross-Reference to Related Applications

[0001] This application claims the benefit of the filing dates of U.S. Provisional Patent Application Number 61/490,693, filed on May 27, 2011, U.S. Provisional Patent Application No. 61/490,873, filed on May 27, 2011, U.S. Provisional Patent Application No. 61/491,723, filed on May 31, 2011, and U.S. Provisional Patent Application No. 61/650,911, filed on May 23, 2012, each of which is hereby incorporated by reference herein in its entirety.

Field of the Invention

[0002] The invention generally relates to sterilized, acellular extracellular matrix compositions and methods of making such compositions. More particularly, the invention relates to methods of contemporaneously sterilizing and decellularizing extracellular matrix compositions, as well as the sterilized, acellular compositions resulting from such methods.

Background of the Invention

[0003] Conventional techniques for sterilizing tissue compositions often alter the properties of the tissue compositions and/or damage important components of the tissue compositions, such as growth factors. Consequently, these conventional sterilization techniques often render tissue compositions unfit for their intended purposes. For example, ethylene oxide is a toxic, mutagenic, and carcinogenic substance that can weaken tissue compositions, reduce the growth factor content of tissue compositions, and denature proteins within tissue compositions. Similarly, conventional steam sterilization techniques are incompatible with the biopolymers of tissue compositions, and gamma radiation causes significant decreases in the integrity of tissue compositions. Although there are known techniques for sterilizing tissue compositions without altering the properties of the tissue compositions, many of these techniques, such as anti-bacterial washes, often fail to completely sterilize the tissue compositions and/or leave residual toxic contaminants in the tissue compositions.

[0004] Additionally, when tissue compositions are designed for implantation within the body of a subject, the tissue compositions must often be exposed to a separate, time-consuming decellularization process. This decellularization process is intended to remove cells from the tissue compositions, thereby decreasing the likelihood that the subject's immune system will reject the implanted tissue compositions and/or generate a significant inflammatory response.

However, conventional decellularization techniques merely decellularize portions of the tissue compositions such that native cells remain in the tissue compositions following the decelluarization process.

[0005] U.S. Patent No. 7,108,832 (the '832 patent), which is assigned to NovaSterilis, Inc., discloses a method that sterilizes various materials through the use of supercritical carbon dioxide. However, as with other known sterilization methods, tissue compositions that are sterilized using the process disclosed in the '832 patent must be exposed to a separate decellularization process, as described above.

[0006] Accordingly, there is a need in the art for a single method of sterilizing and decellularizing a tissue composition, such as an extracellular matrix composition. More particularly, there is a need in the art for a single method of (a) sterilizing a tissue composition while maintaining the native properties of the tissue composition and (b) decellularizing the tissue composition such that the tissue composition is acellular. There is still a further need for a method of incorporating additives into a tissue composition during sterilization and/or decellularization of the tissue composition.

SUMMARY

[0007] Methods for sterilizing and decellularizing an extracellular matrix (ECM) material are disclosed. In one aspect, the methods include harvesting of a selected ECM tissue, freezing the selected ECM tissue, thawing the selected ECM tissue, and isolating an ECM material. The isolated ECM material is subjected to incubation and rinsing before it is processed in supercritical carbon dioxide and subsequently exposed to rapid depressurization. During or after the rapid depressurization of the ECM material, one or more additives can be incorporated into the ECM material to impart desired characteristics to the resulting ECM composition. Sterilized, acellular ECM compositions produced using the disclosed methods are also disclosed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] These and other features of the preferred embodiments of the invention will become more apparent in the detailed description in which reference is made to the appended drawings wherein:

[0009] Figures 1-2 depict the results of an experiment in which DNA content was measured for small intestinal submucosa (SIS) compositions following various sterilization

methods, including the sterilization methods described herein. Figure 1 shows the DNA content of each SIS composition following sterilization. Figure 2 shows the percentage of DNA that was removed from each SIS composition following sterilization, as compared to raw, unprocessed SIS.

[0010] Figures 3-4 depict the results of an experiment in which native growth factor content was measured for SIS compositions following various sterilization methods, including the sterilization methods described herein. Figure 3 shows the bFGF content of each SIS composition (normalized by dry weight of samples) following sterilization. Figure 4 shows the active TGF-β content of each SIS composition (normalized by dry weight of samples) following sterilization.

[0011] Figure 5 depicts the results of an experiment in which bFGF was incorporated into SIS compositions during rapid depressurization, as described herein. Figure 5 shows the bFGF content for each SIS composition (normalized by dry weight of samples) following rapid depressurization.

[0012] Figure 6 depicts the results of an experiment in which the tensile strength of two-ply SIS compositions was measured following various sterilization methods, including the sterilization methods described herein. Figure 6 shows the tensile strength measured for each SIS composition following sterilization.

[0013] Figure 7 depicts the results of an experiment in which native growth factor content was measured for SIS compositions following various sterilization and/or decellularization methods, including the sterilization and decellularization methods described herein. Figure 7 shows the bFGF enzyme-linked immunosorbent assay (ELISA) results for each SIS composition (normalized by dry weight of samples) following sterilization and/or decellularization.

[0014] Figure 8 shows the DNA content in SIS after it is processed in various ways. The baseline measurement is raw. The tissue was then exposed to supercritical CO_2 followed by rapid depressurization (RDP) to facilitate enhanced removal of DNA and cellular debris. After the RDP, the tissue was placed in supercritical CO_2 with peracetic acid (PAA) for sterilization. The comparison is to processed SIS either unsterilized or sterilized with ethylene oxide (ETO).

[0015] Figure 9 shows the Percent removal of DNA from SIS after it is processed in various ways. The baseline measurement is raw. The tissue was then exposed to supercritical CO_2 followed by rapid depressurization (RDP) to facilitate enhanced removal of DNA and

cellular debris. After the RDP, the tissue was placed in supercritical CO_2 with peracetic acid (PAA) for sterilization. The comparison is to processed SIS either unsterilized or sterilized with ethylene oxide (ETO).

[0016] Figure 10 shows the variable active Transforming Growth Factor (TGF-beta) content in SIS after it is processed in various ways. The baseline measurement is raw, or unprocessed SIS followed by processing with only Triton X-100 (TX-100) detergent. The tissue was then exposed to supercritical CO_2 followed by rapid depressurization (RDP) to facilitate enhanced removal of DNA and cellular debris. After the RDP, the tissue was placed in supercritical CO_2 with peracetic acid (PAA) for sterilization. The comparison is to processed SIS either unsterilized or sterilized with ethylene oxide (ETO).

[0017] Figure 11 shows the variable basic Fibroblast Growth Factor (bFGF) content in SIS after it is processed in various ways. The baseline measurement is raw, or unprocessed SIS followed by processing with only Triton X-100 (TX-100) detergent. The tissue was then exposed to supercritical CO_2 followed by rapid depressurization (RDP) to facilitate enhanced removal of DNA and cellular debris. After the RDP, the tissue was placed in supercritical CO_2 with peracetic acid (PAA) for sterilization. The comparison is to processed SIS either unsterilized or sterilized with ethylene oxide (ETO).

[0018] Figure 12 shows the addition of basic Fibroblast Growth Factor (bFGF) content to SIS using rapid depressurization. The baseline measurement is raw, or unprocessed SIS. The comparison is to processed SIS either unsterilized or sterilized with ethylene oxide (ETO).

DETAILED DESCRIPTION

[0019] The present invention may be understood more readily by reference to the following detailed description, examples, and claims, and their previous and following description. However, before the present devices, systems, and/or methods are disclosed and described, it is to be understood that this invention is not limited to the specific devices, systems, and/or methods disclosed unless otherwise specified, as such can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to be limiting.

[0020] As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for

example, reference to a "leaflet" can include two or more such leaflets unless the context indicates otherwise.

[0021] Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

[0022] As used herein, the terms "optional" and "optionally" mean that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

[0023] The word "or" as used herein means any one member of a particular list and also includes any combination of members of that list.

[0024] Unless otherwise expressly stated, it is in no way intended that any method or aspect set forth herein be construed as requiring that its steps be performed in a specific order. Accordingly, where a method claim does not specifically state in the claims or descriptions that the steps are to be limited to a specific order, it is in no way intended that an order be inferred, in any respect. This holds for any possible non-express basis for interpretation, including matters of logic with respect to arrangement of steps or operational flow, plain meaning derived from grammatical organization or punctuation, or the number or type of aspects described in the specification.

[0025] Without the use of such exclusive terminology, the term "comprising" in the claims shall allow for the inclusion of any additional element--irrespective of whether a given number of elements is enumerated in the claim, or the addition of a feature could be regarded as transforming the nature of an element set forth in the claims. Except as specifically defined herein, all technical and scientific terms used herein are to be given as broad a commonly understood meaning as possible while maintaining claim validity.

[0026] As used herein, a "subject" is an individual and includes, but is not limited to, a mammal (e.g., a human, horse, pig, rabbit, dog, sheep, goat, non-human primate, cow, cat, guinea pig, or rodent), a fish, a bird, a reptile or an amphibian. The term does not denote a

particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be included. A "patient" is a subject afflicted with a disease or disorder. The term "patient" includes human and veterinary subjects. As used herein, the term "subject" can be used interchangeably with the term "patient."

[0027] As used herein, the term "acellular" is meant to describe extracellular matrix compositions that are at least 80 % decellularized such that the extracellular matrix composition is 80 % without cells and/or cellular remnants. In some exemplary aspects described herein, the term "acellular" can refer to extracellular matrix compositions that are at least 90 % decellularized such that the extracellular matrix composition is at least 90 % without cells and/or cellular remnants. In other exemplary aspects described herein, the term "acellular" can refer to extracellular matrix compositions that are at least 95 % decellularized such that the extracellular matrix composition is at least 95 % without cells and/or cellular remnants. In other exemplary aspects described herein, the term "acellular" can refer to extracellular matrix compositions that are at least 96 % decellularized such that the extracellular matrix composition is at least 96 % without cells and/or cellular remnants. In still other exemplary aspects described herein, the term "acellular" can refer to extracellular matrix compositions that are at least 97 % decellularized such that the extracellular matrix composition is at least 97 % without cells and/or cellular remnants. In further exemplary aspects described herein, the term "acellular" can refer to extracellular matrix compositions that are at least 98 % decellularized such that the extracellular matrix composition is at least 98 % without cells and/or cellular remnants. In still further exemplary aspects described herein, the term "acellular" can refer to extracellular matrix compositions that are at least 99 % decellularized such that the extracellular matrix composition is at least 99 % without cells and/or cellular remnants. Thus, as used herein, the term "acellular" can refer to extracellular matrix compositions that are decellularized at levels of 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, 100 %, and any percentages falling between these values.

[0028] As used herein, the term "additive" refers to materials that can be selectively incorporated into the disclosed ECM materials to impart predetermined properties to the sterilized, acellular ECM compositions disclosed herein. Such additives can include, for example and without limitation, growth factors, cytokines, proteoglycans, glycosaminoglycans (GAGs), proteins, peptides, nucleic acids, small molecules, cells and pharmaceutical agents, such

as statin drugs, corticosterioids, anti-arrhythmic drugs, nonsteroidal anti-inflammatory drugs, other anti-inflammatory compounds, nanoparticles, and metallic compounds.

[0029] As used herein, the term "contemporaneously" refers to the simultaneous and/or overlapping occurrence of events, as well as the sequential occurrence of events within about thirty minutes before or after one another. Thus, if a first event occurs, then a second event can be said to have occurred contemporaneously with the first event if it occurred concurrently with the first event or within thirty minutes before or after the first event. For example, if a first method step is performed, then a second method step performed five minutes after the first method step can be said to be performed "contemporaneously" with the first method step. Similarly, if the second method step was performed ten minutes before performance of a third method step, then the second method step can be said to be performed "contemporaneously" with the third method step.

[0030] As used herein, the term "emulsion" refers to a mixture in which a first ECM material is dispersed within a second ECM material, with the first ECM material being immiscible with the second ECM material. The "emulsions" described herein can refer to either oil-in-water type emulsions or water-in-oil type emulsions.

[0031] As used herein, the term "suspension" refers to mixture in which a solid ECM material, such as, for example and without limitation, particulate ECM, is dispersed (suspended) in a fluid ECM material, such as, for example and without limitation, ECM gel or ECM liquid.

[0032] As used herein, the term "supercritical" refers to a fluid state of a material when it is held at or above its critical temperature and critical pressure. When a material is held at or above its critical temperature and critical pressure, then it typically adopts functional properties of both a gas and a liquid and is said to function as a supercritical fluid. Thus, for example, when carbon dioxide is held at or above its critical temperature (31.1°C) and its critical pressure (1,071 psi), it behaves as a supercritical carbon dioxide fluid and can, for example, exhibit the expansion properties of a gas while having the density of a liquid.

[0033] Described herein are sterilized, acellular extracellular matrix (ECM) compositions and methods for making such compositions. As described herein, the disclosed extracellular matrix compositions are formed by contemporaneously sterilizing and decellularizing an isolated ECM material. More particularly, the disclosed methods contemporaneously accomplish desired sterilization and decellularization of the isolated ECM material such that the native properties of the tissue composition are maintained and the ECM material is rendered sterile and acellular.

[0034] As further described herein, the disclosed methods make use of rapid depressurization of the ECM material to decellularize the ECM material such that it is acellular. This rapid depressurization of the ECM material occurs at depressurization rates that are significantly higher than the depressurization rates applied in previously known methods. In addition to decellularizing the ECM material as described herein, the rapid depressurization of the ECM material also can be used to incorporate desired sterilants and additives into the ECM material.

ECM Compositions

[0035] In exemplary aspects, a sterilized, acellular ECM composition can comprise any known ECM component or material, including, for example and without limitation, mucosal layers and components, submucosal layers and components, muscularis layers and components, and/or basement membrane layers and components. It is contemplated that a disclosed sterilized, acellular ECM composition can comprise an ECM material obtained from any mammalian tissue source, including, for example and without limitation, stomach tissue (*e.g.*, stomach submucosa (SS)), small intestinal tissue (*e.g.*, small intestinal submucosa (SIS)), large intestinal tissue, bladder tissue (*e.g.*, urinary bladder submucosa (UBS)), liver tissue (*e.g.*, liver basement membrane (LBM)), heart tissue (*e.g.*, pericardium), lung tissue, kidney tissue, pancreatic tissue, prostate tissue, mesothelial tissue, fetal tissue, a placenta, a ureter, veins, arteries, tissue surrounding the roots of developing teeth, and tissue surrounding growing bone. It is further contemplated that a disclosed sterilized, acellular ECM composition can comprise an ECM material obtained from ECM components or materials of one or more mammals including, for example and without limitation, humans, cows, pigs, dogs, sheep, cats, horses, rodents, and the like. Thus, it is contemplated that a disclosed sterilized, acellular ECM composition can comprise ECM components or materials from two or more of the same mammalian species, such as, for example and without limitation, two or more cows, two or more pigs, two or more dogs, or two or more sheep. It is further contemplated that a disclosed sterilized, acellular ECM composition can comprise ECM components or materials from two or more different mammalian species, such as, for example and without limitation, a pig and a cow, a pig and a dog, a pig and a sheep, or a cow and a sheep. It is still further contemplated that a disclosed sterilized, acellular ECM composition can comprise ECM components or materials obtained from a first tissue source, such as, for example and without limitation, SIS, from a first mammal, as well as ECM

components or materials obtained from a second tissue source, such as, for example and without limitation, SS, from a second mammal.

[0036] In various aspects, a disclosed sterilized, acellular ECM composition can be produced in any suitable shape, including, for example and without limitation, a substantially flat sheet, a cylindrical tube, a substantially spherical structure, or a multi-laminate structure. It is contemplated that a disclosed sterilized, acellular ECM composition can also be produced in any suitable form, including, for example and without limitation, a solid, liquid, gel, particulate, emulsion, or suspension form. In one exemplary aspect, it is contemplated that a disclosed sterilized, acellular ECM composition can comprise an outer layer of solid ECM material that encloses an inner layer of liquid, particulate, emulsion, suspension, and/or gel ECM material.

[0037] In another exemplary aspect, it is contemplated that a disclosed sterilized, acellular ECM composition can comprise one or more types of particulate ECM materials that are suspended within an ECM gel to form an ECM suspension. In this aspect, it is contemplated that the particulates within a disclosed ECM suspension can have a diameter ranging from about 5 μm to about 300 μm, with an average diameter ranging from about 90 μm to about 100 μm. It is further contemplated that the percentage of gel within a disclosed ECM suspension can range from about 5% to about 50%, while the percentage of particulate within a disclosed ECM suspension can range from about 50% to about 95%. Thus, it is contemplated that the percentage of gel within a disclosed ECM suspension can be about 10%, while the percentage of particulate within the ECM suspension can be about 90%. It is further contemplated that the percentage of gel within a disclosed ECM suspension can be about 15%, while the percentage of particulate within the ECM suspension can be about 85%. More preferably, the percentage of gel within a disclosed ECM suspension can range from about 20% to about 30%, while the percentage of particulate within a disclosed ECM suspension can range from about 70% to about 80%. Thus, in an exemplary aspect, the percentage of gel within a disclosed ECM suspension can be about 20%, while the percentage of particulate within the ECM suspension can be about 80%. In another exemplary aspect, the percentage of gel within a disclosed ECM suspension can be about 25%, while the percentage of particulate within the ECM suspension can be about 75%. In an additional exemplary aspect, the percentage of gel within a disclosed ECM suspension can be about 30%, while the percentage of particulate within the ECM suspension can be about 70%. Although the above ranges refer to particular beginning point values and end point values, it is

contemplated that a disclosed ECM suspension can be formed from gel percentages and particulate percentages falling within any of the ranges disclosed above.

[0038] In a further aspect, it is contemplated that a disclosed ECM suspension can comprise sterilized, decellularized ECM. In exemplary aspects, the ECM gel of a disclosed ECM suspension can be a hydrolyzed ECM. In these aspects, it is contemplated that the ECM gel of a disclosed ECM suspension can comprise ECM that is greater than about 50% hydrolyzed, more preferably, greater than about 70% hydrolyzed, and, most preferably, greater than about 90% hydrolyzed. In one exemplary aspect, the ECM gel of a disclosed ECM suspension can comprise ECM that is about 100% hydrolyzed. It is still further contemplated that the ECM components of the suspension can comprise at least one of: glycoproteins, such as, for example and without limitation, fibronectin and laminan; glycosaminoglycans, such as, for example and without limitation, heparan, hyaluronic acid, and chondroitin sulfate; and growth factors, thereby providing additional bioavailability for native cellular components. It is contemplated that the ECM components of the suspension can provide a structural and biochemical microenvironment that promotes cell growth and stem cell attraction following implantation of a disclosed ECM suspension within a subject. It is further contemplated that the ECM gel of a disclosed ECM suspension can function as a bulking agent that preserves a desired biomechanical environment until the cells of the subject can begin producing their own ECM.

[0039] It is still further contemplated that the desired biomechanical environment that is preserved by the ECM gel can substantially correspond to a biomechanical environment in native tissue. Thus, it is contemplated that the ECM gel of a disclosed ECM suspension can have an elastic modulus that is substantially equal to the elastic modulus of a target site within a subject. In exemplary aspects, the elastic modulus of the ECM gel of a disclosed ECM suspension can range from about 5 kPa to about 50 kPa, and, more preferably, from about 10 kPa to about 15 kPa.

[0040] In one non-limiting exemplary aspect, it is contemplated that, when a disclosed ECM suspension is configured for injection at a target site on or within the heart of a subject, the elastic modulus of the ECM gel of the disclosed ECM suspension can be about 11.5 kPa, which is the elastic modulus of cardiac muscle. As used herein, the term "on or within the heart" refers to locations that are, for example and without limitation, on or within the pericardium,

epicardium, myocardium, endocardium, ventricles, atria, aorta, pulmonary arteries, pulmonary veins, vena cavae, and the like. In another aspect, it is further contemplated that a disclosed ECM suspension can be injected at a target site on or within the heart of the subject to therapeutically prevent or reverse left ventricular wall negative remodeling that occurs following acute myocardial infarction and/or chronic coronary heart disease. As used herein, the term "negative remodeling" refers to the detrimental and/or undesired changes in the heart that occur in response to myocardial injury; such undesired changes include, for example and without limitation, alterations in myocyte biology, myocyte loss, extracellular matrix degradation, extracellular matrix replacement fibrosis, alterations in left ventricular chamber geometry, increased wall stress (afterload), afterload mismatch, episodic subendocardial hypoperfusion, increased oxygen utilization, sustained hemodynamic overloading, and worsening activation of compensatory mechanisms. It is still further contemplated that a disclosed ECM suspension can be injected at a target site on or within the heart of the subject to therapeutically treat heart failure.

[0041] In an exemplary aspect, it is contemplated that a disclosed ECM suspension can be injected at a target site on or within the heart of a subject, such as, for example and without limitation, on or within the pericardium, epicardium, myocardium, endocardium, ventricles, atria, aorta, and the like. Optionally, in one aspect, a disclosed ECM suspension can be injected in a grid-like pattern. In this aspect, it is contemplated that a disclosed ECM suspension can be injected as a first series of spaced, substantially parallel lines and a second series of spaced, substantially parallel lines that are substantially perpendicular to the first series of spaced, substantially parallel lines, thereby defining the grid-like pattern.

[0042] In another aspect, it is contemplated that a disclosed ECM suspension can be applied to a target site on or within the heart of a subject to create a film of a disclosed ECM suspension having a thickness ranging from about 0.1 mm to about 10 mm, more preferably, from about 1 mm to about 5 mm, and, most preferably, from about 2 mm to about 4 mm. In one exemplary aspect, it is contemplated that a disclosed ECM suspension can be applied to a target site on or within the heart of the subject to create a film of the ECM suspension having a thickness of about 3 mm.

[0043] In a further exemplary aspect, it is contemplated that a disclosed ECM suspension can be injected at a target site positioned within the myocardium or scar tissue of the heart of a

subject. In this aspect, it is contemplated that a disclosed ECM suspension can be injected into the myocardium or scar tissue within the heart of the subject at a desired depth relative to an outer surface of the pericardium. It is further contemplated that the desired depth at which a disclosed ECM suspension is injected can range from about 0.5 mm to about 5 mm, more preferably, from about 1 mm to about 3 mm, and most preferably, from about 1.5 mm to about 2.5 mm. In one exemplary aspect, it is contemplated that the desired depth at which a disclosed ECM suspension is injected can be about 2 mm. In this aspect, it is contemplated that the desired depth at which a disclosed ECM suspension is injected can correspond to a position proximate the junction between the epicardium and the myocardium. It is further contemplated that the desired depth at which a disclosed ECM suspension is injected can correspond to a position proximate ischemic and/or inflamed and/or injured heart tissue. In an exemplary aspect, it is contemplated that the desired depth at which a disclosed ECM suspension is injected can correspond to a position proximate necrotic and/or infarcted myocardium.

[0044] In exemplary aspects, when a disclosed ECM suspension is to be injected at a target site within the myocardium and/or one or more chambers of the heart of a subject following the occurrence of a myocardial infarction, it is contemplated that the ECM suspension should be injected at the target site during one of two possible time periods: prior to full onset of the inflammatory response of the subject or after the inflammatory response of the subject has decreased. In one aspect, when the ECM suspension is injected at the target site prior to full onset of the inflammatory response of the subject, it is contemplated that the ECM suspension should be injected at the target site substantially immediately after occurrence of the myocardial infarction up to the time of therapeutic revascularization of the heart (using, for example, a coronary artery bypass graft or a stent). In another aspect, when the ECM suspension is injected at the target site after the inflammatory response of the subject has decreased, it is contemplated that the ECM suspension should be injected at the target site after the acute phase of the myocardial infarction, during which negatively remodeling and scar tissue formation occur. In various aspects, it is contemplated that, following injection of a disclosed ECM suspension on or within the heart of a subject, the ECM suspension will not disperse but will instead attract stem cells to the target site, thereby promoting desired positive remodeling of the heart. As used herein, the term "positive remodeling" refers to beneficial regeneration and/or restructuring of damaged heart tissue; such positive remodeling promotes growth of new cells while preserving the functionality of the heart and preventing formation of scar tissue.

Sterilization and Decellularization of the ECM Compositions

[0045] Optionally, it is contemplated that the disclosed extracellular matrix compositions can be sterilized using a known sterilization system, such as, for example and without limitation, the system described in U.S. Patent No. 7,108,832, assigned to NovaSterilis, Inc., which patent is expressly incorporated herein by reference in its entirety. Thus, in some aspects, the system used to perform the disclosed methods can comprise a standard compressed storage cylinder and a standard air compressor used in operative association with a booster (*e.g.*, a Haskel Booster AGT 7/30). In other aspects, the air compressor and booster can be replaced with a single compressor. In exemplary aspects, the compressed storage cylinder can be configured to receive carbon dioxide, and the booster can be a carbon dioxide booster.

[0046] The system can further comprise an inlet port, which allows one or more additives contained in a reservoir to be added to a reactor vessel through a valve and an additive line. As used herein, the term "reactor vessel" refers to any container having an interior space that is configured to receive an ECM material and permit exposure of the ECM material to one or more sterilants and additives, as disclosed herein. In exemplary aspects, the reactor vessel can be, without limitation, a basket, a bucket, a barrel, a box, a pouch, and other known containers. It is contemplated that the reactor vessel can be re-sealable. In one aspect, it is contemplated that the reactor vessel can be a syringe that is filled with an ECM material. In an exemplary aspect, the reactor vessel can be a pouch comprising Tyvek® packaging (E.I. du Pont de Nemours and Company).

[0047] It is contemplated that a selected primary sterilant, such as, for example and without limitation, carbon dioxide, can be introduced to the reactor vessel from a header line via a valve and a supply line. It is further contemplated that a filter, such as, for example and without limitation, a 0.5 μm filter, can be provided in the supply line to prevent escape of material from the vessel. In exemplary aspects, a pressure gauge can be provided downstream of a shut-off valve in the header line to allow the pressure to be visually monitored. A check valve can be provided in the header line upstream of the valve to prevent reverse fluid flow into the booster. In order to prevent an overpressure condition existing in the header line, a pressure relief valve can optionally be provided.

[0048] In one aspect, depressurization of the reactor vessel can be accomplished using an outlet line and a valve in communication with the reactor vessel. In this aspect, it is contemplated that the depressurized fluid can exit the vessel via the supply line, be filtered by a

filter unit, and then be directed to a separator, where filtered fluid, such as carbon dioxide, can be exhausted via an exhaust line. It is further contemplated that valves can be incorporated into the various lines of the apparatus to permit fluid isolation of upstream components.

[0049] In one exemplary aspect, the reactor vessel can comprise stainless steel, such as, for example and without limitation, 316 gauge stainless steel. In another exemplary aspect, the reactor vessel can have a total internal volume sufficient to accommodate the materials being sterilized, either on a laboratory or commercial scale. For example, it is contemplated that the reactor vessel can have a length of about 8 inches, an inner diameter of about 2.5 inches, and an internal volume of about 600 mL. In additional aspects, the reactor vessel can comprise a vibrator, a temperature control unit, and a mechanical stirring system comprising an impeller and a magnetic driver. In one optional aspect, it is contemplated that the reactor vessel can contain a basket comprising 316 gauge stainless steel. In this aspect, it is contemplated that the basket can be configured to hold materials to be sterilized while also protecting the impeller and directing the primary sterilant in a predetermined manner.

[0050] It is contemplated that the reactor vessel can be operated at a constant pressure or under continual pressurization and depressurization (pressure cycling) conditions without material losses due to splashing or turbulence, and without contamination of pressure lines via back-diffusion. It is further contemplated that the valves within the system can permit easy isolation and removal of the reactor vessel from the other components of the system. In one aspect, the top of the reactor vessel can be removed when depressurized to allow access to the interior space of the reactor vessel.

[0051] Optionally, the system can comprise a temperature control unit that permits a user to adjustably control the temperature within the reactor vessel.

[0052] In use, the disclosed apparatus can be employed in a method of producing a sterilized, acellular ECM composition, such as disclosed herein. However, it is understood that the disclosed apparatus is merely exemplary, and that any apparatus capable of performing the disclosed method steps can be employed to produce the sterilized, acellular ECM composition. Thus, the claimed method is in no way limited to a particular apparatus.

[0053] It is contemplated that significant reductions in colony-forming units (CFUs) can be achieved in accordance with the disclosed methods by subjecting an isolated ECM material to sterilization temperature and pressure conditions using a primary sterilant. Optionally, it is

contemplated that the primary sterilant can be combined with one or more secondary sterilants to achieve desired sterilization. Optionally, it is further contemplated that selected additives can be incorporated into an ECM material to impart desired characteristics to the resulting ECM composition. It is still further contemplated that the disclosed methods can be employed to produce sterilized, acellular ECM compositions for implantation within the body of a subject.

[0054] As described herein, the disclosed methods make use of rapid depressurization of an isolated ECM material to render the ECM material acellular. This rapid depressurization of the ECM material occurs at depressurization rates that are significantly higher than the depressurization rates applied in previously known methods. In addition to rendering acellular the ECM material as described herein, the rapid depressurization of the ECM material also can be used to enhance the incorporation of desired sterilants and additives into the ECM material. Further, it is contemplated that the rapid depressurization of the ECM material can render the ECM material acellular while also improving retention of native growth factors, as compared to previously known decellularization methods. Still further, it is contemplated that the rapid depressurization of the ECM material can be used to improve retention of the tensile strength of the ECM material, as compared to previously known decellularization methods.

[0055] The disclosed methods not only do not significantly weaken the mechanical strength and bioptric properties of the ECM compositions, but also the methods are more effective in decellularizing the ECM compositions and in enhancing the incorporation of various additives into the ECM compositions. Thus, the disclosed sterilization and decellularization methods provide ECM compositions that are more decellularized and have a greater capacity to incorporate and then deliver more additives than ECM compositions known in the art. Moreover, the disclosed sterilization and decellularization methods provide ECM compositions that have greater amounts and/or concentrations of retained native growth factors and that have greater tensile strength than sterilized and decellularized ECM compositions known in the art.

[0056] In exemplary aspects, the primary sterilant can be carbon dioxide at or near its supercritical pressure and temperature conditions. However, it is contemplated that any conventional sterilant, including, for example, gas,liquid, or powder sterilants that will not interfere with the native properties of the ECM material, can be used as the primary sterilant.

[0057] In one exemplary aspect, the disclosed sterilization process can be practiced using carbon dioxide as a primary sterilant at pressures ranging from about 1,000 to about 3,500 psi and at temperatures ranging from about 25°C. to about 60°C. More preferably, when

supercritical carbon dioxide is used, it is contemplated that the sterilization process can use carbon dioxide as a primary sterilant at pressures at or above 1,071 psi and at temperatures at or above 31.1°C. In this aspect, the ECM material to be sterilized can be subjected to carbon dioxide at or near such pressure and temperature conditions for times ranging from about 10 minutes to about 24 hours, more preferably from about 15 minutes to about 18 hours, and most preferably, from about 20 minutes to about 12 hours. Preferably, the carbon dioxide employed in the disclosed systems and methods can be pure or, alternatively, contain only trace amounts of other gases that do not impair the sterilization properties of the carbon dioxide. For ease of further discussion below, the term "supercritical carbon dioxide" will be used, but it will be understood that such a term is non-limiting in that carbon dioxide within the pressure and temperature ranges as noted above can be employed satisfactorily in the practice of the disclosed methods. Within the disclosed pressure and temperature ranges, it is contemplated that the carbon dioxide can be presented to the ECM material in a gas, liquid, fluid or plasma form.

[0058] The secondary sterilants employed in the disclosed methods can, in some aspects, include chemical sterilants, such as, for example and without limitation, peroxides and/or carboxylic acids. Preferred carboxylic acids include alkanecarboxylic acids and/or alkanepercarboxylic acids, each of which can optionally be substituted at the alpha carbon with one or more electron-withdrawing substituents, such as halogen, oxygen and nitrogen groups. Exemplary species of chemical sterilants employed in the practice of the disclosed methods include, for example and without limitation, hydrogen peroxide (H_2O_2), acetic acid (AcA), peracetic acid (PAA), trifluoroacetic acid (TFA), and mixtures thereof. In one exemplary aspect, the chemical sterilants can include Sporeclenz® sterilant, which is a mixture comprising acetic acid, hydrogen peroxide, and peracetic acid.

[0059] It is contemplated that the secondary sterilants can be employed in a sterilization-enhancing effective amount of at least about 0.001 vol. % and greater, based on the total volume of the primary sterilant. It is further contemplated that the amount of secondary sterilant can be dependent upon the particular secondary sterilant that is employed. Thus, for example, it is contemplated that peracetic acid can be present in relatively small amounts of about 0.005 vol. % and greater, while acetic acid can be employed in amounts of about 1.0 vol. % and greater. Thus, it is contemplated that the concentration of the secondary sterilants can range from about 0.001 vol. % to about 2.0 vol. % and can typically be used as disclosed herein to achieve a

sterilization-enhancing effect in combination with the disclosed primary sterilants, such as, for example and without limitation, supercritical carbon dioxide.

[0060] In one aspect, the method of producing a sterilized, acellular ECM composition can comprise harvesting a selected tissue from a mammal and rinsing the selected tissue in sterile saline or other biocompatible liquid, including, for example and without limitation, Ringer's solution or a balanced biological salt solution. In this aspect, the selected tissue can be, for example and without limitation, stomach tissue (*e.g.*, stomach submucosa (SS)), small intestinal tissue (*e.g.*, small intestinal submucosa (SIS)), large intestinal tissue, bladder tissue (*e.g.*, urinary bladder submucosa (UBS)), liver tissue (*e.g.*, liver basement membrane (LBM)), heart tissue (*e.g.*, pericardium, epicardium, endocardium, myocardium), lung tissue, kidney tissue, pancreatic tissue, prostate tissue, mesothelial tissue, fetal tissue, a placenta, a ureter, veins, arteries, heart valves with or without their attached vessels, tissue surrounding the roots of developing teeth, and tissue surrounding growing bone. In another aspect, the method can comprise freezing the selected tissue for a period ranging from about 12 to about 36 hours, more preferably, from about 18 to about 30 hours, and most preferably, from about 22 to about 26 hours. For example, it is contemplated that the period during which the selected tissue is frozen can be 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 25 hours, 26 hours, 27 hours, 28 hours, 29 hours, 30 hours, 31 hours, 32 hours, 33 hours, 34 hours, 35 hours, 36 hours, and any other period of time falling between the preceding values. In an additional aspect, the method can comprise thawing the selected tissue in cold hypotonic tris buffer. Optionally, in this aspect, the method can comprise thawing the selected tissue in cold hypotonic tris buffer on ice with 5 mM ethylenediaminetetraacetic acid (EDTA). In exemplary aspects, it is contemplated that the steps of freezing and thawing the selected tissue can be cyclically repeated up to six times.

[0061] In another aspect, the method can comprise isolating an ECM material from the selected tissue. In this aspect, the ECM material can be any material comprising known extracellular matrix components, including, for example and without limitation, stomach tissue (*e.g.*, stomach submucosa (SS)), small intestinal tissue (*e.g.*, small intestinal submucosa (SIS)), large intestinal tissue, bladder tissue (*e.g.*, urinary bladder submucosa (UBS)), liver tissue (*e.g.*, liver basement membrane (LBM)), heart tissue (*e.g.*, pericardium, epicardium, endocardium, myocardium), lung tissue, kidney tissue, pancreatic tissue, prostate tissue, mesothelial tissue, fetal tissue, a placenta, a ureter, veins, arteries, heart valves with or without their attached vessels, tissue surrounding the roots of developing teeth, and tissue surrounding growing bone..

In one exemplary, non-limiting aspect, the step of isolating an ECM material can comprise isolating SIS from a mammalian tissue source. In this aspect, the method can comprise: incising a wall of a small intestine along a path that is substantially parallel to the longitudinal axis of the small intestine; opening the small intestine along the path of the incision such that the small intestine lies flat on a surface; rinsing the small intestine with sterile saline or other biocompatible fluid; mechanically stripping the SIS of the small intestine from the surrounding smooth muscle and serosal layers and from the tunica mucosa, leaving essentially the submucosal and basement membrane layers. However, it is contemplated that the ECM material can be isolated using any conventional technique, including those described in: U.S. Patent No. 4,902,508; U.S. Patent No. 5,275,826; U.S. Patent No. 5,281,422; U.S. Patent No. 5,554,389; U.S. Patent No. 6,579,538; U.S. Patent No. 6,933,326; U.S. Patent No. 7,033,611; Voytik-Harbin *et al.*, "Identification of Extractable Growth Factors from Small Intestinal Submucosa," *J. Cell. Biochem.*, Vol. 67, pp. 478-491 (1997); Hodde *et al.*, "Virus Safety of a Porcine-Derived Medical Device: Evaluation of a Viral Inactivation Method," *Biotech. & Bioeng.,* Vol. 79, No. 2, pp. 211-216 (2001); Badylak *et al.*, "The Extracellular Matrix as a Scaffold for Tissue Reconstruction," *Cell & Developmental Biology*, Vol. 13, pp. 377-383 (2002); Robinson *et al.*, "Extracelular Matrix Scaffold for Cardiac Repair," *Circulation*, Vol. 112, pp. I-135-I-143 (2005); Hodde *et al.*, "Effects of Sterilization on an Extracellular Matrix Scaffold: Part I. Composition and Matrix Architecture," *J. Mater. Sci.: Mater. Med.*, Vol. 18, pp. 537-543 (2007); and Hodde *et al.*, "Effects of Sterilization on an Extracellular Matrix Scaffold: Part II. Bioactivity and Matrix Interaction," *J. Mater. Sci.: Mater. Med.*, Vol. 18, pp. 545-550 (2007), each of which is expressly incorporated herein by reference in its entirety.

[0062] In an additional aspect, the method can comprise incubating the isolated ECM material for 24 to 48 hours in 0.5-1% Triton X-100/0.5-1% Deoxycholic acid with 5 mM EDTA in Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza Walkersville, Inc.). In this aspect, it is contemplated that flat or sheet-like ECM materials, such as stomach submucosa (SS), small intestinal submucosa (SIS), and bladder submucosa (UBS), can be incubated in a stretched configuration. It is further contemplated that ECM material conduits or other lumenal ECM materials, such as ureters, arteries, veins, and tubular SIS, can be perfused with the various disclosed solutions through soaking and by use of a peristaltic pump.

[0063] In a further aspect, after incubation, the method can comprise rinsing the ECM material with DPBS. In this aspect, it is contemplated that the step of rinsing the ECM material

can comprise rinsing the ECM material up to six times, including one, two, three, four, five, or six times, with each rinse lasting for about thirty minutes. In an exemplary aspect, it is contemplated that the step of rinsing the ECM material can comprise rinsing the ECM material three times, with each rinse lasting for about thirty minutes.

[0064] Optionally, in exemplary aspects, the method can further comprise a second incubation procedure. In these aspects, the second incubation procedure can comprise incubating the ECM material in isotonic tris buffer containing 10-50 μg/mL of RNAase/0.2-0.5 μg/mL DNAase with 5 mM EDTA. It is contemplated that the step of incubating the ECM material in isotonic tris buffer can be performed at a temperature of about 37°C, substantially corresponding to the temperature of a human body. It is further contemplated that the step of incubating the ECM material in isotonic tris buffer can be performed for a period ranging from about 30 minutes to about 24 hours, more preferably, from about 1 hour to about 18 hours, and most preferably, from about 2 hours to about 12 hours. In an additional aspect, the second incubation procedure can further comprise rinsing the ECM material with DPBS. In this aspect, it is contemplated that the step of rinsing the ECM material can comprise rinsing the ECM material three times, with each rinse lasting for about thirty minutes.

[0065] In yet another aspect, whether or not the second incubation procedure is performed, the method can comprise storing the ECM material at a temperature ranging from about 1°C to about 10°C, more preferably, from about 2°C to about 6°C, and, most preferably, from about 3°C to about 5°C. In an exemplary aspect, the ECM material can be stored at 4°C.

[0066] In an additional aspect, the method can comprise introducing the ECM material into the interior space of the reactor vessel. Optionally, in this aspect, one or more secondary sterilants from the reservoir can be added into the interior space of the reactor vessel along with the ECM material. In these aspects, it is contemplated that the one or more secondary sterilants from the reservoir can be added into the interior space of the reactor vessel before, after, or contemporaneously with the ECM material. It is further contemplated that the temperature control unit can be selectively adjusted to produce a desired temperature within the interior space of the reactor vessel. In a further aspect, the method can comprise equilibrating the pressure within the reactor vessel and the pressure within the storage cylinder. For example, in this aspect, it is contemplated that the pressure within the reactor vessel and the pressure within the storage cylinder can be substantially equal to atmospheric pressure. In yet another aspect, after equilibration of the pressures within the apparatus, the method can comprise operating the

magnetic driver to activate the impeller of the reactor vessel. In still a further aspect, the method can comprise selectively introducing the primary sterilant from the storage cylinder into the reactor vessel until a desired pressure within the reactor vessel is achieved. In this aspect, it is contemplated that the step of selectively introducing the primary sterilant into the reactor vessel can comprise selectively activating the air compressor and the booster to increase flow of the primary sterilant into the reactor vessel. In exemplary aspects, the air compressor and booster can be activated to subject the ECM material to supercritical pressures and temperatures, such as, for example and without limitation, the pressures and temperatures necessary to produce supercritical carbon dioxide, for a time period ranging from about 20 minutes to about 60 minutes.

[0067] In a further aspect, the method can comprise rapidly depressurizing the reactor vessel. In this aspect, a predetermined amount of primary sterilant, such as, for example and without limitation, supercritical carbon dioxide, can be released from the reactor vessel through the depressurization line. It is contemplated that the primary sterilant can be released from the reactor vessel through opening of the valve coupled to the reactor vessel to thereby rapidly reduce the pressure within the reactor vessel. As used herein, the term "rapid depressurization" refers to depressurization of the reactor vessel at a rate greater than or equal to 400 psi/min. For example, it is contemplated that the reactor vessel can be depressurized at a depressurization rate ranging from about 2.9 MPa/min. to about 18.0 MPa/min. (about 400 psi/min. to about 2,600 psi/min.), more preferably from about 5.0 MPa/min. to about 10.0 MPa/min. (700 psi/min. to about 1,500 psi/min.), and, most preferably, from about 7.0 MPa/min. to about 8.0 MPa/min. (about 1,000 psi/min. to about 1,200 psi/min.). Thus, these rapid depressurizations are significantly greater than the 300 psi/min. depressurization rate disclosed in U.S. Patent No. 7,108,832. Without being bound by any particular theory, it is believed that the disclosed rapid depressurization rates increase the level of decellularization achieved in the ECM material. For example, the rapid depressurization of a disclosed ECM material can lead to levels of decellularization in the ECM material of greater than about 96%, including 96.1%, 96.2%, 96.3%, 96.4%, 96.5%, 96.6%, 96.7%, 96.8%, 96.9%, 97.0%, 97.1%, 97.2%, 97.3%, 97.4%, 97.5%, 97.6%, 97.7%, 97.8%, 97.9%, 98.0%, 98.1%, 98.2%, 98.3%, 98.4%, 98.5%, 98.6%, 98.7%, 98.8%, 98.9%, 99.0%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9%, and 100.0%.

[0068] In exemplary aspects, the method can further comprise the step of incorporating one or more additives into the ECM material. In these aspects, it is contemplated that the one or more additives can be provided in either a powder or a liquid form. In one optional aspect, the step of incorporating the one or more additives can comprise introducing the one or more additives into the reactor vessel during the step of rapidly depressurizing the reactor vessel. In this aspect, it is contemplated that the introduction of the one or more additives can be characterized as a conventional foaming process. In another optional aspect, the step of incorporating the one or more additives can comprise introducing the one or more additives into the reactor vessel after the step of rapidly depressurizing the reactor vessel. In this aspect, it is contemplated that the one or more additives can be added to the ECM material after the rapid depressurization of the reactor vessel has caused the ECM material to swell and/or expand, thereby permitting improved penetration of the additives into the ECM material. It is further contemplated that, in an exemplary aspect, the one or more additives can be added to the ECM material within about thirty minutes after the rapid depressurization of the reactor vessel. In a further optional aspect, the step of incorporating the one or more additives can comprise introducing the one or more additives into the reactor vessel both during and after the step of rapidly depressurizing the reactor vessel. In this aspect, it is contemplated that the one or more additives can be released into the reactor vessel in both a quick manner and a slow, extended manner. In still a further optional aspect, the step of incorporating the one or more additives can comprise introducing the one or more additives into the reactor vessel before the step of rapidly depressurizing the reactor vessel.

[0069] The disclosed additives can be incorporated into the ECM material to impart selected properties to the resulting sterilized, acellular ECM composition. Thus, it is contemplated that the one or more additives can be selected to replace or supplement components of the ECM material that are lost during processing of the ECM material as described herein. For example, and as described below, the one or more additives can comprise growth factors, cytokines, proteoglycans, glycosaminoglycans (GAGs), proteins, peptides, nucleic acids, small molecules, drugs, or cells. It is further contemplated that the one or more additives can be selected to incorporate non-native components into the ECM material. For example, the one or more additives can comprise, for example and without limitation, growth factors for recruiting stem cells, angiogenic cytokines, and anti-inflammatory cytokines. It is still further contemplated that the one or more additives can be pharmaceutical agents, such as statins, corticosteroids, non-steroidal anti-inflammatory drugs, anti-inflammatory compounds,

anti-arrhythmic agents, and the like. It is still further contemplated that the one or more additives can be nanoparticles, such as, for example and without limitation, silver nanoparticles, gold nanoparticles, platinum nanoparticles, iridium nanoparticles, rhodium nanoparticles, palladium nanoparticles, copper nanoparticles, zinc nanoparticles, and other metallic nanoparticles. It is still further contemplated that the one or more additives can be metallic compounds. In one exemplary aspect, the one or more additives can be selected to pharmaceutically suppress the immune response of a subject following implantation of the resulting ECM composition into the body of a subject.

[0070] In one aspect, the one or more additives can comprise one or more growth factors, including, for example and without limitation, transforming growth factor-β -1, -2, or -3 (TGF-β -1, -2, or -3), fibroblast growth factor-2 (FGF-2), also known as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), placental growth factor (PGF), connective tissue growth factor (CTGF), hepatocyte growth factor (HGF), Insulin-like growth factor (IGF), macrophage colony stimulating factor (M-CSF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor-α (TGF-α).

[0071] In another aspect, the one or more additives can comprise one or more cytokines, including, for example and without limitation, stem cell factor (SCF), stromal cell-derived factor-1 (SDF-1), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-gamma), Interleukin-3, Interleukin-4, Interleukin-10, Interleukin-13, Leukemia inhibitory factor (LIF), amphiregulin, thrombospondin 1, thrombospondin 2, thrombospondin 3, thrombospondin 4, thrombospondin 5, and angiotensin converting enzyme (ACE).

[0072] In an additional aspect, the one or more additives can comprise one or more proteoglycans, including, for example and without limitation, heparan sulfate proteoglycans, betaglycan, syndecan, decorin, aggrecan, biglycan, fibromodulin, keratocan, lumican, epiphycan, perlecan, agrin, testican, syndecan, glypican, serglycin, selectin, lectican, versican, neurocan, and brevican.

[0073] In a further aspect, the one or more additives can comprise one or more glycosaminoglycans, including, for example and without limitation, heparan sulfate, hyaluronic acid, heparin, chondroitin sulfate B (dermatan sulfate), and chondroitin sulfate A.

[0074] In still a further aspect, the one or more additives can comprise one or more proteins, peptides, or nucleic acids, including, for example and without limitation, collagens,

elastin, vitronectin, versican, laminin, fibronectin, fibrillin-1, fibrillin-2, plasminogen, small leucine-rich proteins, cell-surface associated protein, cell adhesion molecules (CAMs), a matrikine, a matrix metalloproteinase (MMP), a cadherin, an immunoglobin, a multiplexin, cytoplasmic domain-44 (CD-44), amyloid precursor protein, tenascin, nidogen/entactin, fibulin I, fibulin II, integrins, transmembrane molecules, and osteopontin.

[0075] In yet another aspect, the one or more additives can comprise one or more pharmaceutical agents, including, for example and without limitation, statin drugs, for example, cerevastatin, atorvastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin, corticosteroids, non-steroidal anti-inflammatory drugs, anti-inflammatory compounds, anti-arrhythmic agents, antimicrobials, antibiotics, and the like.

[0076] In exemplary aspects, the steps of introducing the one or more additives into the reactor vessel can comprise opening the valve to allow the one or more additives to flow from the reservoir into the inlet port. Prior to pressurization, it is contemplated that the one or more additives can be introduced directly into the reactor vessel prior to sealing and/or via the inlet port.

[0077] It is contemplated that the disclosed rapid depressurization and repressurization of the reactor vessel, with or without the addition of the one or more additives, can be repeated for any desired number of cycles. It is further contemplated that the cycles of depressurization and repressurization, as well as the introduction of the primary sterilants and/or secondary sterilants and/or additives, can be automatically controlled via a controller that is configured to selectively open and/or close the various valves of the system to achieve desired pressure conditions and cycles.

[0078] In some aspects, the disclosed methods can further comprise the step of agitating the contents of the reactor vessel. In these aspects, it is contemplated that the step of agitating the contents of the reactor vessel can comprise periodically agitating the contents of the reactor vessel using a vibrator. It is further contemplated that the agitation of the reactor vessel can be intermittent, continual, or continuous. In exemplary aspects, the step of agitating the contents of the reactor vessel can occur during the step of introducing the primary sterilant into the reactor vessel. It is contemplated that the agitation of the contents of the reactor vessel can enhance the mass transfer of the sterilants and/or additives by eliminating voids in the fluids within the reactor vessel to provide for more complete contact between the ECM material and the sterilants and/or additives. It is further contemplated that the step of agitating the contents of the reactor

vessel can comprise selectively adjusting the intensity and duration of agitation so as to optimize sterilization times, temperatures, and pressurization/depressurization cycles.

[0079] In a further aspect, after the sterilization and decellularization of the ECM material is complete, the method can further comprise depressurizing the reactor vessel and deactivating the magnetic drive so as to cease movement of the stirring impeller. Finally, the method can comprise the step of removing the resulting sterilized, acellular ECM composition through the top of the reactor vessel.

[0080] It is contemplated that the duration of the disclosed steps, as well as the temperatures and pressures associated with the disclosed steps, can be selectively varied to account for variations in the characteristics of the ECM material. For example, when the ECM material is a multi-laminate structure, has an increased thickness, or is positioned within a syringe, it is contemplated that the duration of the disclosed steps can be increased.

[0081] In one optional aspect, in order to make the sterilized, acellular ECM composition into a particulate form, the method can comprise cutting the ECM composition into pieces having desired lengths. In another aspect, the method can optionally comprise freeze-drying the pieces of the ECM composition. In an additional aspect, the method can optionally comprise grinding the frozen, hydrated pieces of the ECM composition and then passing the pieces of the ECM composition through a sizer screen until ECM particulate of a desired size is isolated. In a further optional aspect, the method can comprise rehydrating the ECM particulate with sterile saline or other sterile, biocompatible fluid to form an ECM suspension, as described herein.

Methods of Enhancing the Incorporation of an Additive Into an ECM Material

[0082] In exemplary aspects, a method for enhancing the incorporation of an additive into an extracellular matrix (ECM) material can comprise: positioning an ECM material within an interior space of a reactor vessel; introducing carbon dioxide into the interior space of the reactor vessel at supercritical pressure and temperature conditions, thereby sterilizing the ECM material; rapidly depressurizing the interior space of the reactor vessel at a depressurization rate sufficient to render the ECM material acellular and to increase the capacity of the ECM material for incorporation of an additive; and introducing one or more additives into the interior space of the reactor vessel, whereby at least a portion of each additive of the one or more additives is incorporated into the sterilized and acellular ECM material.

[0083] It is contemplated that the depressurization rate can range from about 400 psi/minute to about 2,600 psi/minute, more preferably, from about 700 psi/minute to about 1,500 psi/minute and, most preferably, from about 1,000 psi/minute to about 1,200 psi/minute. It is further contemplated that the method for enhancing the incorporation of an additive into the ECM material can further comprise introducing at least one secondary sterilant into the interior space of the reactor vessel. It is still further contemplated that the one or more additives can comprise at least one growth factor. It is still further contemplated that the one or more additives can comprise at least one cytokine. It is still further contemplated that the one or more additives can comprise at least one proteoglycan. It is still further contemplated that the one or more additives can comprise at least one glycosaminoglycan (GAG). It is still further contemplated that the one or more additives can comprise at least one of a protein, a peptide, and a nucleic acid. It is still further contemplated that the one or more additives can comprise at least one pharmaceutical agent. It is still further contemplated that the one or more additives can comprise nanoparticles.

[0084] Optionally, it is contemplated that the step of introducing the one or more additives into the interior space of the reactor vessel is performed contemporaneously with the step of rapidly depressurizing the interior space of the reactor vessel.

[0085] It is further contemplated that a sterilized, acellular ECM composition can be produced using the disclosed method for enhancing the incorporation of an additive into an ECM material. In exemplary aspects, the sterilized, acellular ECM composition can comprise at least one tissue, and each tissue of the at least one tissue can be from a respective tissue source. In these aspects, it is contemplated that the tissue source of each tissue of the at least one tissue can be selected from the group consisting of small intestinal submucosa, stomach submucosa, large intestinal tissue, urinary bladder submucosa, liver basement membrane, pericardium, epicardium, endocardium, myocardium, lung tissue, kidney tissue, pancreatic tissue, prostate tissue, mesothelial tissue, fetal tissue, a placenta, a ureter, veins, arteries, heart valves with or without their attached vessels, tissue surrounding the roots of developing teeth, and tissue surrounding growing bone. In one aspect, the sterilized, acellular ECM composition can be at least about 96% decellularized, as set forth herein.

EXAMPLES

[0086] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles,

devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Example 1

[0087] In exemplary applications of the disclosed sterilization and decellularization methods, selected tissues were harvested and rinsed in sterile saline. The selected tissues were then frozen for 24 hours. The frozen tissues were thawed in cold hypotonic tris buffer on ice with 5 mM ethylenediaminetetraacetic acid (EDTA). An extracellular matrix material was then isolated from each selected tissue, as described herein.

[0088] The isolated extracellular matrix materials were incubated for 24 to 48 hours in 0.5-1% Triton X-100/0.5-1% Deoxycholic acid with 5 mM EDTA in Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza Walkersville, Inc.). Flat extracellular matrix materials, such as stomach submucosa (SS), small intestinal submucosa (SIS), and bladder submucosa (UBS), were incubated in a stretched configuration. Tubular extracellular matrix materials, such as ureters, arteries, veins, and tubular SIS, were perfused with the solutions through soaking and by use of a peristaltic pump.

[0089] After incubation, each extracellular matrix material was rinsed three times with DPBS. Each rinsing with DPBS lasted 30 minutes. Some extracellular matrix materials were then incubated for 2 to 12 hours at 37°C in isotonic tris buffer containing 10-50 μg/mL of RNAse/0.2-0.5 μg/mL DNAse with 5 mM EDTA. Following this incubation step, the extracellular matrix materials were again rinsed three times with DPBS. Each rinsing with DPBS lasted 30 minutes. The extracellular matrix materials were stored at 4°C.

[0090] Within 48 hours of storage, the extracellular matrix materials were processed in supercritical carbon dioxide as disclosed herein for 20-60 minutes at temperatures at or greater than 31.1°C and pressures at or greater than 1,071 psi. After this sterilization step, the extracellular matrix materials were rapidly depressurized at a rate of 2.7 MPa/10 sec. (391.6 psi/10 sec.) for a minute and 19 seconds. During this time, the pressure applied to the extracellular matrix materials rapidly decreased from 9.9 MPa to 0.69 MPa.

[0091] The extracellular matrix materials were then processed in supercritical carbon dioxide and peracetic acid (PAA) as disclosed herein for 30 minutes to 6 hours to achieve terminal sterilization. In this processing step, the pressure applied to the extracellular matrix materials was increased to 9.9 MPa. The resulting sterilized, acellular extracellular matrix materials were then packaged in Tyvek® (E.I. du Pont de Nemours & Company) pouches that were sealed within plastic pouches to prevent fluid leakage.

[0092] Table 1 summarizes the sterilization and decellularization of porcine ureter, bovine pericardium, and porcine mesothelium.

Table 1

Material	Triton X-100 Conc.	Deoxycholic Acid Conc.	TX-100/Deoxy incubation	RNAse/ DNAse incubation	Supercritical CO_2/PAA time
Porcine ureters	0.5%	0.5%	24 hours	2 hours	120 minutes
Bovine pericardium	0.5%	0.5%	24 hours	2 hours	180 minutes
Porcine mesothelium	0.5%	0.5%	24 hours	2 hours	120 minutes

Example 2

[0093] The DNA content of ECM material samples was measured as an indicator of decellularization of the respective ECM material samples using various sterilization and decellularization techniques. The measured DNA content was evaluated with a pico green assay in which DNA was labeled with a fluorescent label that was detected with a spectrophotometer. The measured DNA content was normalized by the dry weight of the samples. DNA content was measured and evaluated for the following treatment groups: (1) Lyophilized, non-sterile SIS; (2) Ethylene Oxide (EtO)-sterilized SIS; (3) Lyophilized, non-sterile SIS that was sterilized through a 60 minute treatment with PAA and supercritical CO_2, as disclosed herein; (4) Lyophilized, non-sterile SIS that was sterilized through a 20 minute treatment with PAA and supercritical CO_2, as disclosed herein; and (5) Raw, unprocessed SIS.

[0094] Figure 1 shows the total DNA content for the respective samples, as normalized by dry weight. Figure 2 shows the percent of DNA that was removed from each respective sample, as compared to raw, unprocessed SIS. These results indicated that by sterilizing the non-sterile SIS using a 60 minute treatment with PAA and supercritical CO_2, as disclosed herein, over 96%

of the DNA found in raw SIS was removed, as compared to only 94% when the SIS was sterilized by EtO and only 93% when the SIS was not sterilized by any method.

Example 3

[0095] Ureters were processed with a gentle detergent (0.5% Triton X-100/0.5% Sodium Deoxycholate in 5mM EDTA in DPBS) for 24 hours and then rinsed three times in DPBS as disclosed herein. After this pretreatment, the ureters were decellularized and sterilized using rapid depressurization and treatment with PAA and supercritical CO_2, as disclosed herein. Hematoxylin and Eosin (H&E) Stains were prepared for one sample ureter at the following stages of treatment: (A) native ureter; (B) pretreated ureter; and (C) pretreated ureter with rapid depressurization and treatment with PAA and supercritical CO_2, as disclosed herein. These stains indicated that DNA content was significantly reduced with rapid depressurization.

Example 4

[0096] The growth factor content of ECM material samples was measured. Enzyme-linked immunosorbent (ELISA) assays were performed on the ECM material samples to quantify the content of bFGF and the active form of TGF-β in each respective sample. The following treatment groups were evaluated: (1) Lyophilized, non-sterile SIS; (2) Ethylene Oxide (EtO)-sterilized SIS; (3) Lyophilized, non-sterile SIS that was sterilized through a 60 minute treatment with PAA and supercritical CO_2, as disclosed herein; (4) Lyophilized, non-sterile SIS that was sterilized through a 20 minute treatment with PAA and supercritical CO_2, as disclosed herein; and (5) Raw, unprocessed SIS. The bFGF content and TGF-β content measurements were normalized by dry weight of each respective sample. These results are shown in Figures 3 and 4. These results indicated that the concentration of both growth factors was reduced by exposure to EtO. However, the concentration of the growth factors was not affected by sterilization with PAA and supercritical CO_2.

Example 5

[0097] Using the methods disclosed herein, supercritical CO_2 was used as a primary sterilant and as a carrier for adding bFGF into SIS sheets. First, the respective SIS sheets were placed into Tyvek® pouches along with varying amounts of bFGF. The pouches were exposed to supercritical CO_2 for 60 minutes at 9.6 MPa. The pouches were rapidly depressurized at a rate of 7.20 MPa/min. Samples were directly processed in 16 mL PAA in supercritical CO_2 for 20

minutes. The following treatment groups were evaluated: (1) No bFGF added; (2) 5 μL bFGF added; and (3) 15 μL bFGF added. Each μL of bFGF contained 0.1 μg of bFGF. Thus, since each SIS sheet weighed approximately 0.5 g, the maximum concentrations of bFGF for the 5 μL and 15 μL groups were about 4170 pg/mg dry weight and about 12,500 pg/mg dry weight, respectively. The bFGF content for these groups is shown in Figure 5, as measured with respect to the dry weight of the respective samples. These results indicated that the measured concentrations of bFGF did not reach the maximum concentrations and that the sample to which 15 μL of bFGF was added did not have a measured concentration of bFGF that was three times greater than the measured concentration of bFGF in the sample to which 5 μL of bFGF was added.

Example 6

[0098] The tensile strengths of two-ply SIS samples were measured. The following treatment groups were evaluated: (1) EtO Treatment; (2) PAA/supercritical CO_2 treatment for 20 minutes; (3) PAA/supercritical CO_2 treatment for 60 minutes; and (4) PAA/supercritical CO_2 treatment for 120 minutes. The tensile strength test results are shown in Figure 6. These results indicated that the SIS samples that were processed with PAA/supercritical CO_2 for 20 or 120 minutes, as disclosed herein, were significantly stronger than the SIS samples that were processed with EtO.

Example 7

[0099] Rapid depressurization was used following gentle detergent soaks or perfusion of the ECM materials listed in Table 2 (below) at the noted concentrations and for the noted time periods. Tissues were harvested and rinsed in saline. The tissues were frozen for at least 24 hours. The tissues were thawed in cold hypotonic tris buffer on ice with 5 mM EDTA. The ECM of interest was isolated. For flat tissues (e.g., stomach submucosa, small intestine submucosa, and bladder submucosa), the tissue was stretched on a tissue stretching device and incubated in solutions in a stretched configuration. For tubular tissues (e.g., ureters, arteries, veins, and tubular SIS), the tissue was perfused with solutions using a peristaltic pump and were soaked during incubation. The tissues were incubated for 2 to 24 hours in 0.5% Triton X-100/0.5% Deoxycholic acid with 5 mM EDTA in DPBS. The tissues were rinsed 3 times for 15-30 minutes each time in DPBS. The tissues were stored at 4° C. Within 48 hours of tissue storage, the tissues were processed in supercritical CO_2 for 20-120 minutes followed by rapid

depressurization (RDP)(decrease in pressure from 9.9 MPa to 0.69 MPa in 1 min 19 sec, corresponding to a depressurization of 2.7 MPa/10sec).

Table 2

Material	Triton X-100 Conc.	Deoxycholic Acid Conc.	TX-100/Deoxy incubation	Supercritical CO_2 time
Porcine ureters	0.5%	0.5%	24 hours	60 minutes
Bovine pericardium	0.5%	0.5%	24 hours	60 minutes
Porcine mesothelium	0.5%	0.5%	2 hours	60 minutes
SIS	0.5%	0.5%	2 hours	60 minutes

[00100] The results showed that supercritical CO_2 exposure followed by rapid depressurization ($SCCO_2$+RDP) did aid in the removal of cell remnants and DNA while preserving growth factors in the ECMs.

Example 8

[00101] The growth factor content of various ECM compositions was analyzed using basic fibroblast growth factor (bFGF) as a representative growth factor. bFGF was selected because it is a prevalent growth factor in native ECM tissues. An enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN) was used to measure the bFGF content in the following samples: (1) Unprocessed (Raw) SIS; (2) SIS after detergent soak (TX-deoxy) only; (3) SIS after TX-deoxy and RDP (includes $SCCO_2$); (4) SIS after TX-deoxy, RDP, and PAA ($SCCO_2$ with PAA for sterilization); (5) SIS after TX-deoxy, and PAA; (6) SIS sterilized by EtO (supplied by Cook Biotech, Inc.); and (7) non-sterile SIS (supplied by Cook Biotech, Inc.).

[00102] In these studies, SIS was used to compare an ECM composition processed with and without RDP to SIS provided by Cook Biotech, Inc. Some of the processed SIS was also sterilized using the described $SCCO_2$+ PAA method after decellularization. The measured growth factor content of the respective ECM compositions is shown in Figure 7.

[00103] These results indicate that the rapid depressurization process was more effective than other decellularization processes at preserving the bFGF content and that the additional RDP processing to remove residual DNA and cell fragments results in only a small loss of bFGF. By comparison, the PAA sterilization process appeared to remove almost all of the remaining bFGF, even in the absence of RDP. Additionally, the rapid depressurization process preserved more of the bFGF content in the native SIS than the Cook decellularization methods. For

purposes of these results, when the bFGF content was reduced, it is assumed that all other growth factor content was similarly reduced since the growth factors are all bound to the ECM compositions in a similar manner.

[00104] Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

[00105] It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

What is claimed is:

1. A method for sterilizing and rendering acellular an extracellular matrix (ECM) material, comprising:

positioning an ECM material within an interior space of a reactor vessel;

introducing carbon dioxide into the interior space of the reactor vessel at supercritical pressure and temperature conditions, thereby sterilizing the ECM material; and

rapidly depressurizing the interior space of the reactor vessel at a depressurization rate of at least 400 psi/minute, thereby rendering the ECM material acellular.

2. The method of Claim 1, wherein the depressurization rate ranges from about 400 psi/minute to about 2,600 psi/minute.

3. The method of Claim 1, wherein the depressurization rate ranges from about 700 psi/minute to about 1,500 psi/minute.

4. The method of Claim 1, wherein the depressurization rate ranges from about 1,000 psi/minute to about 1,200 psi/minute.

5. The method of Claim 1, further comprising introducing at least one secondary sterilant into the interior space of the reactor vessel.

6. A sterilized, acellular extracellular matrix (ECM) composition produced using the method of Claim 1.

7. The sterilized, acellular ECM composition of Claim 6, wherein the ECM composition comprises at least one tissue, each tissue of the at least one tissue being from a respective tissue source, wherein the tissue source of each tissue of the at least one tissue is selected from the group consisting of small intestinal submucosa, stomach submucosa, large intestinal tissue, urinary bladder submucosa, liver basement membrane, pericardium, epicardium, endocardium, myocardium, lung tissue, kidney tissue, pancreatic tissue, prostate tissue, mesothelial tissue, fetal tissue, a placenta, a ureter, veins, arteries, heart valves with or without their attached vessels, tissue surrounding the roots of developing teeth, and tissue surrounding growing bone.

8. The sterilized, acellular ECM composition of Claim 6, wherein the ECM composition is at least about 96% decellularized.

9. A method for sterilizing, rendering acellular, and incorporating an additive into an extracellular matrix (ECM) material, comprising:

positioning an ECM material within an interior space of a reactor vessel;

introducing carbon dioxide into the interior space of the reactor vessel at supercritical pressure and temperature conditions, thereby sterilizing the ECM material;

rapidly depressurizing the interior space of the reactor vessel at a depressurization rate of at least 400 psi/minute, thereby rendering the ECM material acellular; and

introducing one or more additives into the interior space of the reactor vessel, whereby at least a portion of each additive of the one or more additives is incorporated into the sterilized and acellular ECM material.

10. The method of Claim 9, wherein the depressurization rate ranges from about 400 psi/minute to about 2,600 psi/minute.

11. The method of Claim 9, wherein the depressurization rate ranges from about 700 psi/minute to about 1,500 psi/minute.

12. The method of Claim 9, wherein the depressurization rate ranges from about 1,000 psi/minute to about 1,200 psi/minute.

13. The method of Claim 9, further comprising introducing at least one secondary sterilant into the interior space of the reactor vessel.

14. The method of Claim 9, wherein the one or more additives comprises at least one growth factor.

15. The method of Claim 9, wherein the one or more additives comprises at least one cytokine.

16. The method of Claim 9, wherein the one or more additives comprises at least one proteoglycan.

17. The method of Claim 9, wherein the one or more additives comprises at least one glycosaminoglycan (GAG).

18. The method of Claim 9, wherein the one or more additives comprises at least one of a protein, a peptide, and a nucleic acid.

19. The method of Claim 9, wherein the one or more additives comprises at least one pharmaceutical agent.

20. The method of Claim 9, wherein the one or more additives comprises nanoparticles.

21. The method of Claim 9, wherein the step of introducing the one or more additives into the interior space of the reactor vessel is performed contemporaneously with the step of rapidly depressurizing the interior space of the reactor vessel.

22. A sterilized, acellular extracellular matrix (ECM) composition produced using the method of Claim 9.

23. The sterilized, acellular ECM composition of Claim 22, wherein the ECM composition comprises at least one tissue, each tissue of the at least one tissue being from a respective tissue source, wherein the tissue source of each tissue of the at least one tissue is selected from the group consisting of small intestinal submucosa, stomach submucosa, large intestinal tissue, urinary bladder submucosa, liver basement membrane, pericardium, epicardium, endocardium, myocardium, lung tissue, kidney tissue, pancreatic tissue, prostate tissue, mesothelial tissue, fetal tissue, a placenta, a ureter, veins, arteries, heart valves with or without their attached vessels, tissue surrounding the roots of developing teeth, and tissue surrounding growing bone.

24. The sterilized, acellular ECM composition of Claim 22, wherein the ECM composition is at least about 96% decellularized.


DNA ug/g dry weight
0
2
4
6
8
10
12
14
16
18
non-sterile lyo
EtO sterilized
60 min SCO2/PAA
20 min SCO2/PAA
raw SIS

FIGURE 1

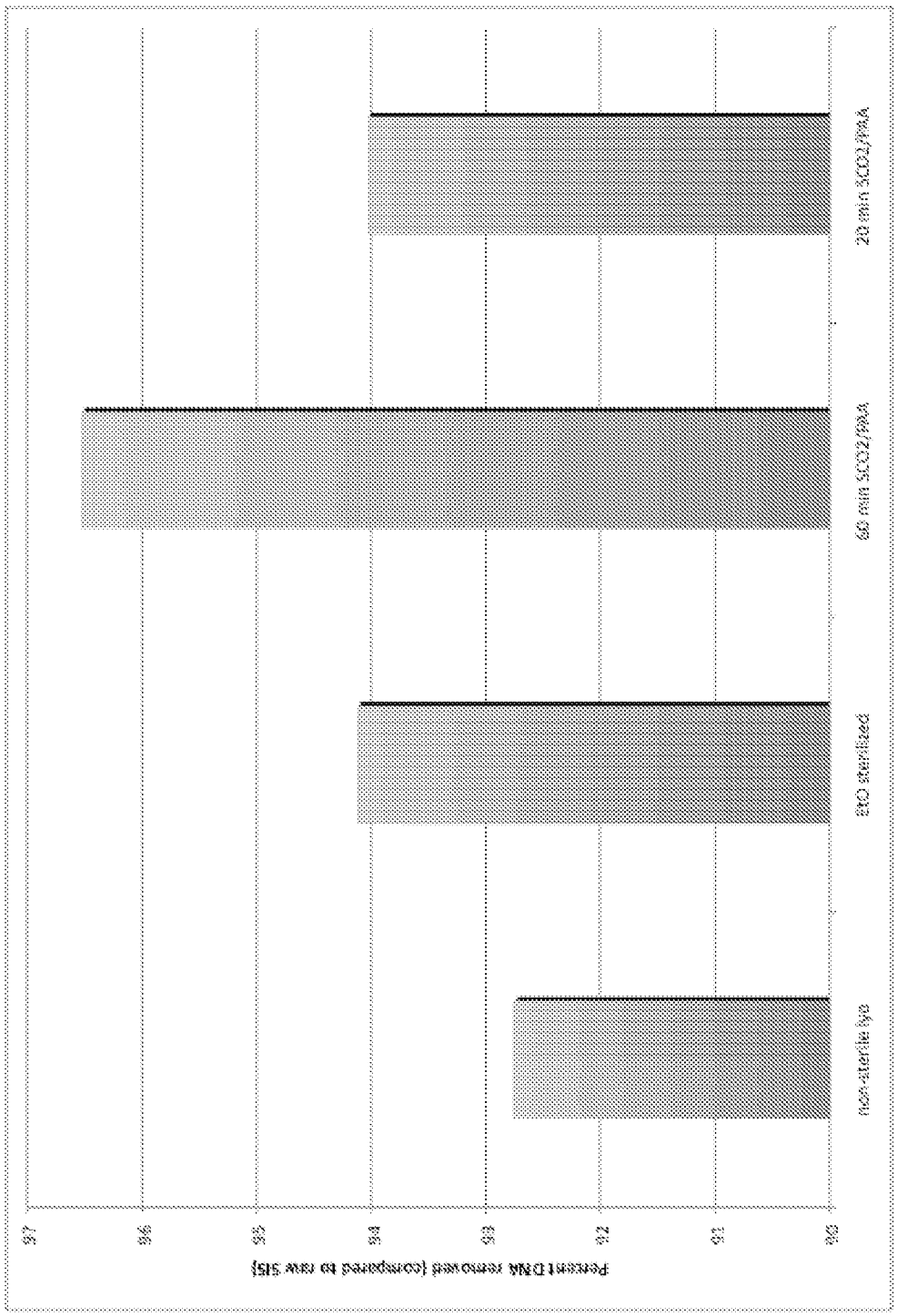
Percent DNA removed (compared to raw SIS)
97
96
95
94
93
92
91
90
non-sterile lyo
EtO sterilized
60 min SCO2/PAA
20 min SCO2/PAA

FIGURE 2

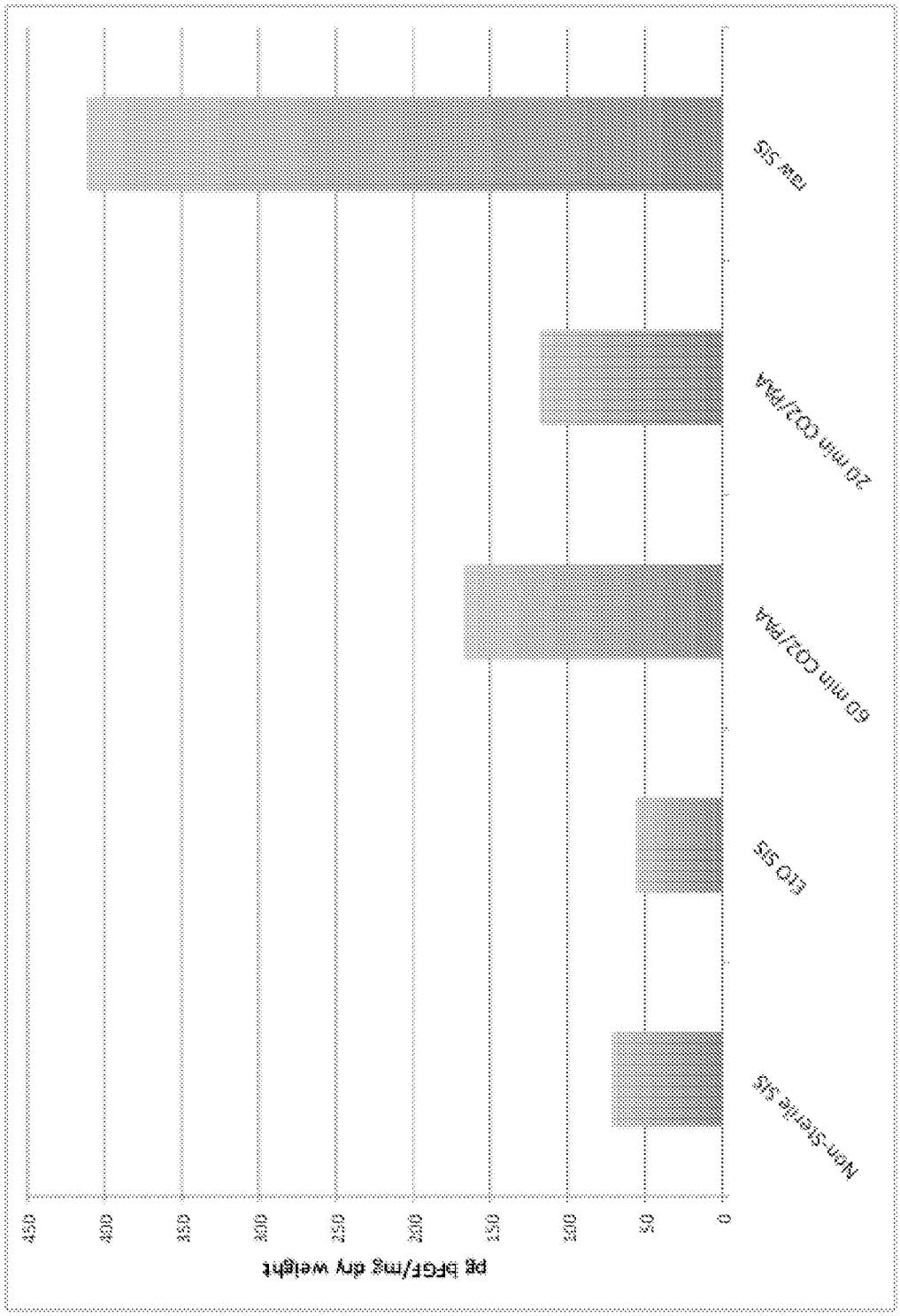
pg bFGF/mg dry weight
450
400
350
300
250
200
150
100
50
0
Non-Sterile SIS
ETO SIS
60 min CO2/PAA
20 min CO2/PAA
raw SIS

FIGURE 3

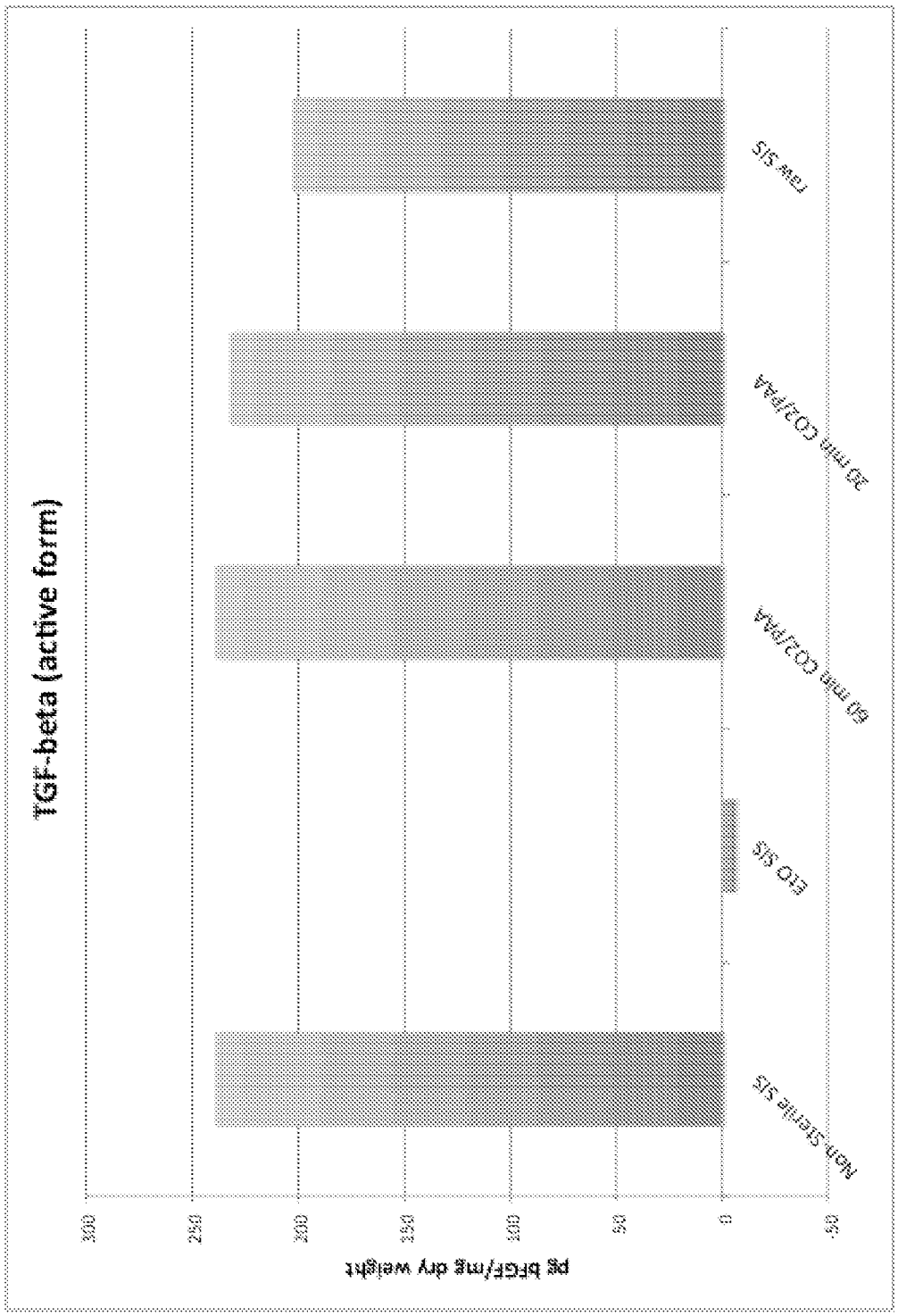
TGF-beta (active form)
pg bFGF/mg dry weight
300
250
200
150
100
50
0
-50
Non-Sterile SIS
ECO SIS
60 min CO2/PAA
30 min CO2/PAA
Raw SIS

FIGURE 4

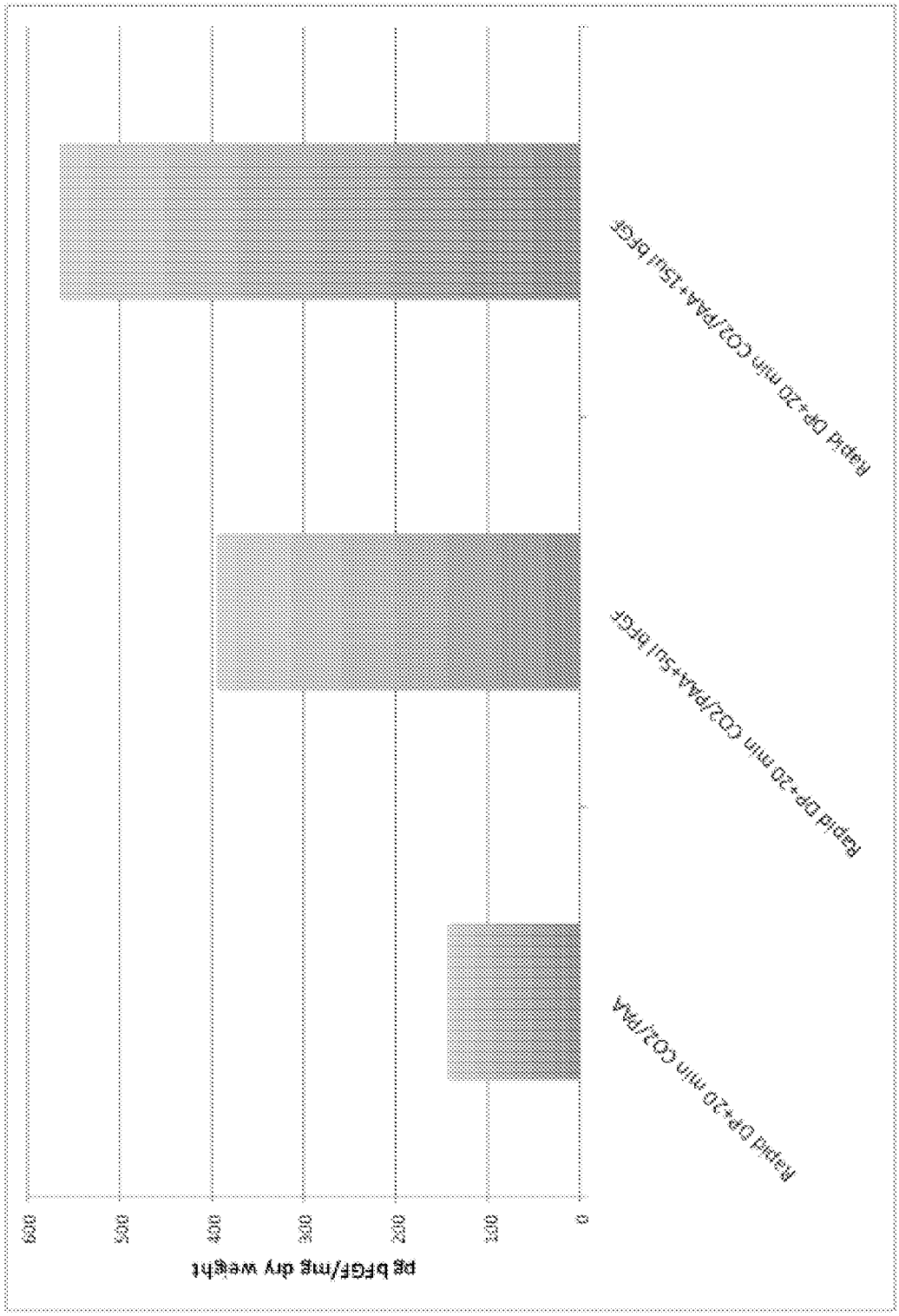
Rapid DP+20 min CO2/PAA
Rapid DP+20 min CO2/PAA+5U/ bFGF
Rapid DP+20 min CO2/PAA+15U/ bFGF
0
200
400
600
800
pg bFGF/mg dry weight

FIGURE 5

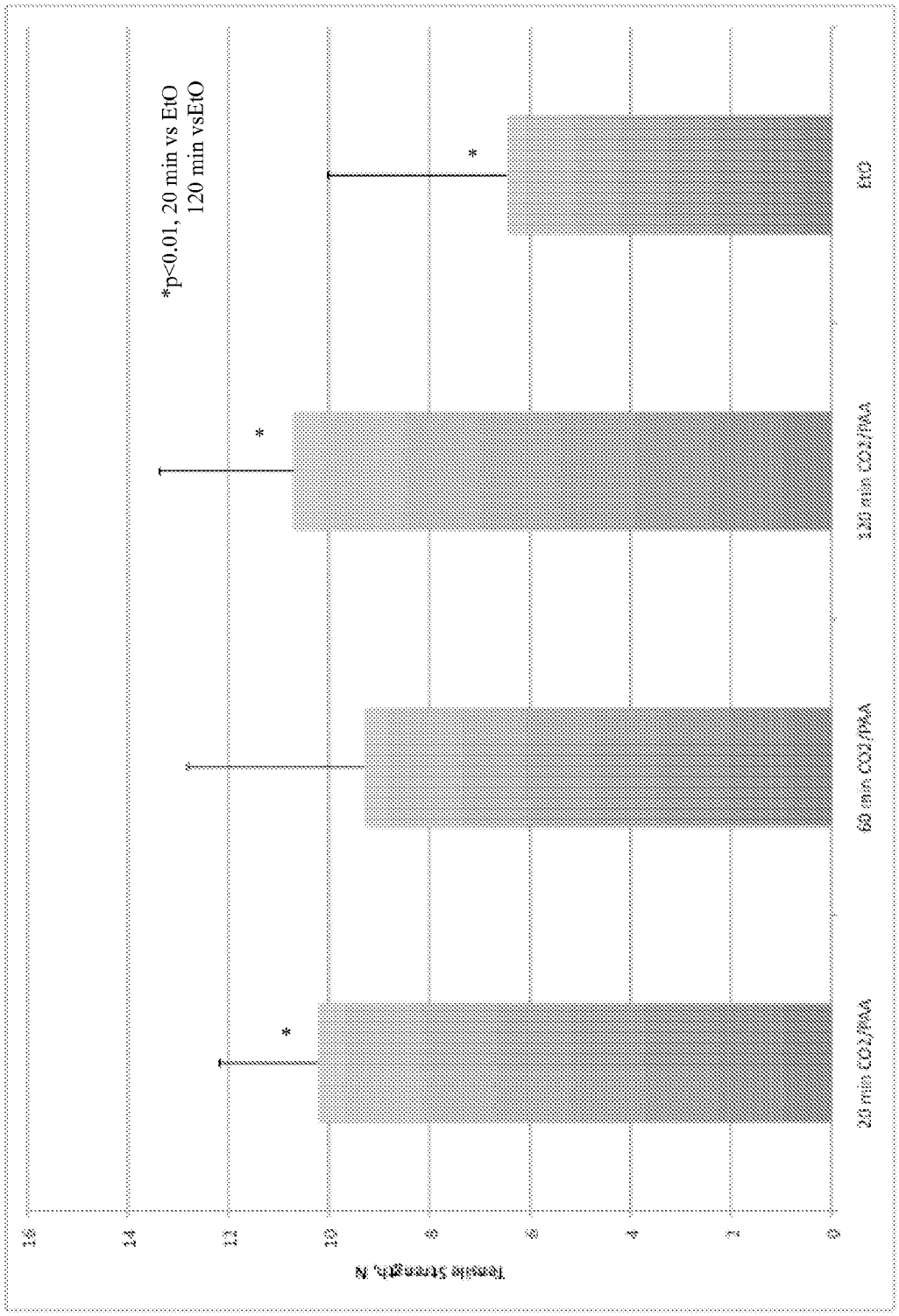
*p<0.01, 20 min vs EtO
120 min vsEtO
Tensile Strength, N
16
14
12
10
8
6
4
2
0
*
*
*
20 min CO2/PAA
60 min CO2/PAA
120 min CO2/PAA
EtO

FIGURE 6

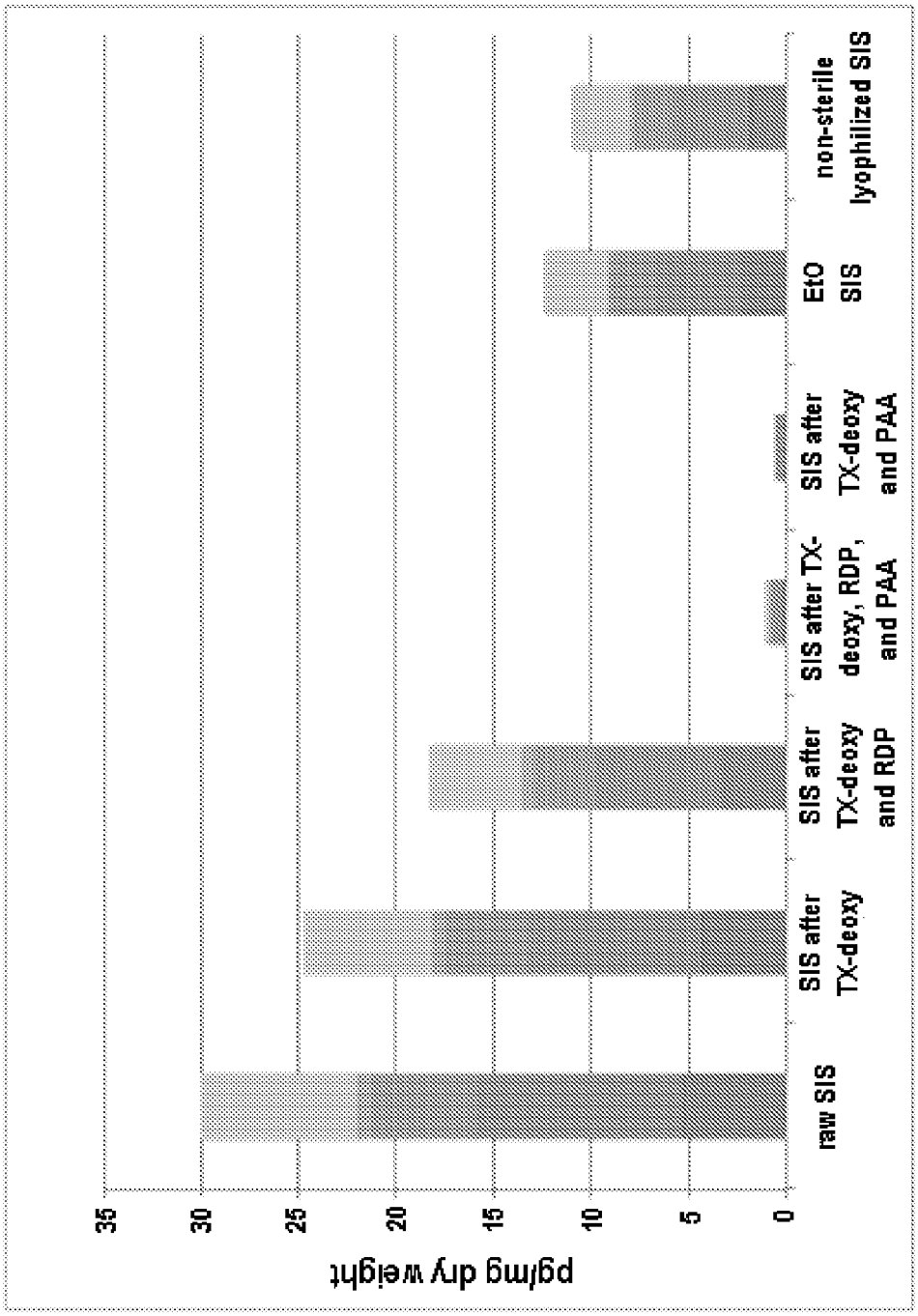
pg/mg dry weight
35
30
25
20
15
10
5
0
raw SIS
SIS after TX-deoxy
SIS after TX-deoxy and RDP
SIS after TX-deoxy, RDP, and PAA
SIS after TX-deoxy and PAA
EtO SIS
non-sterile lyophilized SIS

FIGURE 7

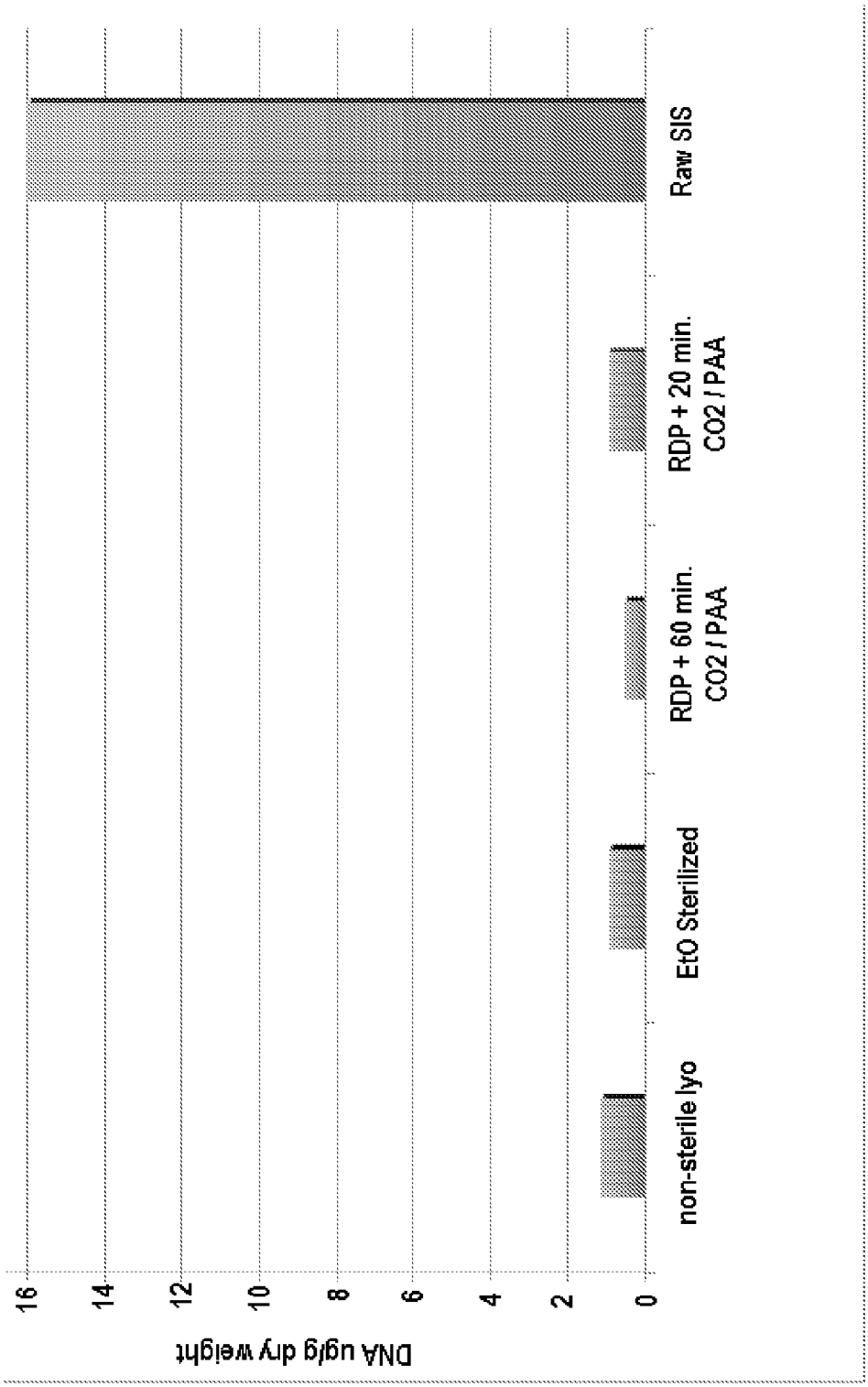
DNA ug/g dry weight
0
2
4
6
8
10
12
14
16
non-sterile lyo
EtO Sterilized
RDP + 60 min. CO2 / PAA
RDP + 20 min. CO2 / PAA
Raw SIS

FIGURE 8

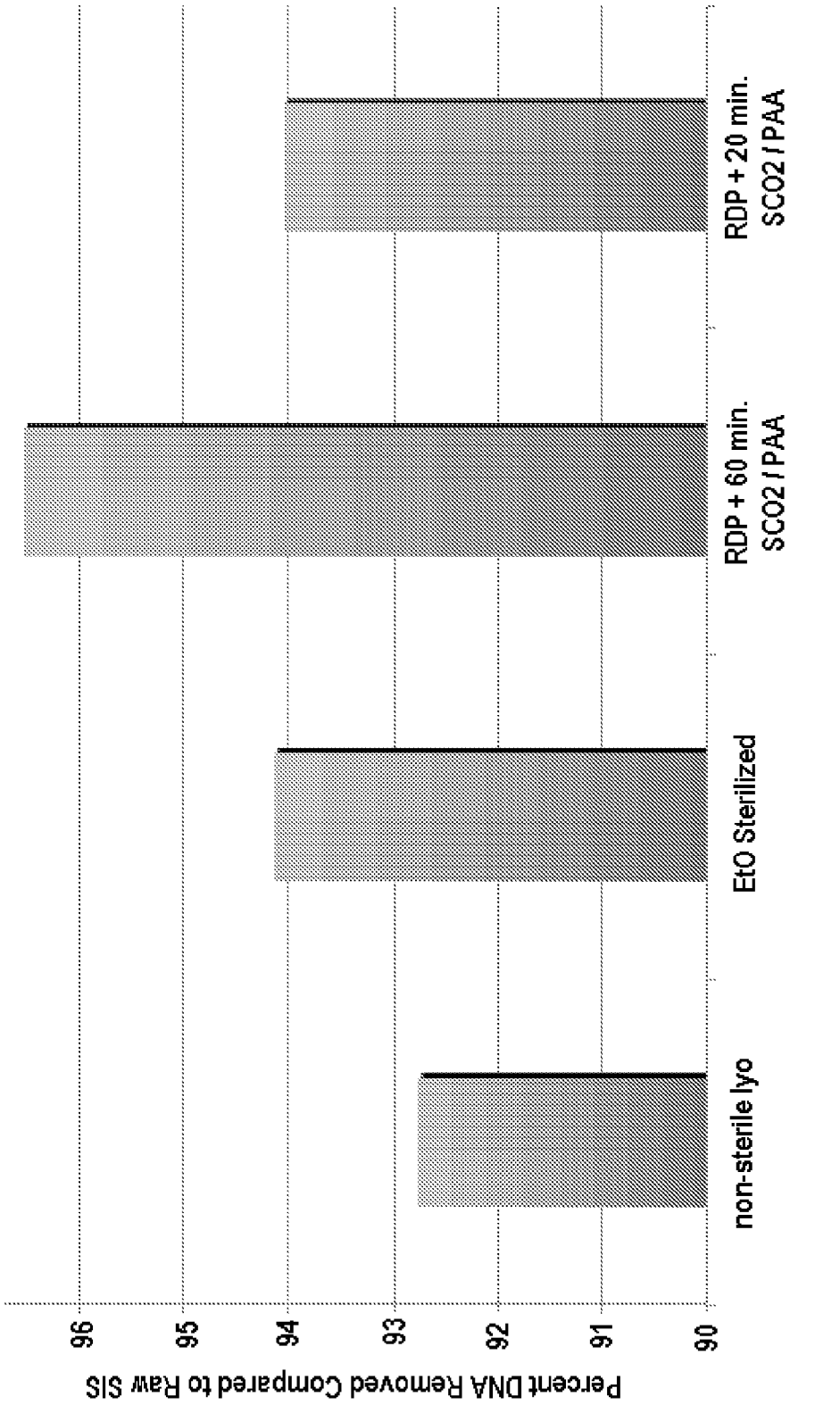
Percent DNA Removed Compared to Raw SIS
96
95
94
93
92
91
90
non-sterile lyo
EtO Sterilized
RDP + 60 min. SCO2 / PAA
RDP + 20 min. SCO2 / PAA

FIGURE 9

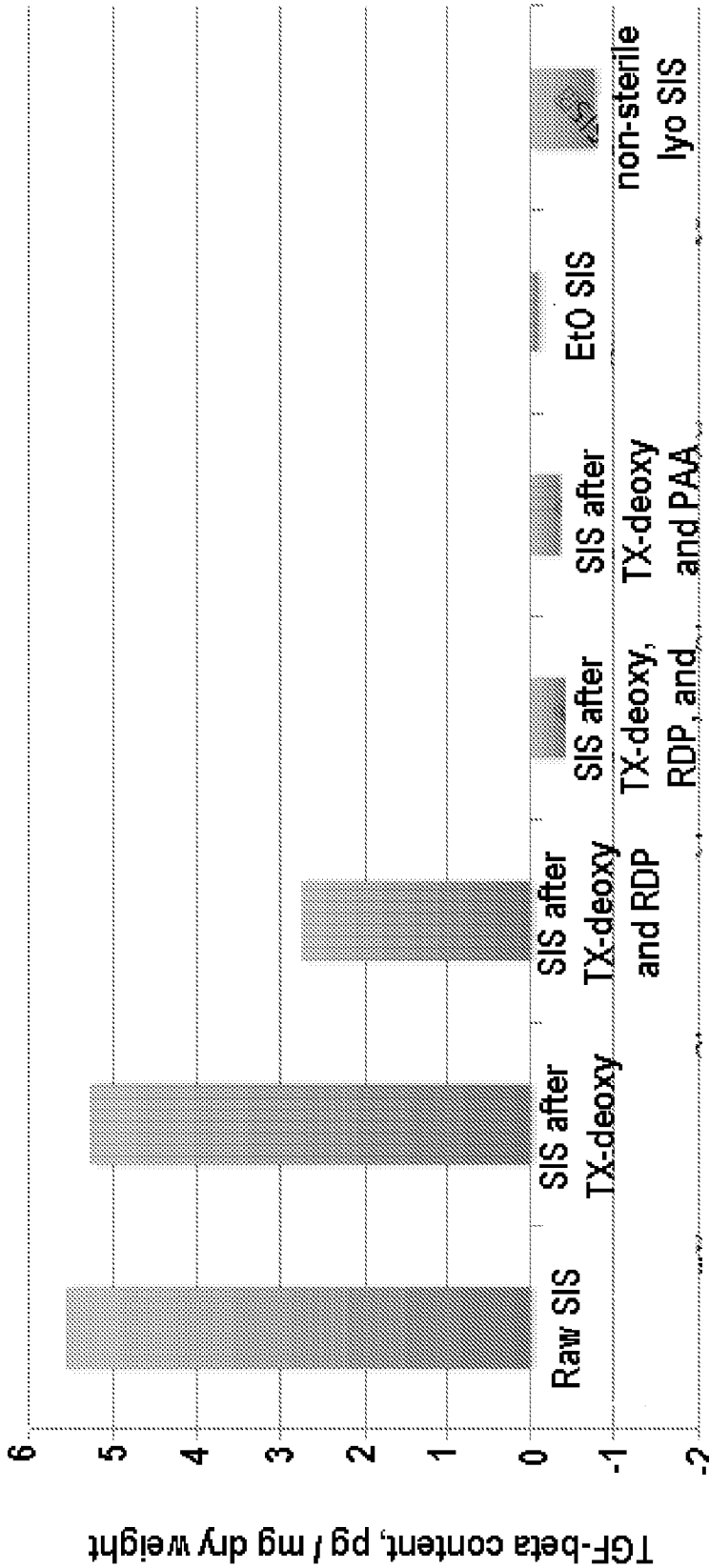
TGF-beta content, pg / mg dry weight
6
5
4
3
2
1
0
-1
-2
Raw SIS
SIS after TX-deoxy
SIS after TX-deoxy and RDP
SIS after TX-deoxy, RDP, and PAA
SIS after TX-deoxy and PAA
EtO SIS
non-sterile lyo SIS

FIGURE 10

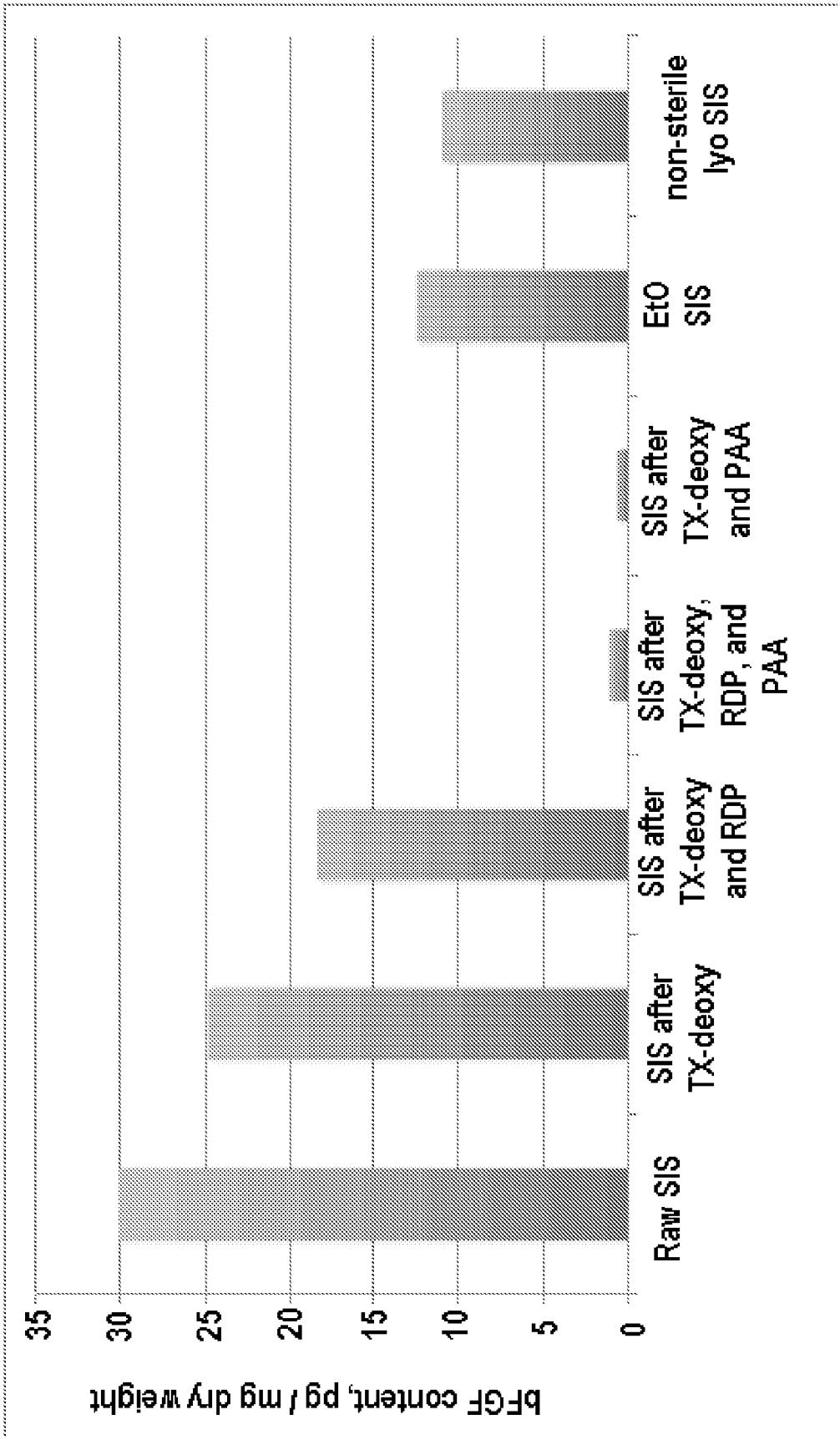
bFGF content, pg / mg dry weight
35
30
25
20
15
10
5
0
Raw SIS
SIS after TX-deoxy
SIS after TX-deoxy and RDP
SIS after TX-deoxy, RDP, and PAA
SIS after TX-deoxy and PAA
EtO SIS
non-sterile lyo SIS

FIGURE 11

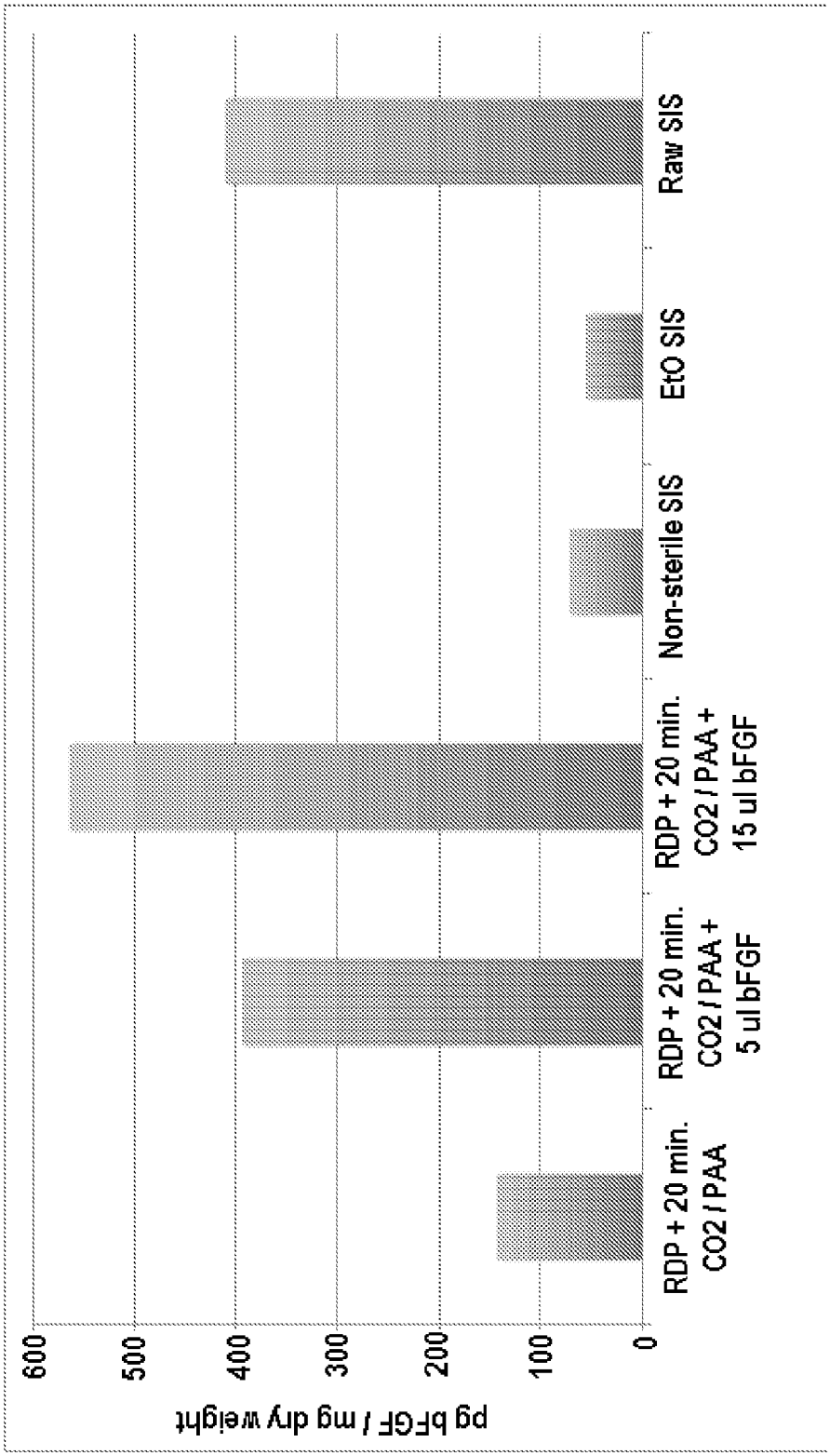
pg bFGF / mg dry weight
600
500
400
300
200
100
0
RDP + 20 min. CO2 / PAA
RDP + 20 min. CO2 / PAA + 5 ul bFGF
RDP + 20 min. CO2 / PAA + 15 ul bFGF
Non-sterile SIS
EtO SIS
Raw SIS

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US12/39413

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61L 2/00, 2/18, 2/20, 9/00 (2012.01)
USPC - 422/28, 31, 33; 516/9
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61L 2/00, 2/18, 2/20, 9/00 (2012.01)
USPC: 422/28, 31, 33; 516/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-Granted, US-Applications, EP-A, EP-B, WO, JP (bibliographic data only), DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; ScienceDirect; DialogPRO; supercritical, carbon dioxide, CO2, acellular, decellular, cell, remove, extracellular matrix, ECM, tissue, additive, decompress, depressurize

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAWADA, K, et al. Cell Removal With Supercritical Carbon Dioxide For Acellular Artificial Tissue. Journal of Chemical Technology and Biotechnology. 2008, Vol. 83, pp 943–949; page 944, column 1, paragraph 3; page 944, column 2, paragraph 3; page 945, figure 2, column 2, paragraphs 1-2; page 947, figure 6, column 1, paragraph 2; page 948, column 2, paragraph 2.	1-24
Y	US 2010/0080790 A1 (MATTHEWS, MA et al.) April 1, 2010, abstract, paragraphs [0035], [0053], [0077], [0125]	1-24
Y	WO 2011/031827 A2 (LIU, J-L et al.) March 17, 2011, page 3, line 14; page 7, lines 10-22; page 11, lines 21-31; page 13, lines 13-20; page 14, lines 3-5; page 15, lines 4-6; page 15, line 27; page 16, lines 27-31; page 17, lines 4-20; page 18, lines 5-7; page 22, lines 12-20	9-24
Y	US 5877005 A (CASTOR, TP et al.) March 2, 1999, column 9, lines 30-35	4, 12
Y	WO 2011/022369 A2 (KIBALO, B) February 24, 2011, paragraphs [0029]-[0030]	8, 24
Y	US 7771652 B2 (CHRISTOPHER, RA et al.) August 10, 2010, column 5, lines 29-46	21

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 2012 (20.08.2012)	06 SEP 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)