Sterilization and disinfection methods for decellularized matrix materials: Review, consideration and proposal

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ABSTRACT

Sterilization is the process of killing all microorganisms, while disinfection is the process of killing or removing all kinds of pathogenic microorganisms except bacterial spores. Biomaterials involved in cell experiments, animal experiments, and clinical applications need to be in the aseptic state, but their physical and chemical properties as well as biological activities can be affected by sterilization or disinfection. Decellularized matrix (dECM) is the low immunogenicity material obtained by removing cells from tissues, which retains many inherent components in tissues such as proteins and proteoglycans. But there are few studies concerning the effects of sterilization or disinfection on dECM, and the systematic introduction of sterilization or disinfection for dECM is even less. Therefore, this review systematically introduces and analyzes the mechanism, advantages, disadvantages, and applications of various sterilization and disinfection methods, discusses the factors influencing the selection of sterilization and disinfection methods, summarizes the sterilization and disinfection methods for various common dECM, and finally proposes a graphical route for selecting an appropriate sterilization or disinfection method for dECM and a technical route for validating the selected method, so as to provide the reference and basis for choosing more appropriate sterilization or disinfection methods of various dECM.

1. Introduction

Sterilization is the process of killing or removing all microorganisms, including bacterial spores [1]. Disinfection is the process of killing or removing all kinds of pathogenic microorganisms except bacterial spores [2]. Asepsis is no living microorganism in the object, which is the premise for biomaterials to be used in cell experiments, animal experiments and clinical applications. Implants such as artificial blood vessels and heart valves must be sterilized before clinical applications [3]. The most common sterilization methods are irradiation and ethylene oxide (EO). However, if biomaterials are only used in experimental research rather than clinical application, and the aseptic state of biomaterials meets the needs of cell experiments and animal experiments, there are many other sterilization and disinfection methods, such as peracetic acid, alcohol, and ultraviolet, etc., could be used besides irradiation and

EO. The sterilization and disinfection often affect the physic-chemical properties and biological activity of biomaterials [4–6], for example high temperature [7] or high-dose irradiation [8,9] can induce protein denaturation [2], remained EO is toxic, etc.

Decellularized matrix (dECM) is a low immunogenicity scaffold material obtained by removing cells from tissues with physical or chemical methods. DECM is a complex network of macromolecules interacting with each other and has complex components. It retains many inherent components in tissues, such as collagen, elastin, proteoglycan, hyaluronic acid and other macromolecules, as well as growth factors [10]. With its complex composition and structure close to natural tissues, good biocompatibility and low immunogenicity, dECM has gradually become a research hotspot of biomaterials in the past two decades, such as decellularized pericardium [11], decellularized blood vessels [12], decellularized corneas [13], decellularized bone [14],
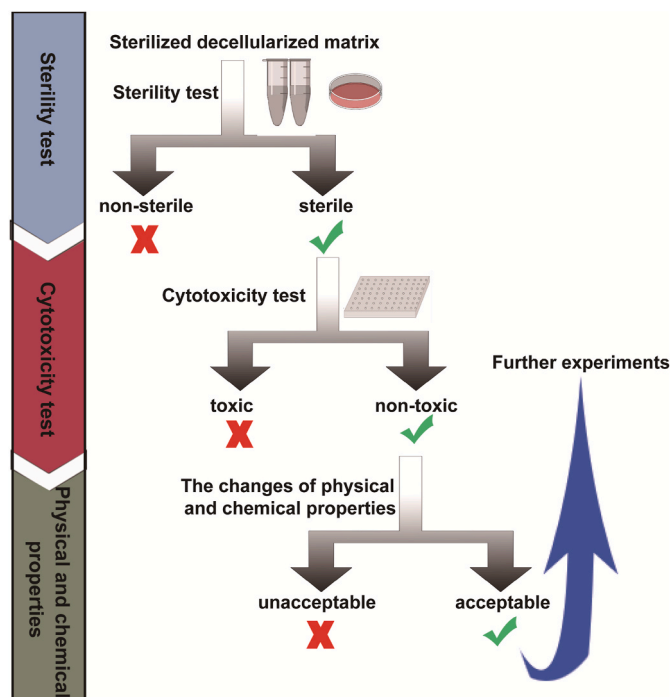
Fig. 1. The technical route for validating the sterilization or disinfection method for dECM.

decellularized lung [15], and decellularized nerve [10].

There are various final states of dECM. Some decellularized matrix materials maintain the natural morphology of tissues, such as decellularized trachea prepared by M Den Hondt [16] and decellularized kidney prepared by Jeremy J Song [17]. Some decellularized matrix materials are further processed to obtain new forms, such as decellularized pericardial matrix hydrogel [18], decellularized perivascular matrix hydrogel [19], bovine cancellous bone granules [20], and human placental derived extracellular matrix sponge [21], as well as

decellularized matrix as coating for 3D printing polycaprolactone (PCL) tubes [22].

The ideal sterilization or disinfection for dECM can not only effectively remove microorganisms, but also ensure that the sterilized material is non-toxic, and maintain the physical and chemical properties and biological activity of the biomaterial. Therefore, the selection of sterilization or disinfection method is very important. The technical route to verify an appropriate sterilization or disinfection method for dECM is as follows. First of all, to determine whether the dECM is sterile with the sterility test; after effective sterilization or disinfection, to test whether there are toxic and harmful substances in the biomaterials with the cytotoxicity test; finally, after ensuring the materials are non-toxic and harmless, to evaluate whether the changes of physical and chemical properties of biomaterials after sterilization or disinfection affect the desired function of dECM, such as whether the materials used to promote tissue repair effectively retain growth factors, and whether the membranes used to wrap wounds have appropriate hardness and elasticity. After the above conditions are satisfied, the sterilized dECM can be used for further experiments and applications (Fig. 1).

Although being in the aseptic state is very important for dECM, there are few studies concerning the effects of sterilization or disinfection on dECM, and the systematic introduction to the sterilization or disinfection for dECM is even less.

In the present paper, the mechanism, advantages, disadvantages and applications of various sterilization or disinfection methods are systematically introduced and analyzed, then the factors influencing the selection of sterilization and disinfection methods are discussed, and the sterilization and disinfection methods for various common dECM are summarized. Finally, the graphical route for selecting the sterilization or disinfection method for dECM is proposed. This review aims to provide the reference basis for choosing appropriate sterilization or disinfection methods of various dECM.

2. Sterilization and disinfection methods of dECM

2.1. Irradiation

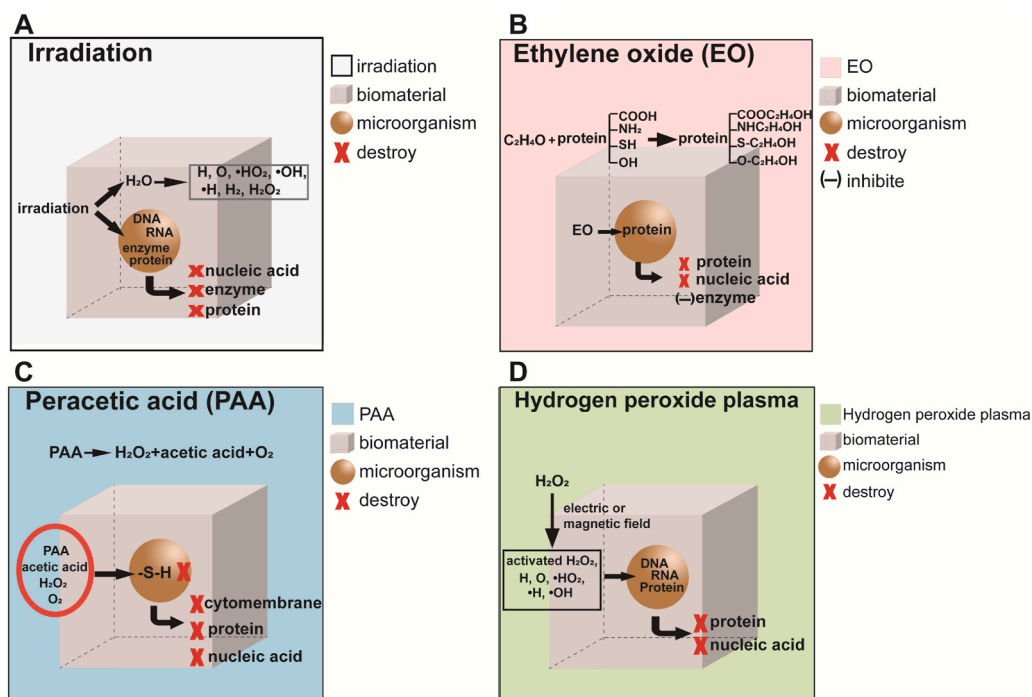Irradiation is a physical sterilization method. The common

Fig. 2. Sterilization and disinfection mechanisms of irradiation (A), ethylene oxide (B), peracetic acid (C), and hydrogen peroxide plasma (D).
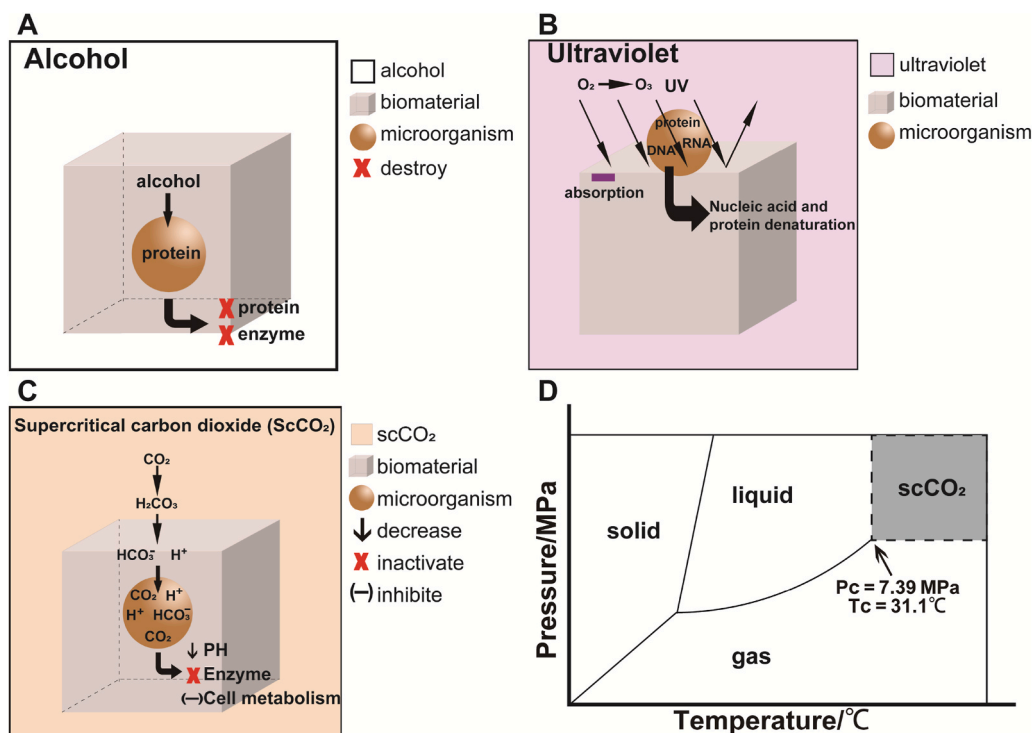
Fig. 3. Sterilization and disinfection mechanisms of alcohol (A), ultraviolet (B), and supercritical carbon dioxide (C). Three-phase diagram of supercritical carbon dioxide (D).

Table 1
Summary of sterilization and disinfection methods of dECM.

Methods	Sterilization/Disinfection	Advantages	Disadvantages	Time required and accessibility
Irradiation	sterilization	- no residual toxicity - strong penetrability	- changes the physical and chemical properties, and bioactivity	- hours to days - require radiation devices
EO	sterilization	- strong penetrability - less damage	- toxic - react with water or chlorine to produce toxic substances - require EO residue test	- several weeks - require EO devices
PAA	disinfection; sterilization (under certain conditions)	- nontoxic decomposition products	- strong oxidation and acidity	- dozens of minutes - soaking in PAA solution
H_2O_2	disinfection; sterilization (under certain conditions)	- nontoxic decomposition products	- strong oxidation	- dozens of minutes - soaking in H_2O_2 solution
HPLP	sterilization	- nontoxic decomposition products	- strong oxidation - poor permeability	- dozens of minutes - require HPLP devices
Alcohol	disinfection	- less damage to structure	- no killing effect on bacterial spores - decrease content of collagen	- dozens of minutes - soaking in alcohol solution
UV	disinfection	- simple and convenient	- weak penetrability. - not suitable for deep disinfection	- dozens of minutes - require UV devices
$ScCO_2$	disinfection/sterilization (uncertain)	- no toxicity and residue - decellularization effect	- rare research on its disadvantages - may affect the physical and chemical properties	- dozens of minutes -require $ScCO_2$ devices

irradiation sterilization mainly includes gamma ray (GI) produced by ^{60}Co device and electron beam (E-beam) produced by electron accelerator. Comparing between them, the penetrability of gamma ray is stronger than that of electron beam, and gamma ray is more often used for sterilization of biomaterials [2]. According to the different dose rate of radiation source, the required time of radiation sterilization is different, and usually the sterilization can be completed within hours to days.

The effect of irradiation on materials is mainly related to radiation dose [23,24]. For the same material, at relatively low dose, radiation-induced crosslinking increases tensile strength and stiffness of dECM; while at relatively high dose, tissue denaturation and degradation decrease tensile strength and stiffness [24]. Moreover, due to the

differences in shape and size of various biomaterials, the appropriate dose is set according to the specific conditions.

In addition to the radiation dose, the gas state around the materials (such as nitrogen, oxygen, or air) [25,26] and water content [27] may affect the effectiveness of irradiation sterilization and the physic-chemical properties of materials. However, the comparative studies about the effects of above factors on the irradiation sterilization of dECM have not been found, which need to be further studied.

Sterilization mechanism: Irradiation directly destroys the nucleic acids, proteins and enzymes of microorganisms. At the same time, the water molecules within the organism are radiated to produce peroxides and free radicals, which destroy the nucleic acids, enzymes and proteins of the microorganisms and make the microorganisms lose their
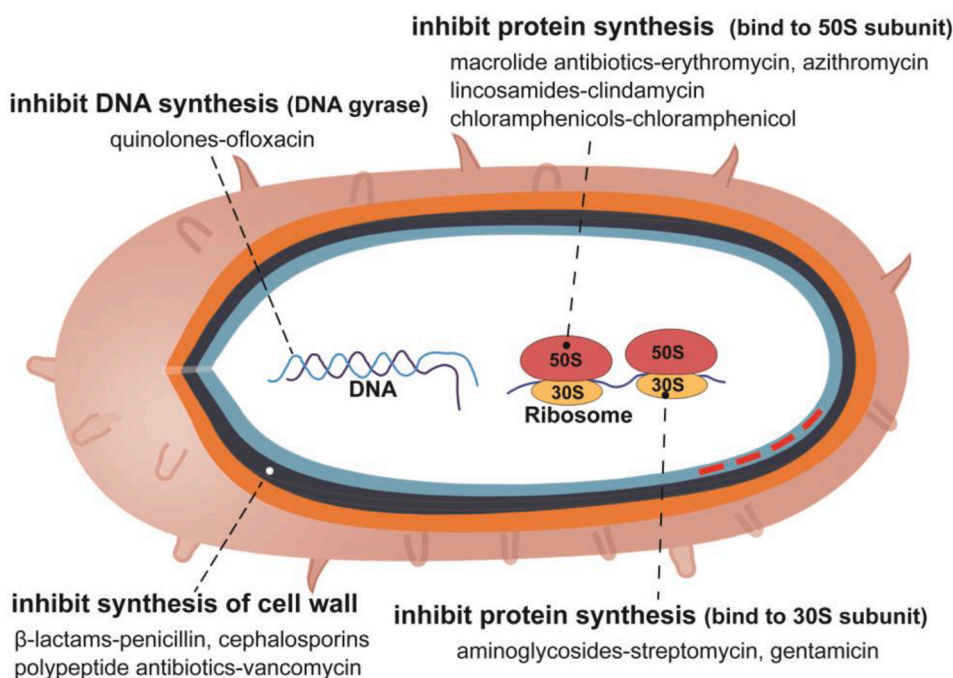
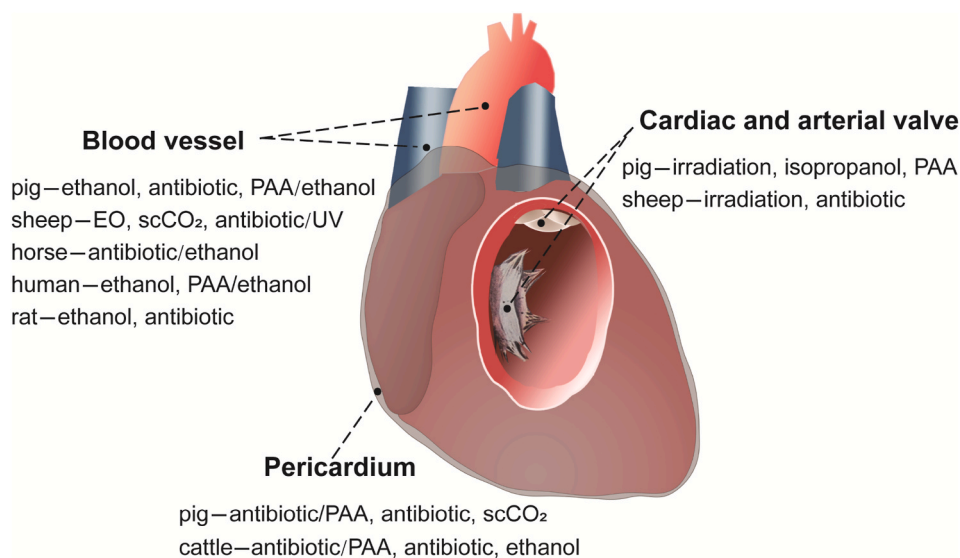**Fig. 4.** The action mechanism of antibiotics.

Fig. 5. Summary of sterilization and disinfection methods for decellularized pericardium, valves, and blood vessels.

metabolic function [2,28] (Fig. 2A).

Advantages: Irradiation sterilization is carried out at room temperature, with no residual toxicity, and its strong permeability could result in thorough sterilization.

Disadvantages: Irradiation may lead to changes in physical and chemical properties and biocompatibility, such as protein denaturation, and changes in material color and mechanical strength [29].

Applications: Irradiation is a widely used sterilization method. It is often used for sterilization of disposable medical supplies, drugs, food and daily necessities. The dECM sterilized by irradiation mentioned in the existing literature are mainly derived from valve [30], blood vessel [31], liver [32], stomach [33], pancreas [34,35], esophagus [36], small intestine [37,38], lung [15,39], trachea [40–42], bladder [43–46], kidney [23,47,48], bone [14,49–53], tendon [27,54–56] and nerve [57–59].

2.2. Ethylene oxide

Ethylene oxide (EO) sterilization is a mature sterilization method. EO is a toxic organic compound. It is a colorless and transparent liquid below 10.8 °C and a colorless gas with ether-like pungent odor at room temperature. Because dECM has the strong adsorption capacity for EO, it is necessary to extend the time of removing EO residue after sterilization, so EO sterilization often takes several weeks.

Sterilization mechanism: EO can alkylate with sulfhydryl, amino and carboxyl groups in proteins and nucleic acid molecules, which make these macromolecules of microorganisms lose their activity and result in sterilization [60]. For example, EO can inhibit the activities of various enzymes, hinder the normal metabolism and then lead to the death of microorganisms [2] (Fig. 2B).

Advantages: EO sterilization is carried out at room temperature

Table 2

Summary of sterilization and disinfection methods for decellularized pericardium, valves and blood vessels.

Tissue type	Origins	Methods	Appearance and Size	Details and References
Pericardium	pig	antibiotic/PAA	piece (12 mm diameter)	antibiotic, 37 °C, 24 h. Then, 0.05% (v/v) or 0.1% (v/v) PAA (pH 7.3), 27 °C, 3 h [182]
		antibiotic	overall shape	antibiotic, 4 °C, 72 h [160]
		ScCO$_2$	overall shape	ScCO$_2$ [149]
	bovine	antibiotic/PAA	piece (12 mm diameter)	antibiotic, 37 °C, 24 h. Then, 0.05% (v/v) or 0.1% (v/v) PAA (pH 7.3), 27 °C, 3 h [182]
		antibiotic	piece (100 mm × 100 mm)	antibiotic [151]
			overall shape	antibiotic, 4 °C, 72 h [160]
		ethanol	overall shape	ethanol, 20%–75% (v/v), 4 h [11]
Blood vessel	pig	ethanol	segment	80% (v/v) ethanol, 3 days [121]
		antibiotic	segment	antibiotic, 4 °C [161]
		PAA/ethanol	aortic adventitia	0.1% (v/v) PAA in 4% (v/v) ethanol [19]
	sheep	EO	segment (60 mm length, 3–4 mm diameter)	EO, room temperature [62]
		ScCO$_2$	segment (15 mm length, 10 mm diameter)	ScCO$_2$, 1 h [148]
		antibiotic/UV	segment (10 mm × 40 mm)	antibiotic, 10 min. Then, UV, 30 min [146]
	horse	antibiotic/ethanol	segment	antibiotic. Then, 70% (v/v) ethanol, 20 min [183]
	human	ethanol	segment	70% (v/v) ethanol [125]
		PAA/ethanol	segment	0.1% (v/v) PAA in 4% (v/v) ethanol [19]
	rat	ethanol	segment (30–40 mm length)	ethanol [126]
		antibiotic	segment	antibiotic [162]
Cardiac and arterial valves	pig	irradiation	overall shape	gamma irradiation, 1.5 kGy and 3 kGy [30]
			overall shape	gamma irradiation, 0.15 kGy [31]
		isopropanol	overall shape	10% (v/v) isopropanol [123]
		PAA	overall shape	0.1% (v/v) PAA, 37 °C, 3 h [84]
	sheep	irradiation	overall shape	gamma irradiation, 0.15 kGy [31]
		antibiotic	overall shape	antibiotic [152]

which has strong permeability and cause no damage to most of materials.

Disadvantages:

(1) EO is toxic and its residues cause adverse reactions [61]. Therefore, EO is not recommended for sterilization of materials which are easy to absorb EO such as loose and porous lyophilized decellularized matrix materials, unless EO can be effectively removed.

(2) EO is easily soluble in water to form toxic ethylene glycol and harmful chloroethanol is produced in the presence of chlorine. Therefore, the materials containing water and chlorine should not be sterilized with EO.

Applications: EO is usually used in sterilization of medical devices, including high polymer materials such as catheters of medical instruments, instruments with high sharpness, etc. The dECM materials sterilized by EO mentioned in the existing literature are mainly derived from blood vessel [62], stomach [63], esophagus [64,65], bladder [66–75], kidney [76], bone [77] and tendon [78–80].

2.3. Peroxide

Peroxide is a strong oxidant containing the peroxy group "-O-O-" in the chemical molecular structure. Common peroxides include peracetic acid and hydrogen peroxide.

2.3.1. Peracetic acid

Peracetic acid (PAA) is a common disinfectant, but it can achieve sterilization effect under certain conditions, so it is also used as a chemical sterilization agent. For example, 1% PAA can kill the spores of *Bacillus anthracis* in 30 min [81]. It is an organic peroxide with strong oxygenation. The pH value of PAA is less than 5. The stronger the acidity, the better is the sterilization effect. In addition, the experiment of Jason Hodde showed that exposure to 0.18% (v/v) PAA/4.8% (v/v) ethanol aqueous solution for more than 0.5 h could effectively inactivate the virus of porcine small intestine [82]. The sterilization and disinfection of PAA is usually to soak the material in PAA solution for dozens of minutes [4].

Sterilization and disinfection mechanism: Peracetic acid is produced by the reaction of hydrogen peroxide and acetic acid, which has poor stability and is easy to decompose. The strong oxidation of PAA and the synergistic effect of hydrogen peroxide with acetic acid destroy the cytoderm of microorganisms, oxidize the sulfhydryl (-S-H) in proteins and enzymes, and destroy the enzyme system [2,83] (Fig. 2C).

Advantages: The decomposition products of PAA are acetic acid, water and oxygen, which are non-toxic.

Disadvantages: PAA has strong oxidation and acidity which may affect the physical and chemical properties of some materials. For example, the young's modulus of the decellularized porcine bladder sterilized by PAA was decreased [66]; sterilization of the decellularized small intestine with PAA resulted in the fraying and flattening of the edge at the villous portions [5].

Applications: PAA is mainly used for sterilization and disinfection of non-metal objects and environment. The dECM materials sterilized by PAA mentioned in the existing literature are mainly derived from valve [84], liver [85,86], small intestine [5,87,88], spleen [89,90], lung [91], bladder [66,92,93], kidney [48,94], tendon [95,96] and nerve [97]. In addition, the decellularized matrix materials sterilized with PAA/ethanol solution are mainly derived from blood vessel [19], esophagus [65, 98], liver [4,99], small intestine [38,100], trachea [40,42,101], lung [15,102,103], bladder [104–109], kidney [48,110], spleen [111–113] and nerve [114].

2.3.2. Hydrogen peroxide and hydrogen peroxide low-temperature plasma

Hydrogen peroxide (H_2O_2) is a strong oxidant, and usually used as a disinfectant. Under certain conditions, hydrogen peroxide can also kill bacterial spores to achieve sterilization effect, for example exposure to liquid H_2O_2 at 30 °C for 20 min can effectively inactivate the *Bacillus anthracis* spores [115]. Hydrogen peroxide low-temperature plasma (HPLP) sterilization is a commonly used low-temperature sterilization method in hospitals. In HPLP devices, the working temperature of sterilization is generally 45–55 °C and does not exceed 60 °C. Both H_2O_2 and HPLP need dozens of minutes to achieve the effects of disinfection or sterilization [116].
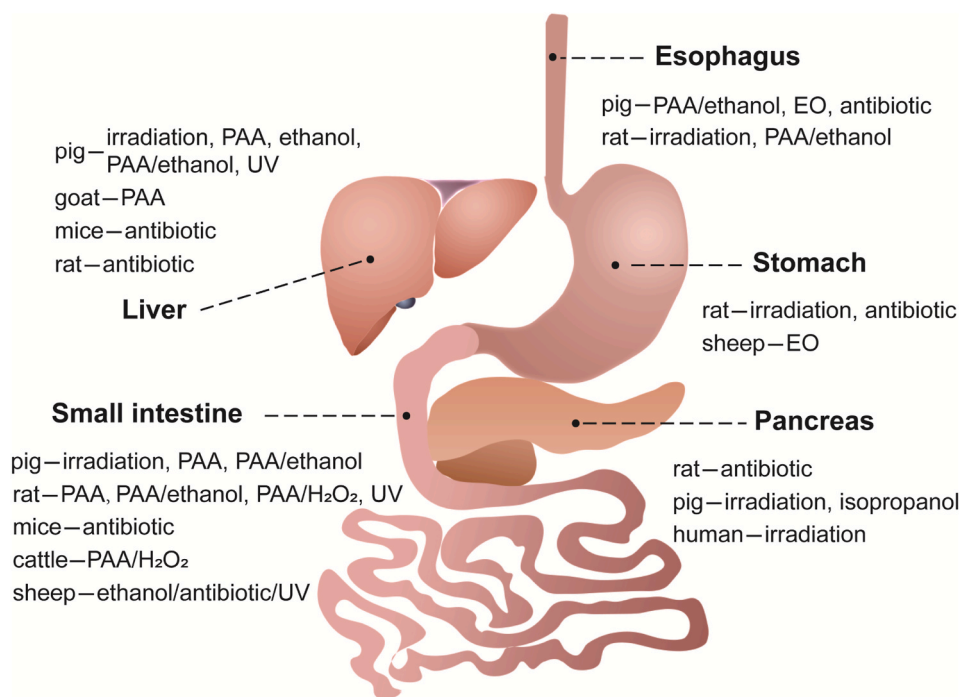
Fig. 6. Summary of sterilization and disinfection methods for decellularized tissues and organs in digestive system.

Sterilization and disinfection mechanism: H_2O_2 and its active derivatives produced after decomposition have strong oxidation effects which directly cause the destruction of cytomembrane and the denaturation of nucleic acids and proteins [117].

The sterilization mechanism of HPLP is that H_2O_2 generates a large number of active oxygen and high-energy free radicals through magnetic field excitation, such as hydroxyl radical (•OH), hydroxyl peroxide radical (•HO$_2$), activated oxygen atom (O), activated hydrogen atom (H) and activated H_2O_2 molecule, which can denature nucleic acids and proteins in microorganisms and destroy the balance of potential inside and outside of microbial cytomembrane [2] (Fig. 2D).

Advantages: Both of H_2O_2 and HPLP can be decomposed into nontoxic water and oxygen.

Disadvantages:

(1) The most serious problem of H_2O_2 and HPLP is that H_2O_2 can produce oxygen under the catalysis of catalase *in vivo* which is easy to cause gas embolism [118]. Therefore, whether they are suitable for application in the sterilization of dECM needs further exploration. It is suggested that H_2O_2 residue detection should be carried out before further experiments.
(2) They have strong oxidation which may affect the physical and chemical properties of some materials, such as changes in the tertiary structure of proteins [119].
(3) In addition, because HPLP sterilization needs to be in vacuum and has poor permeability, the object material is required to be in a dry state. Moreover, HPLP is not suitable for the sterilization of materials which cannot bear vacuum or with one end blocked.

Applications: H_2O_2 and HPLP are mainly used for the disinfection and sterilization of nonmetal objects and environment, but rarely used for disinfection and sterilization of dECM. Occasionally, H_2O_2 was used in combination with other sterilization methods in dECM sterilization [120].

2.4. Alcohol

Alcohol is a common disinfectant, which cannot kill spores. And the most commonly used alcohol disinfectants are ethanol and isopropanol. Alcohol disinfection method is to soak the materials in alcohol solution for dozens of minutes [11,121].

Disinfection mechanism: Alcohols can denature protein and destroy the enzyme system of microorganisms (Fig. 3A).

Advantages: Alcohol has less damage to the tissue structure of dECM materials. For example, after 1 h of alcohol disinfection, the structure of renal tubule and glomerulus of pig kidney had no obvious change [48].

Disadvantages: Alcohol has no killing effect on bacterial spores and can only be used for disinfection, but not for sterilization [122]. And alcohol can denature protein and significantly decrease the content of collagen [4].

Applications: Alcohol is commonly used in disinfection of skin and precision instruments, etc. There are many reports on the use of alcohol to disinfect dECM, which are mainly derived from pericardium [11], valve [123,124], blood vessel [12,121,125,126], liver [127–129], pancreas [130], trachea [131], bladder [130,132–135], kidney [48, 136], bone [137,138], tendon [139–141] and nerve [142].

2.5. Ultraviolet ray

Ultraviolet ray (UV) is mainly used for physical disinfection. Generally, the UV wavelength is 200–300 nm and the strongest disinfection effect is at 253.7 nm for dozens of minutes. UV is transmitted along a straight line and can be reflected or absorbed by the surface of an object. The penetrability of UV is weak, but it is easy to penetrate air and pure water.

Disinfection mechanism: UV can not only denature nucleic acids and proteins directly, but also make oxygen in the air produce ozone, and ozone also has bactericidal effects. Therefore, UV could complete disinfection together with ozone [28,143] (Fig. 3B).

Advantages: UV is a simple and easy method to disinfect the surface and environment.

Disadvantages: Because of weak penetrability of UV, it is not suitable for liquid medicine disinfection and internal disinfection of solid objects.

Applications: UV ray is mainly used for the disinfection of object surface and environment. The dECM materials disinfected with UV

Table 3

Summary of sterilization and disinfection methods for decellularized tissues and organs in digestive system.

Tissue type	Origins	Methods	Appearance and Size	Details and References
Liver	pig	irradiation	slice (1 mm thick)	(placed in PBS) gamma irradiation, 10 kGy [32]
		PAA	powder (<250 μm)	0.1% (v/v) PAA, 2 h [85]
			slice (14 mm diameter, 3 mm thick)	0.1% (v/v) PAA, 2 h [184]
			slice (25 mm × 15 mm × 2 mm)	0.1% (v/v) PAA [4]
		PAA/ ethanol	slice (3 mm thick)	0.1% (v/v) PAA in 4% (v/v) ethanol for 2 h [99]
		ethanol	powder	70% (v/v) ethanol [127,128]
			slice (5 mm × 5 mm × 5 mm)	70% (v/v) ethanol [129]
		UV	slice (4 mm diameter, 0.25 mm thick)	UV, 2 h [144]
	goat	PAA	slice (5 mm × 5 mm × 2 mm)	lyophilized, PAA, 2–3 h [86]
	mice	antibiotic	overall shape	antibiotic [163]
	rat	antibiotic	overall shape	antibiotic [164]
Small intestine	pig	irradiation	segment (10 cm length)	lyophilized, gamma irradiation, 25 kGy [38]
			segment	gamma irradiation, 0.15 kGy [185]
		EO	flocculent piece	EO [100]
		PAA	segment (30 cm length)	0.1% (v/v) PAA, room temperature, 3 h [87]
	rat	PAA	segment	0.1% (v/v) PAA, 3 h [5,88]
		PAA/ ethanol	segment	0.18% (v/v) PAA with 4.8% (v/v) ethanol, 30 min [5]
		PAA/H_2O_2	segment	0.08% (v/v) PAA with 1% (v/v) H_2O_2, 3 h [5]
		UV	segment	UV at 254 nm, 90 s [5]
	mice	antibiotic	segment	antibiotic [168]
	bovine	PAA/H_2O_2	segment (10 cm length)	0.15% (v/v) PAA in 20% (v/v) H_2O_2, 16 h [120]
Esophagus	pig	EO	segment	EO [65]
		antibiotic	segment	antibiotic, overnight [167]
	rat	irradiation	segment	gamma irradiation [36]
		PAA/ ethanol	segment	0.1% (v/v) PAA in a 4% (v/v) ethanol, 4 °C, 24 h [98]
Stomach	rat	irradiation	overall shape	gamma irradiation, 12 kGy [33]
		antibiotic	overall shape	antibiotic [154]
	sheep	EO	uncertain	EO [63,64]
Pancreas	rat	antibiotic	overall shape	antibiotic, 72 h [165, 166]
	pig	irradiation	slice (7 mm diameter, 5 mm thick)	gamma irradiation, 10 kGy [34]
		isopropanol	slice (5 mm × 5 mm)	70% isopropanol, 1 h [130]
	human	irradiation	overall shape	gamma irradiation, 12 kGy [35]

usually has a thin shape and large specific surface area, such as decellularized small intestine [5], liver slice [144] and bladder [145]. Chen et al. sliced the decellularized liver into 250 μm thickness and disinfected it with UV rays for 2 h [144]. UV rays are rarely used alone for disinfection, and are often used in combination with other disinfection methods. For example, Selcan Guler immersed the decellularized sheep aorta in 3% (w/v) AA (antibiotic/antifungal) solution for 10 min, and then exposed it to UV for 30 min [146].

2.6. Supercritical carbon dioxide

In the field of disinfection or sterilization, supercritical carbon dioxide (ScCO₂) technology is still in the new stage, and its killing effect on spores remains to be further studied, so it is uncertain whether it is sterilization or disinfection.

There are three kinds of phase states: gas phase, liquid phase and solid phase. The point of equilibrium between liquid phase and gas phase is called the critical point (P_C, T_C). Fluid is the general term of liquid and gas. When the temperature and pressure of the fluid is higher than the critical point, it is in supercritical state. The properties of supercritical fluid are between liquid and gas, with similar density of liquid, viscosity between liquid and gas, and diffusivity similar to gas, so that it has strong permeability and solubility. Carbon dioxide (CO_2) has a lower critical temperature ($T_C = 31.1$ °C) and a critical pressure ($P_C = 7.39$ MPa) (Fig. 3D). ScCO₂ technology is not only widely used in extraction separation, but also a friendly green disinfection and sterilization method. In ScCO₂ devices, it takes dozens of minutes to achieve disinfection or sterilization.

Sterilization and disinfection mechanism: The mechanism of ScCO₂ is not completely clear, and many theories have been proposed, among which acidification is the main mechanism [147]. CO_2 dissolves and transforms into carbonic acid, and then decomposes into bicarbonate ions (HCO_3^-) and hydrogen ions (H^+), which increases the permeability of cytomembrane. CO_2, H^+ and HCO_3^- penetrate the cytomembrane and accumulate in cells. Hence, the intracellular pH decreases which results in the inactivation of enzymes, inhibition of cell metabolism, and final death of microorganisms (Fig. 3C).

Advantages: ScCO₂ has no toxicity and no residue, and it also has the function of decellularization [148].

Disadvantages: There are few studies on ScCO₂ for disinfection and sterilization. ScCO₂ may have effects on the physical and chemical properties of the materials. For example, after ScCO₂ sterilization, stiffness of human tendon was significantly reduced [74], and the tensile strength of decellularized porcine pericardium was significantly increased [149]. Moreover, ScCO₂ can cause acidification, which may lead to the partial dissolution of proteins.

Applications: ScCO₂ is rarely used for disinfection and sterilization. The decellularized matrix materials sterilized by ScCO₂ mentioned in the existing literature are mainly derived from pericardium [149], blood vessel [148], lung [150] and tendon [55].

Table 1 summarizes the advantage, disadvantage, and accessibility of the sterilization and disinfection methods of dECM, in order to benefit researchers to compare various methods and choose the appropriate method for their materials.

2.7. Antibiotics

Although the use of antibiotics is not the typical method of disinfection or sterilization, antibiotics are also used alone for the treatment of dECM, without combining with other sterilization methods, in order to achieve the aseptic state [151–155]. Therefore, this paper introduces and analyzes the mechanism, advantages and disadvantages of antibiotics acting on microorganisms and their applications for dECM, so as to provide a possible selection for sterilization methods of dECM.

Action mechanism: Antibiotics have a variety of action mechanisms [156]. Antibiotics inhibit or kill microorganisms through the following
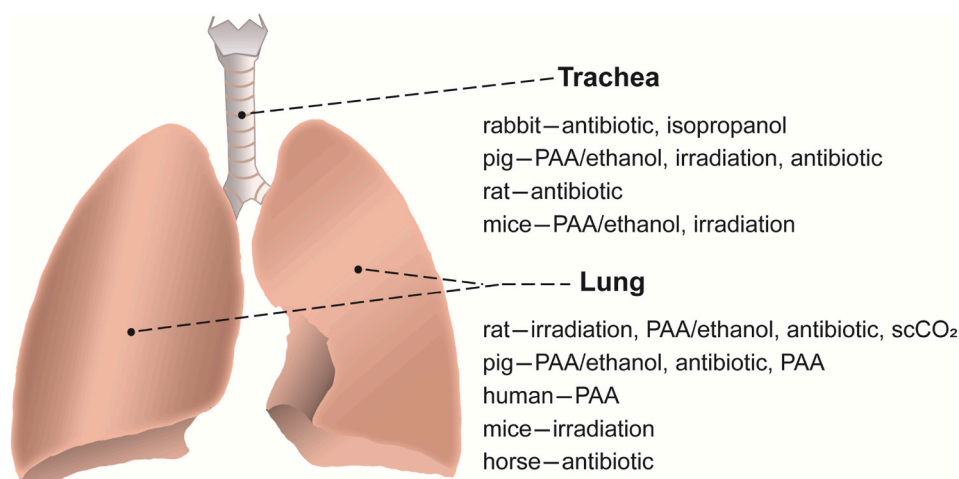
Trachea

rabbit—antibiotic, isopropanol
pig—PAA/ethanol, irradiation, antibiotic
rat—antibiotic
mice—PAA/ethanol, irradiation

Lung

rat—irradiation, PAA/ethanol, antibiotic, scCO₂
pig—PAA/ethanol, antibiotic, PAA
human—PAA
mice—irradiation
horse—antibiotic

Fig. 7. Summary of sterilization and disinfection methods for decellularized lung and trachea.

Table 4
Summary of sterilization and disinfection methods for decellularized lung and trachea.

Tissue type	Origins	Methods	Appearance and Size	Details and References
Lung	rat	irradiation	overall shape	irradiation, 5 Gy/min, 12 min [15]
		PAA/ethanol	overall shape	0.1% (v/v) PAA in 4% (v/v) ethanol, 2 h [15]; 1 h [102]
		antibiotic	overall shape	antibiotic, 37 °C, 48 h [169]; 37 °C, 72 h [171]; 4 °C [172]
		ScCO₂	overall shape	ScCO₂, 1440 psi, 35 °C, 30–45 min [150]
	pig	PAA/ethanol	overall shape	0.1% (v/v) PAA in 4% (v/v) ethanol [103]
		antibiotic	overall shape	antibiotic [173]
		PAA	overall shape	PAA [91]
	human	PAA	overall shape	PAA [91]
	mice	irradiation	overall shape	gamma irradiation, 31 kGy (at room temperature; frozen) [39]
	horse	antibiotic	overall shape	antibiotic [186]
Trachea	rabbit	antibiotic	segment	antibiotic [16,174,187]
		isopropanol	segment (0.75 cm length)	isopropanol, overnight [131]
	pig	PAA/ethanol	segment	0.1% (v/v) PAA in 4% (v/v) ethanol, 6 h [101]
		irradiation	segment	gamma irradiation, 20 kGy [40]
		antibiotic	segment	antibiotic, 7 days [188]
	rat	antibiotic	segment	antibiotic [176,187,189]
	mice	irradiation	segment	(in PBS) gamma irradiation, 5 kGy, 25 kGy [41] (in 0.9% NaCl) gamma irradiation, 20 kGy [42]

mechanisms: ①β-lactams such as penicillin, and polypeptide antibiotics such as vancomycin inhibit the synthesis of bacterial cell wall; ②Aminoglycosides such as streptomycin and gentamicin can bind to the 30S subunit of ribosome, so as to prevent protein translation of bacteria; ③Macrolide antibiotics such as erythromycin and azithromycin, and lincosamides such as clindamycin can bind to the 50S subunit of the ribosome to interfere with bacterial protein synthesis; ④Quinolones such as ofloxacin can interfere with DNA gyrase and inhibit DNA synthesis of bacteria; ⑤Amphotericin B and other polyene antifungal antibiotics can affect the permeability of cell membrane and inhibit the growth of fungi [157] (Fig. 4).

Advantages: Antibiotics have no obvious effect on mechanical properties, structure and components of dECM.
Disadvantages:

(1) The antimicrobial spectrum of each antibiotic is limited. Even if multiple antibiotics are used in combination, some microorganisms are not sensitive to these antibiotics, such as mycoplasma, chlamydia, anaerobic bacteria, viruses, and so on. Therefore, it is suggested to carry out sterility test after treatment of dECM with antibiotics.

(2) Some antibiotics have adverse effects on cells. For example, Gilda Kalinec evaluated the response of HEI-OC1 cells to several antibiotics, and found that tobramycin could induce significant cell death, gentamicin could stimulate autophagy, and penicillin and streptomycin could reduce cell viability and increase senescence [158]. Some antibiotics may cause adverse reactions such as hypersensitivity reactions (HSRs) and toxic reactions. The most common antibiotics causing HSRs are β - lactams, followed by quinolones, and the clinical manifestations of HSRs are maculopapular rash and even anaphylactic shock [159]. In addition, some antibiotics can cause toxic reactions in the human body, such as streptomycin and other aminoglycosides have ototoxicity and nephrotoxicity. Therefore, in view of the possible toxic and side effects of antibiotics on cells and human, it is suggested to detect antibiotic residues and conduct the cytotoxicity test.

Applications: The decellularized matrix materials incubated in antibiotics without other sterilization methods mentioned in the existing literature are mainly derived from pericardium [151,160], valve [152], blood vessel [161,162], liver [163,164], stomach [154], pancreas [165, 166], esophagus [167], small intestine [168], lung [169–173], trachea [16,174–176], kidney [17,177,178], bone [20], tendon [153,179] and nerve [155].

3. Factors influencing the selection of sterilization and disinfection methods

3.1. Application purpose of materials

When selecting a sterilization or disinfection method for a material, first of all, we need to determine its application purpose. If it is to be used for clinical application, the material needs to be sterilized, so only sterilization methods should be selected, such as irradiation and EO. If the material is only used for experimental research, all disinfection and sterilization methods can be considered.
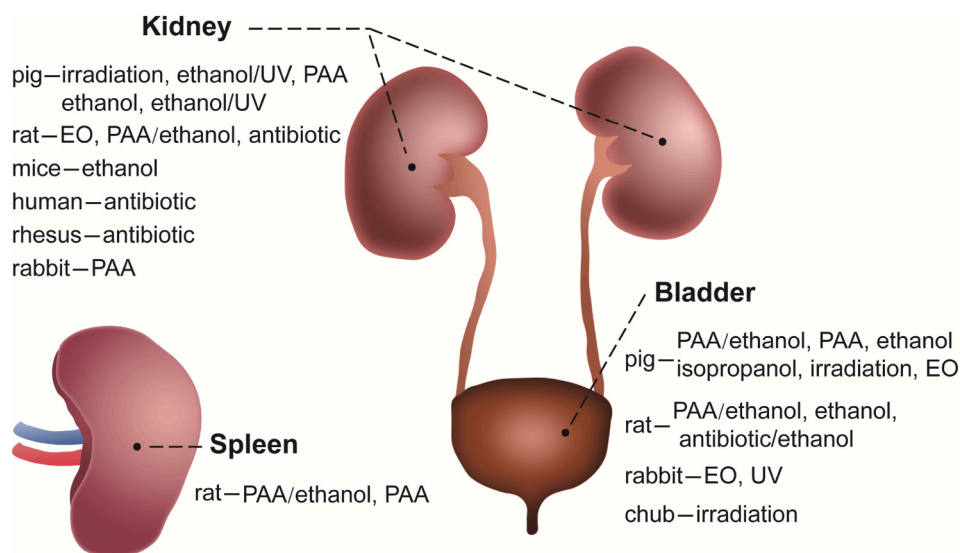
Fig. 8. Summary of sterilization and disinfection methods for decellularized bladder, kidney and spleen.

3.2. Physical and chemical properties of materials

The chemical properties of biomaterials mainly depend on their composition, and the physical properties mainly include physical state, appearance and size.

3.2.1. Chemical properties

The chemical properties of the main components in the material need to be considered. Most of the dECM materials mainly retain organic substance, in which protein is the main component. Protein is not resistant to high temperature, acid and alkali, strong oxidation and strong irradiation. High temperature and irradiation can denature proteins [8]. PAA and H_2O_2 have strong oxidation and acidity, which may lead to protein denaturation or dissolution [5]. In addition, a small number of materials mainly retain inorganic components, such as some kinds of bone powders, and irradiation is suitable for their sterilization.

3.2.2. Physical properties

(1) Physical state

Most of the dECM materials are solid, which include dry state and wet state, and a small amount of dECM is hydrogel. EO is easily soluble in water and forms toxic ethylene glycol, so it cannot be used in materials containing water. HPLP sterilization requires the sterilized materials to be in dry state. Due to its weak penetrability, UV is only suitable for the disinfection of the surface of objects and distilled water, but not for the disinfection of liquid medicine and the deep disinfection of solid objects. So, the above methods are not suitable for wet solid materials and hydrogel. In addition, selecting a sterilization or disinfection method for dECM hydrogel still face the following problems: ①irradiation [180] and high temperature [181] tend to reduce the molecular weight, resulting in a decrease in viscosity of hydrogels; ②PAA, alcohol and H_2O_2 are dissolved in water and difficult to be removed; ③there are almost no research on the sterilization or disinfection for dECM hydrogel with $ScCO_2$, so its effect of sterilization is uncertain. Therefore, for sterilization or disinfection of dECM hydrogels, it is recommended to first sterilize the solid raw materials of dECM hydrogel, and then carry out the subsequent hydrogel preparation following the aseptic operation principle.

(2) Appearance

Freeze dried dECM is loose and porous which makes it having strong adsorption capacity, so it is not suitable to be sterilized by EO, unless the removal time of EO residue is prolonged to remove the residue effectively. In another scene, the materials with one end blocked cannot to be sterilized by HPLP due to its poor permeability.

(3) Size

Considering size, thickness is the main factor involved in affecting sterilization and disinfection. The influence of thickness on the selection of sterilization and disinfection method is related to the penetrability of sterilization and disinfection agent, for example, the penetrability of irradiation and EO is strong, while the penetrability of HPLP and UV is poor.

3.3. Time required and accessibility

Due to the need to extend the removal time of EO residue after EO sterilization of dECM materials, EO sterilization usually takes several weeks. Irradiation sterilization takes hours to days. Sterilization or disinfection by PAA, H_2O_2, HPLP, alcohol and UV take dozens of minutes (Table 1).

Sterilization with irradiation, EO and HPLP need corresponding devices. UV disinfection requires the UV light source, but UV device is more common. Sterilization or disinfection by PAA, H_2O_2, and alcohol are to soak the materials directly in their solution (Table 1).

4. Sterilization and disinfection methods of various common decellularized matrix materials

Because of the different composition, structure, volume and desired function of the dECM materials, the sterilization or disinfection methods are various. However, for the same tissue or organ, the sterilization or disinfection methods reported in the current literature are often different, mainly due to the lack of ideal selection criteria, and the literature about the effects of sterilization and disinfection methods on dECM materials is scarce.

4.1. Cardiovascular system

There are many dECM materials in cardiovascular system such as decellularized pericardium, aorta, and heart valve (Fig. 5 and Table 2).

Table 5

Summary of sterilization and disinfection methods for decellularized bladder, kidney and spleen.

Tissue type	Origins	Methods	Appearance and Size	Details and References
Bladder	pig	ethanol	overall shape	70% ethanol [132, 191]
			piece (muscle removed)	75% ethanol [135]
		isopropanol	piece (5 mm × 5 mm)	70% isopropanol, 1 h [130]
		PAA/ ethanol	piece	0.1% (v/v) PAA in 4% (v/v) ethanol, 2 h [104–106,109,190]
		irradiation	piece (basement membrane, lamina propria)	gamma irradiation, 20 kGy [43,45]
			piece (1 cm²)	gamma irradiation, 30 kGy [44]
		EO	piece	EO [66–73,134]
			overall shape; piece (8 cm × 10 cm)	EO [67]
			piece (0.2–0.4 mm thick)	EO [70]
			piece (1 cm × 1 cm)	EO [71]
			piece (15 cm × 15 cm)	EO [73]
		PAA	overall shape	0.1% (v/v) PAA (pH 7.5), 37 °C, 3 h [93]
				0.1% (v/v) PAA, room temperature, 3 h or 5 h [66]
			piece (<2 mm thick)	0.1% (v/v) PAA, 1 h [92]
	rat	antibiotic/ ethanol	overall shape	antibiotic; then, 70% ethanol, 10 min [192]
		PAA/ ethanol	overall shape	0.1% (v/v) PAA in 4% (v/v) ethanol, 4 °C, 24 h [98]
		ethanol	piece	70% ethanol [133]
	rabbit	EO	overall shape	EO [75]
		UV	piece (submucosa)	UV, 24 h [145]
	chub	irradiation	piece (10 cm²)	gamma irradiation, 25 kGy [46]
Kidney	pig	irradiation	overall shape	gamma irradiation [47]; (placed in PBS), 10 kGy [23]
			slice (7 mm diameter, 2 mm thick)	gamma irradiation, 1–10 kGy [47]
		ethanol/UV	electrospinning membrane	70% ethanol; then UV, 30 min/side [193]
		ethanol	slice (7 mm diameter, 2 mm thick)	70% ethanol (pH 7.4), 0.5 h, 1 h, 2 h, 3 h, 4 h [48]
		PAA/ ethanol	slice (7 mm diameter, 2 mm thick)	0.2% (v/v) PAA in 4% (v/v) ethanol, (pH 3.0), 0.5 h, 1 h, 2 h, 3 h, 4 h [48]
		PAA	slice (7 mm diameter, 2 mm thick)	0.2% (v/v) PAA (in 1 M NaCl) (pH 3.1), 0.5 h, 1 h, 2 h, 3 h, 4 h [48]
	rat	EO	overall shape	EO [76]
		PAA/ ethanol	overall shape	0.2% (v/v) PAA in 4% (v/v) ethanol, 10 min [110]
		antibiotic	overall shape	antibiotic, 96 h [17]
	mice	ethanol	overall shape	70% ethanol, 30 min [136]
	human	antibiotic	slice (2 mm thick)	antibiotic [177]
	rhesus	antibiotic	transverse sections	antibiotic, 4 °C [178]
	rabbit	PAA	slice (10 mm × 10 mm × 3 mm)	0.5%, 1%, 1.5% (v/v) PAA (pH 7.2–7.4), 2 h [94]

Table 5 (*continued*)

Tissue type	Origins	Methods	Appearance and Size	Details and References
Spleen	rat	PAA/ ethanol	overall shape	0.1% (v/v) PAA in 4% (v/v) ethanol, 2 h [111–113]
		PAA	overall shape	0.1% (v/v) PAA, 3 h [89], 2 h [90]

4.1.1. Pericardium

The pericardium is the membrane that surrounds the heart. The decellularized pericardium is the biomaterial widely used in clinic. The main source species of decellularized pericardium are pig and bovine, and the sterilization and disinfection methods mentioned in current literature mainly include incubation in antibiotics [151,160], combination of antibiotics and PAA [182], ethanol [11] and ScCO₂ [149].

Cátia Fidalgo [182] sterilized porcine and bovine pericardium with the two-step sterilization, which was antibiotics/antimycotic (AA) cocktail and PAA. It was found that the aseptic scaffolds could be obtained after the two-step sterilization, while the structural integrity and biocompatibility of the tissue were preserved. Joshua A Choe [149] sterilized porcine pericardium with ScCO₂, and the compression and tensile strength of the material were improved after sterilization.

4.1.2. Blood vessel

Decellularized vascular materials involve arteries, veins and perivascular tissues. They come from a variety of source species including pig, sheep, horse, human, and rat according to relevant literature. The sterilization and disinfection methods mentioned in current reports mainly include ethanol [121,125,126,183], PAA/ethanol [19], antibiotics [161,162], EO [62], antibiotic/UV [146] and ScCO₂ [148], etc.

George R. Fercana [19] incubated the decellularized adventitia in a solution of 0.1% (v/v) PAA/4% (v/v) ethanol. After sterilization, the decellularized arterial adventitia was freeze-dried, ground and prepared into hydrogels to promote angiogenesis. Selcan Guler [148] sterilized the aorta of sheep with ScCO₂ for 1 h, with the result showing that ScCO₂ could not only get sterilization, but also bring about decellularization. After treatment, the tissue retained its thickness and stiffness, but became drier.

4.1.3. Cardiac and arterial valve

The main source species of valves are pig and sheep, and their sterilization and disinfection methods mainly include irradiation [30,31], isopropanol [123], PAA [84] and antibiotics [152].

Meghana R K Helder [30] irradiated the decellularized porcine heart valves with 1.5 kGy and 3 kGy irradiation, with the result that the valves irradiated with 1.5 kGy were not completely sterilized, while the valves irradiated with 3 kGy showed no signs of infection, and the mechanical strength of the valves decreased with the increase of irradiation dose.

4.2. Digestive system

There are many dECM materials of digestive system such as decellularized liver, small intestinal, esophagus, stomach and pancreas, etc. (Fig. 6 and Table 3)

4.2.1. Liver

Decellularized livers are derived mainly from pigs, and some from goats, rats and mice, etc. The sterilization and disinfection methods of decellularized liver mainly include ethanol [127–129], PAA [85,86, 184], PAA/ethanol [4,99], irradiation [32], UV [144] and antibiotics [163,164].

Kamal H Hussein [4] sterilized pig liver slices with PAA and ethanol for 2 h. The results showed that ethanol could not sterilize materials effectively, but PAA could. The content of glycosaminoglycan after
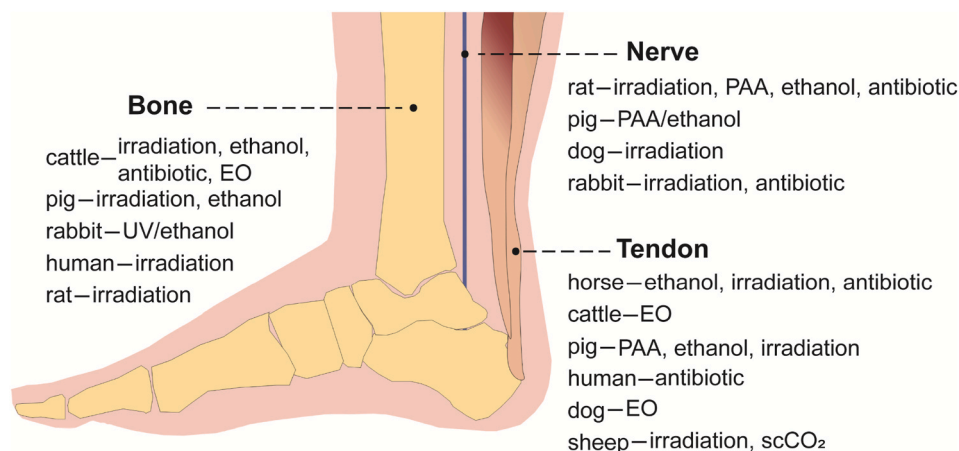
Fig. 9. Summary of sterilization and disinfection methods for decellularized bone, tendon, and nerve.

ethanol treatment was higher than that of PAA treatment, but the content of collagen after ethanol treatment decreased significantly than that of PAA treatment.

4.2.2. Small intestine

Decellularized submucosa of small intestine is a common decellularized material, and there is a lot of related literature. Decellularized small intestine mainly comes from pigs, some from rats and mice, and a few from bovine and sheep. The common sterilization and disinfection methods include irradiation [37,38,185], PAA/ethanol [5,38,100], PAA [5,87,88], antibiotics [168], UV [5], PAA/H$_2$O$_2$ [5,120] and so on.

Carolyn Gosztylal [5] sterilized the decellularized small intestine of rats with four methods, which mainly includes perfusion with 0.1% (v/v) PAA for 3 h, 0.08% (v/v) PAA/1% (v/v) H$_2$O$_2$ solution for 3 h, 0.18% (v/v) PAA/4.8% (v/v) ethanol solution for 3 h, and placed in the UV cross-linker at 254 nm for 90 s. The results showed that all of these sterilization methods were effective, and the sterilized tissues in each group had similar amount of collagen and glycosaminoglycan, and the similar ability to support cell growth. However, chemical sterilization led to the loss of tissue structure with fraying and flattening of the edge at the villous portions, while UV sterilization could keep the integrity of tissue structure.

4.2.3. Esophagus, stomach and pancreas

There are few reports about decellularized esophagus, stomach and pancreas, and most of this literature does not clearly elucidate sterilization and disinfection methods. The decellularized esophagus mainly comes from pigs and rats. The sterilization and disinfection methods mainly include antibiotics [167], PAA/ethanol [65,98], EO [65] and irradiation [36].

The decellularized gastric matrix mentioned in the existing literature mainly comes from sheep and rats. The sterilization methods mainly include EO [63,64], irradiation [33], and immersion in antibiotic solution [154]. Liang Feng [33] irradiated the decellularized rat stomach with 12 kGy, with the result that the spatial structure and elasticity of the tissue did not change significantly.

Decellularized pancreas in the existing reports is derived from rats, pigs and humans. Most of the decellularized pancreas was obtained with aseptic operation, and then washed and soaked in antibiotic solution. The sterilization and disinfection methods mainly include antibiotic [165,166], isopropanol [130] and irradiation [34,35].

4.3. Respiratory system

Decellularized lung and trachea are the most common dECM materials in respiratory system (Fig. 7 and Table 4).

4.3.1. Lung

The common decellularized lung mainly comes from rats, and a few from pigs, humans, mice and horses. The main method to obtain the aseptic state of decellularized lung is antibiotic rinsing [169–172,186]. In addition, there are PAA/ethanol [15,102,103], PAA [91], irradiation [15,39], ScCO$_2$ [150], etc.

Nicholas R Bonenfant [15] compared two sterilization methods of decellularized mouse lungs, one of which was irradiation with 5 Gy/min for 12 min, and the other was to wash the decellularized lung in a mixture of 0.1% (v/v) PAA/4% (v/v) ethanol solution thrice and incubated in the mixture for 2 h. The results showed that PAA/ethanol could dissolve the extracellular matrix, and the overall appearance of PAA/ethanol treated lung was similar to that of natural lung with slight atelectasis in the central region. While in the irradiated lung, alveolar septum thickening, fusion and large emphysema appeared.

Jenna L. Balestrini [150] compared the sterilization effect of ScCO$_2$ for 2 h and 0.1% PAA for 2 h on decellularized lung. The results showed that ScCO$_2$ sterilization had no adverse effect on the mechanical properties and the contents of collagen, elastin and glycosaminoglycan (sGAG) in decellularized lung; while PAA-treated samples had no change in mechanical properties after 24 h, but had higher Young's moduli and lower failure strain after 6 months of storage, and PAA could significantly decrease the contents of elastin and sGAG.

Juan J. Uriarte [39] sterilized the decellularized lung of mice with 31 kGy. It was found that the volume of irradiated lung decreased and the mechanical impedance increased.

4.3.2. Trachea

Decellularized tracheas are mostly from rabbits, while pigs, rats and mice are also relatively common. Most of the decellularized tracheas were obtained in aseptic condition, and incubated in antibiotic solution after decellularization. The sterilization and disinfection methods mainly include antibiotic [16,174–176,187–189], PAA/ethanol [40,42, 101], irradiation [40–42], and isopropanol [131].

Christopher M. Johnson [41] irradiated the mice trachea with 25 kGy gamma radiation, with the result showing that 25 kGy radiation was enough to effectively sterilize the trachea, but the ultrastructure of tissue would be seriously degraded, such as a large number of empty lacunae and matrix disorganization.

4.4. Urinary system

The most common dECM materials of urinary system are decellularized bladder and kidney (Fig. 8 and Table 5).

4.4.1. Bladder

Decellularized bladder is a common dECM material mainly from

Table 6

Summary of sterilization and disinfection methods for decellularized bone, tendon and nerve.

Tissue type	Origins	Methods	Appearance and Size	Details and References
Bone	bovine	irradiation	piece (15 cm × 4 cm × 2 cm)	lyophilized, gamma irradiation, 15 kGy [14]
			piece (2 mm length, 10 mm diameter)	gamma irradiation [49]
		EO	piece (5.5 cm × 0.5 cm × 0.2 cm)	EO [77]
		ethanol	piece (2 mm length, 4 mm diameter)	lyophilized, ethanol [137]
		antibiotic	granule	antibiotic, 4 °C, 24 h [20]
	pig	irradiation	piece (10 mm × 10 mm × 10 mm)	gamma irradiation [50]
			piece (5 mm length, 10 mm diameter)	discs (5 mm thick), gamma irradiation, 20 kGy [51]
		ethanol	piece (5 mm × 5 mm × 3 mm)	70% ethanol [138]
	rabbit	UV/ ethanol	piece	UV, 20 min; then, 70% ethanol [194]
	human	irradiation	granule (1–20 μm)	gamma irradiation, 30 kGy [52]
	rat	irradiation	piece (8 mm diameter)	gamma irradiation, 10 kGy [53]
Tendon	horse	ethanol	piece (natural thick, or 0.3 mm thick)	aseptic working, washed twice in ethanol [139]
			piece (20 mm × 10 mm × 1.2 mm)	70% ethanol, 4 h [139]
		irradiation	piece (100 mm × 15 mm × 3 mm); segment	(placed in PBS) β-irradiation, 15 kGy [54]
			segment	β-irradiation, 15 kGy [56]
		antibiotic	segment	antibiotic, 4 °C, overnight [179]
	bovine	EO	piece (2 mm × 1.5 mm)	lyophilized, EO [78]
	pig	PAA	segment	0.1% (v/v) PAA (pH 7.6), 3 h [95]
				0.1% (v/v) PAA [96]
				0.1% (v/v) PAA (pH 6.0), 3 h [27]
		ethanol	segment	70% ethanol, 5 min × three times [141]
		irradiation	segment	gamma irradiation, 15 kGy, 30 kGy, 55 kGy; E-beam, 15 kGy, 34 kGy, (15 + 15) kGy (fractionated dose) [27]
	human	antibiotic	segment	antibiotic, overnight [153]
	dog	EO	piece (300 μm thick)	EO [79,80]
			piece (40 mm length)	EO [80]
	sheep	irradiation	piece (40 mm length)	(in dry ice) gamma irradiation, 25 kGy [55]
		ScCO$_2$	piece (40 mm length)	ScCO$_2$, 2 h [55]
Nerve	rat	irradiation	segment	gamma irradiation, 2.5 kGy [57]
		PAA	segment	0.1% (v/v) PAA, room temperature, 1 h [97]
		ethanol	porous scaffold	75% ethanol, 1 h [142]
		antibiotic	segment	antibiotic, 7 days [155]
	pig	PAA/ ethanol	piece (<30 mm length)	0.1% (v/v) PAA in 4% (v/v) ethanol, 2 h [114]
	dog	irradiation		gamma irradiation [58]

Table 6 (*continued*)

Tissue type	Origins	Methods	Appearance and Size	Details and References
	rabbit	irradiation	piece (30 mm length; 60 mm length) piece (30 mm length)	gamma irradiation, 25 kGy [59]
		antibiotic	segment	antibiotic, 7 days [155]

pigs, as well as rats, rabbits and chubs. The main sterilization and disinfection methods include PAA/ethanol [104–109,190], EO [66–70, 75], irradiation [43–45], isopropanol [130], ethanol [132,133,191], antibiotic/ethanol [192], PAA [44,66,92] and UV [145].

Derek J Rosario [66] sterilized the decellularized pig bladder with EO and 0.1% (v/v) PAA, with the results showed that the young's modulus of the tissue sterilized by PAA was decreased, while the young's modulus of the tissue sterilized with EO increased.

4.4.2. Kidney

Decellularized kidney has a wide range of tissue sources mainly including pig, rat, mouse, rabbit, human and rhesus monkey in accordance with the relevant literature. In most of the reports, researchers immerse or preserve the tissue in an antibiotic solution after decellularization, without other sterilization or disinfection methods [17,177, 178]. In addition, there are ethanol [48,136], EO [76], irradiation [23, 47,48,94], PAA/ethanol [48,110], ethanol/UV [193], and PAA [48,94].

Lida Moradi [94] sterilized the decellularized rabbit kidney slices with four sterilization methods. ①The rabbit kidney slices were immersed in 0.5% PAA solution with pH value of 7.2–7.4 for 2 h. The result showed that they could be sterilized effectively and retain the mechanical properties of tissues and the main components of matrix. ②The rabbit kidney slices were incubated in the antibiotic mixture composed of penicillin G, amphotericin B and gentamicin for 30 min, with the result that they were effectively sterilized and preserved the mechanical properties of tissues, but no cell adhesion was observed when rabbit adipose mesenchymal stem cells were seeded on the kidney slices. ③The kidney slices were immersed in sterile PBS and irradiated with UV at 320–480 nm for 2 h, with the result that they could not be sterilized effectively. ④After 3–5 min irradiation with 5 kGy gamma radiation, the mechanical strength of kidney slices decreased and the microstructure changed.

Nafiseh Poornejad [48] obtained the optimal conditions of four common sterilization and disinfection methods for decellularized whole porcine kidneys through sterility test. Specifically, porcine kidneys were exposed respectively to 70% (v/v) ethanol for 1 h, 0.2% (v/v) PAA in 1 M NaCl for 1 h, 0.2% (v/v) PAA in 4% PAA ethanol for 1 h, and gamma irradiation at room temperature with 3 kGy, and the sterile state could be obtained. Further experiments showed that the content of collagen decreased by more than 50% after irradiation, while other methods had no significant change. The porosity of the sample irradiated increases, which makes its swelling ratio the largest of all the methods; while the sample with PAA in 1 M NaCl is closest to the normal tissue. Except 70% ethanol, other methods may further damage the structure of glomerulus and renal tubules.

4.5. Immune system

The most common dECM material in the immune system is decellularized spleen, and almost all of it comes from rats. The main sterilization and disinfection method of decellularized spleen is to infuse 0.1% (v/v) PAA/4% (v/v) ethanol [111–113], and some studies have used PAA alone for sterilization [89,90], e.g., Rui Gao [89] sterilized the spleen of rats in 0.1% (v/v) PAA for 3 h (Fig. 8 and Table 5).
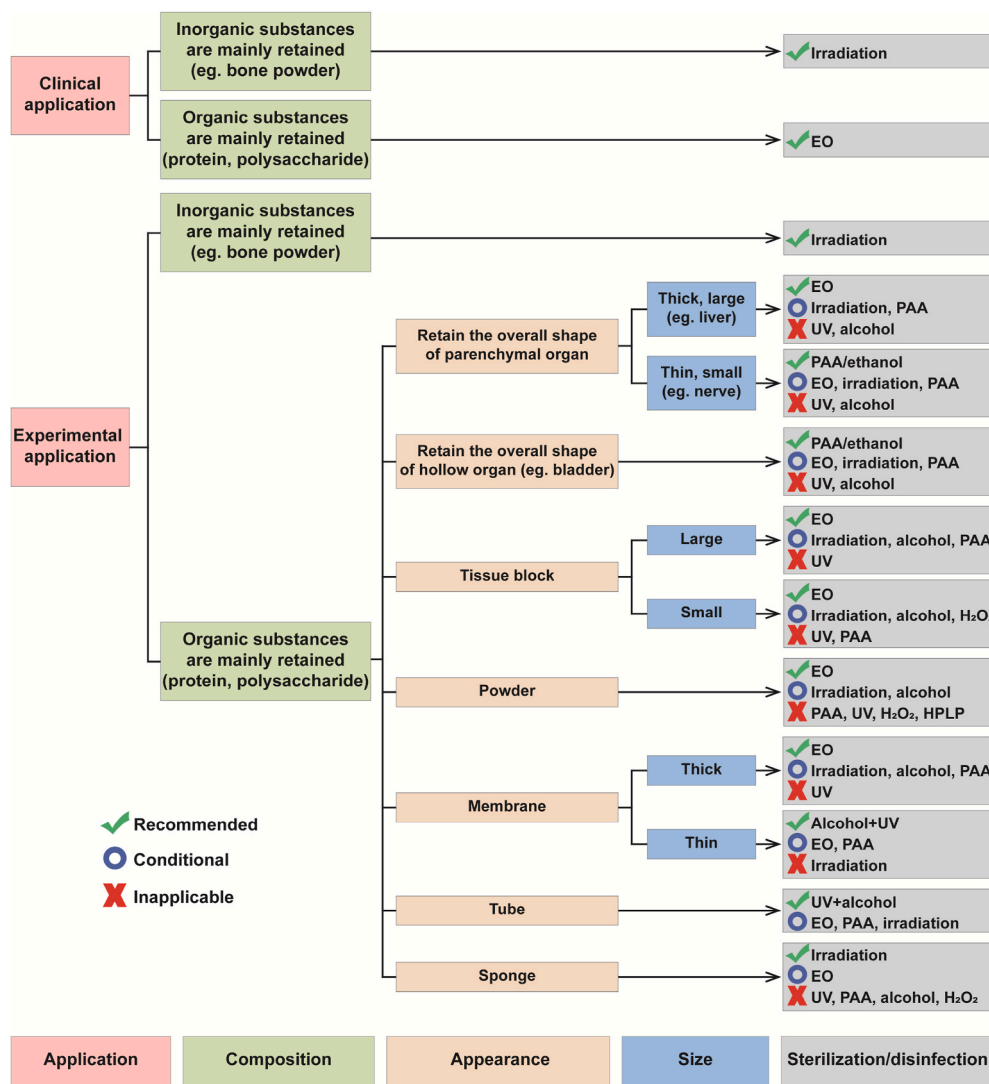
Fig. 10. The graphical route for selecting an appropriate sterilization or disinfection method for dECM.

4.6. Motor and nervous system

The dECM materials of motor and nervous system mainly include decellularized bone, tendon and nerve (Fig. 9 and Table 6).

4.6.1. Bone

Bone repair has been a research hotspot for many years, and there are a large number of research studies about it. However, there are not many reports that could clarify the sterilization and disinfection methods of decellularized bone. Decellularized bone mainly comes from bovine and pigs, and a small amount from human, mice and rabbits. Irradiation [14, 49–53] is the most common sterilization method for decellularized bone, and ethanol [137,138], EO [77], antibiotics [20], UV/antibiotic [194] can also be used for its sterilization or disinfection.

4.6.2. Tendon

Decellularized tendon matrix is a common decellularized biomaterial. Tendons mainly come from bovine, horse and pig, and a small number of tendons are from human, rabbit, sheep, dog. The sterilization and disinfection methods of decellularized tendon mainly include ethanol [139–141], EO [78–80], PAA [95,96], irradiation [27,54–56], antibiotics [153,179] and ScCO₂ [55], etc.

Jennifer H. Edwards [27] studied the sterilization effects of irradiation on decellularized porcine tendon, and the results confirmed that

irradiation changed its biomechanical properties including reduction of the ultimate tensile strength and Young's modulus. Yikan Sun [55] compared the sterilization effects of 25 kGy irradiation and ScCO₂ for 2 h on sheep tendon, with the results showing that the tendons were dried and contracted after irradiation, while the tissue was dehydrated and transparent to a certain extent after ScCO₂. There were obvious holes in the cross section of the irradiated tendon, while the ultrastructure of ScCO₂ treated tendon was intact.

4.6.3. Nerve

There are a lot of reports about decellularized neural matrix, but only few studies have clarified the sterilization and disinfection methods. Decellularized nerves are mainly derived from rats and pigs, and a small number is obtained from dogs and rabbits. The sterilization and disinfection methods mainly include irradiation [57–59], PAA [97], ethanol [142], antibiotic [155], PAA/ethanol [114], etc.

5. Discussion

In order to facilitate researchers to have a clear idea on the selection of sterilization or disinfection methods of dECM after reading this review, a graphical route for then to select an appropriate sterilization or disinfection method were proposed, which is based on the application purpose and physic-chemical properties of various dECM, as well as the

advantages and disadvantages of common sterilization and disinfection methods (Fig. 10).

In Fig. 10, there are three options for the selecting sterilization or disinfection methods, namely "Recommended", "Conditional" and "Inapplicable". "Recommended" means "the sterilization or disinfection method has less impact on the physical and chemical properties of materials under the premise of effective sterilization or disinfection"; "Conditional" means "the sterilization or disinfection method is only applicable under certain conditions, such as appropriate exposure time, solution concentration or radiation dose, etc."; "Inapplicable" refers to "the sterilization or disinfection method has serious problems".

The application purpose should be considered first, while selecting a sterilization or disinfection method for dECM materials. If the material is prepared for clinical application, only sterilization methods should be chosen, such as irradiation and EO sterilization. Irradiation is suitable for dECM with inorganic retention, such as hydroxyapatite-retained bone powder. EO is better for materials with "organic substances are mainly retained". If the material is used only for experimental research, both of sterilization and disinfection methods can be considered.

Appearance and size are also the important concerns while selecting a sterilization or disinfection method for dECM materials. For the thick and large parenchymal organs with overall shape, EO is recommended to be used for sterilization due to its strong penetrability and good sterilization effect. Although EO is toxic, it can be solved by prolonging the removal time of EO residue. Both irradiation and PAA are "Conditional" options for sterilization or disinfection of the thick and large parenchymal organs, but the lowest dose of irradiation and the shortest exposure time of PAA need to be explored. The thin and small parenchymal organs and hollow organs are recommended to be sterilized or disinfected with PAA/ethanol, because it can get an effective sterilization and takes less time, and does not require residue test. The tissue block and thick membrane are recommended to be sterilized by EO. Because the exposed collagen in tissue block may be dissolved in acidic PAA, PAA cannot be used for relatively small tissue block. In addition, PAA is adjusted to neutral and then used for sterilization in some studies [93,95], and its mechanism and sterilization effect still need to be further studied. The powder is recommended to be sterilized by EO. PAA, H_2O_2 and HPLP are not suitable for the sterilization and disinfection of powder. For thin membranes and tubes, it is recommended to combine UV and alcohol immersion to achieve the aseptic effect, especially for tubes prepared by electrospinning, because most electrospinning machines have UV function.

Sponge is a special material with strong adsorption capacity. If toxic EO is used to sterilize the sponge, the removal time of EO residue needs to be extended considerably. Due to the poor penetrability, UV is difficult to effectively sterilize the sponge. After the sponge is sterilized by liquid agents, it is difficult to remove the residues of PAA, alcohols and H_2O_2. Therefore, irradiation may be the solution for sterilization of sponge at present, but irradiation may reduce the porosity of sponge. Perhaps, it is better to sterilize the raw materials, and then process them into sponges under aseptic conditions.

In addition to solid materials, dECM materials also include hydrogels. As mentioned in the section of "factors influencing the selection of sterilization and disinfection methods", irradiation, EO, PAA, alcohols, H_2O_2, HPLP, UV and ScCO$_2$ are not suitable for sterilization and disinfection of dECM hydrogels. Therefore, the sterilization of hydrogels is recommended by sterilizing the solid raw materials of dECM hydrogels, followed by subsequent preparation of hydrogels in a sterile environment.

The aseptic state obtained by effective sterilization and disinfection is the premise of subsequent experiment and application of dECM. In order to achieve more efficient sterilization and disinfection, various methods can be used not only alone, but also in combination. Because of the strong penetrability and stable sterilization effect, irradiation and EO can be used alone to obtain satisfactory sterilization effects. Moreover, PAA, alcohol, H_2O_2, UV and ScCO$_2$ can be used in combination.

For example, "0.1% (v/v) PAA in 4% (v/v) ethanol" has been widely used in various dECM materials [98,114]. There is also the combination of PAA and H_2O_2 [195], ScCO$_2$ and PAA [196], etc. Due to the convenience of operation, UV can be combined with various sterilization or disinfection methods to disinfect the surface of dECM materials.

There are many researchers who incubated dECM in antibiotic solution to achieve aseptic state. For example, trachea and lung were obtained and decellularized in sterile environment, and washed or soaked the decellularized tissue in antibacterial solution, without using other disinfection methods, and their subsequent cell experiments and animal experiments were found successful [169,174,175]. Due to the limited antibacterial spectrum, antibiotic immersion is not a typical sterilization or disinfection method, but under certain circumstances (e.g., low chance of virus contamination), antibiotics have some advantages, such as not affecting the physical and chemical properties of biomaterials, whereas many other methods do not have the advantage. Considering the limited antimicrobial spectrum of antibiotics and some potential adverse effects of residual antibiotics, it is suggested that researchers should carry out the aseptic tests, cytotoxicity tests, and antibiotic residue tests to ensure the biological safety of dECM after antibiotic treatment. Furthermore, the removal of antibiotic residues may be an important issue which need to be paid more attention by researchers. In addition, if the antibiotic-treated dECM is applied in clinical practice, the cross-species infection would be a potential risk.

6. Conclusion

There are many reports on dECM, but only few of them clarified their sterilization and disinfection methods, and these methods are not necessarily correct or the best. Due to the diversity of dECM materials, the suitable sterilization methods are different, and the specific methods need to be determined according to the specific material and relevant conditions. Therefore, basing on the applications and physic-chemical properties of various dECM, and considering the advantages and disadvantages of common sterilization and disinfection methods, this paper proposes a graphical route for selecting a suitable sterilization or disinfection method for dECM (Fig. 10), and the chosen method could be validated according to "the technical route for validating the sterilization or disinfection method for dECM" (Fig. 1).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplement

(1) Sterility test-method 1

According to the European Pharmacopoeia (EP 10.0) [197], the United States Pharmacopoeia (USP 41) [198], and Chinese Pharmacopoeia 2020 (CP 2020) [199], the sterility test and related evaluation indicators are summarized as follows:

The positive control group is the medium containing relative positive bacteria; the negative control group is the medium without materials; and the sample group is the medium with materials.

Fluid Thioglycollate Medium is mainly used for the cultivation of anaerobic bacteria, but can also be used for aerobic bacteria, incubated

at 30–35 °C. Soybean-Casein Digest Medium is suitable for the cultivation of fungi and aerobic bacteria, incubated at 20–25 °C.

During the culture period, the turbidity and the microbial growth are observed by visual examination. After 14 days, if the medium is turbid and the presence or absence of microbial growth cannot be determined visually, transfer 1 ml of medium to the fresh tubes of the same medium and continue to incubate for 4 days, then observe the microbial growth.

Results: the positive control group has obvious microbial growth, while the negative control group has no microbial growth, which is the premise of effective sterility test.

If the sample tube is clear or it is turbid but without microbial growth, the sample complies with sterility test. If any tube is turbid and microbial growth is confirmed in the sample tube, it does not comply with sterility test.

(2) Sterility test-method 2

Agar diffusion can be used as an auxiliary method for sterility test. With the agar diffusion, the microbial growth can be observed within 24, 48, and 72 h, so agar diffusion is suitable for the screening of invalid sterilization of biomaterials.

In brief, Luria-Bertani (LB) agar medium dissolved in distilled water is autoclaved at 121 °C for 15 min. The hot medium is poured into the dish and cooled to solidify the agar. The samples are laid flat in the center of these dishes and the dishes are kept upside down. The positive group is the one in which unsterilized samples are lying in the center of the solid medium, and the negative group contains the solid medium without samples. After incubation at 37 °C for 24, 48, and 72 h, the microbial growth on the solid medium is observed [150,200].

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