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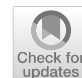
REVIEW



Application of Tissue-Specific Extracellular Matrix in Tissue Engineering: Focus on Male Fertility Preservation

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Abstract

In vitro spermatogenesis and xenotransplantation of the immature testicular tissues (ITT) are the experimental approaches that have been developed for creating seminiferous tubules-like functional structures in vitro and keeping the integrity of the ITTs in vivo, respectively. These strategies are rapidly developing in response to the growing prevalence of infertility in adolescent boys undergoing cancer treatment, by the logic that there is no sperm cryopreservation option for them. Recently, with the advances made in the field of tissue engineering and biomaterials, these methods have achieved promising results for fertility preservation. Due to the importance of extracellular matrix for the formation of vascular bed around the grafted ITTs and also the creation of spatial arrangements between Sertoli cells and germ cells, today it is clear that the scaffold plays a very important role in the success of these methods. Decellularized extracellular matrix (dECM) as a biocompatible, functionally graded, and biodegradable scaffold with having tissue-specific components and growth factors can support reorganization and physiologic processes of originated cells. This review discusses the common protocols for the tissue decellularization, sterilization, and hydrogel formation of the decellularized and lyophilized tissues as well as in vitro and in vivo studies on the use of the testis-derived dECM for testicular organoids.

Keywords Decellularization · Extracellular matrix · Fertility preservation · In vitro spermatogenesis · Xenotransplantation

Introduction

Spermatogenesis is the process that takes place in the seminiferous tubules of the testis, by which sperm cells are produced from spermatogonial stem cells (SSCs) [1, 2]. This process starts at puberty and continues throughout adulthood [1]. The fertility of children undergoing chemotherapy or radiotherapy for pediatric cancers or preconditioning therapies before bone marrow transplantation is severely reduced when they become adults [3]. Sperm cryopreservation is routinely used for post-pubertal patients, but in peripubertal boys who do not have any mature sperm, cryopreservation of immature testicular tissues(ITT) is the only alternative for preservation of fertility [4, 5]. This infertility is due to the loss of SSCs when exposed to cytotoxic agents. Therefore, to prevent prepubertal germ cell loss and subsequent infertility, cryopreservation of testicular tissues containing SSCs has been accepted as the gold standard protocol before oncological treatments in some centers in Europe and the USA [3]. The approaches include in vitro spermatogenesis, SSC transplantation, and testicular tissue grafting have been followed by researchers to restore male fertility in peripubertal boys using cryopreserved ITTs [6, 7]. Autotransplantation of frozen-thawed gonadal tissues has presented a significant option for restoration purposes as approved by over 100 live births worldwide using autotransplantation of the frozen-thawed ovarian cortex [8]. Autotransplantation of gonadal tissues has commonly applied only in the cases where there is no risk of retransmission of cancer cells to the cured patient, such as patients undergoing bone marrow transplantation for nonmalignant diseases like hemoglobinopathies [3]. Xenotransplantation of ITTs to nude mice, which is at a preclinical stage has presented another method suitable for those who suffer from malignant diseases [9–11]. However, further studies should be focused on the important loss of germ cells following transplantation due to hypoxia/reoxygenation injuries [10, 12, 13]. Different protocols based on

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tissue engineering approaches have been followed to limit these events, such as ITTs encapsulation in fibrin hydrogel, alginate, or using a combination of hydrogel and growth factors like vascular growth factor [14–17]. On the other hand, the three-dimensional culture of testicular cells in vitro is another option for sperm production from testicular tissue samples [3]. Since in the structure of seminiferous tubules, germ cells are immersed in the cytoplasm of Sertoli cells, and both of these cells interact with the matrix and the cells around the seminiferous tubules, such a structure cannot be reconstructed with two-dimensional cultures [2]. Therefore, three-dimensional culture has become the main focus of the researches to investigate in vitro spermatogenesis using cryopreserved ITTs. Accordingly, encapsulation of testicular cells in 3D condition using synthetic or biologically-derived matrices such as fibrin [18], matrigel [19], collagen [20], alginate [21], and agarose [22–25] has been studied to provide a condition that mimics the extracellular matrix (ECM). Today, the ECM derived from tissue decellularization, considering its multiple functions has emerged as one of the biomaterials for tissue engineering [26]. This review summarizes the methods used to decellularize tissues and obtain ECM, as well as their use in testicular tissue engineering (Fig. 1). Accordingly, a combination of the following terms without time limitation was applied to search the articles performed on PubMed: [(tissue decellularization) (179) AND (fertility) AND (restoration OR preservation)] [12]. Scientific video protocols [6], reviews [26], guidelines [10], irrelevant articles [27], articles in languages other than English [10], and articles focusing on female fertility [7] were excluded.

Decellularization, Sterilization, and Gel Formation of ECM-Derived Decellularized Tissues

To prevent immune reaction and inflammation, tissue-derived material which are used for tissue engineering or transplantation should be decellularized. Therefore, chemical, enzymatic, physical decellularization methods have been developed to obtain dECM [28] (Fig. 2). These procedures can maintain the composition and architecture of ECM, similar to the original tissue [28]. Quantifying the DNA content of dECM is currently used as a significant tool to evaluate the effectiveness of these protocols in the removal of cellular debris [29]. Chemical agents which are used to solubilize cell membranes are detergents. One of the most common detergents used in decellularization processes is Triton X-100 targeting the lipid-lipid and lipid-protein interactions [30, 31]. It is very useful in cells removal, especially in those tissues where the key components of ECM are primarily proteins despite the limitation of that in decellularization of tissues with high content of glycosaminoglycans (GAGs) [32]. Sodium dodecyl sulfate (SDS) can also be used as a detergent with Triton but with more efficiency in solubilizing cell membranes. The main side effects of using SDS are related to the degradation of proteins that will change their structure and function, therefore, it is necessary to limit the duration of using SDS in the decellularization protocols [33, 34]. Enzymes are also applied in most decellularization protocols. Of course, the process of removing enzymes after the decellularization process, as well as the side effects that they can have on the structure and function of the tissue should not be ignored [35]. Trypsin attacking the C-side bonds in arginine and lysine can be used along with a breaking agent of cell–matrix interactions such as EDTA [36]. Of note, changes in the structure of ECM due to the removal of proteins such as laminin, and removing GAGs have been observed in cases of prolonged exposure to trypsin–EDTA treatment [37, 38]. Pepsin is another enzyme used for long-term with low-concentration in decellularization protocols [39]. Exposing cells to osmotic pressure using hypoosmotic and hyperosmotic solutions can be an effective way to disintegrate cells. Of course, it should be noted that in this method, the cellular residues can still be attached to the extracellular matrix. Accordingly, this method can be used as a complementary method with detergents [40, 41]. Freeze-thawing of tissues is The most commonly used physical technique in eliminating cells. This is done by forming ice crystals and then disrupting the plasma membranes which makes it a useful method for tissues that have a loose extracellular matrix structure [41]. Finally, it needs to be mentioned that a combination of these methods should be used to optimize decellularization, since, in a recent study, pre-frozen-thawed pig testicular tissues were exposed to hypoosmotic and hyperosmotic solutions and detergents [42]. Today, for sterilization, physical and chemical protocols are used to remove pathogens from the extracellular matrix, especially when they are to be used in vivo. In using this method, exposure time, preservation of matrix structure, and pathogen removal efficiency should be considered [43]. Physical protocols can be divided into two categories: heat and irradiation techniques. The use of the heat method is limited because it causes changes in the structure of the protein, but the irradiation method with gamma rays or ultraviolet light is more efficient such that gamma rays are used as an optimal method for sterilizing medical products today. Liquid chemicals such as alcohol, peracetic acid, and aldehydes are included in the list of chemical methods for sterilizing extracellular matrix [44]. In addition to altering the structure of the protein, these substances can be toxic. Therefore, they should be removed as much as possible after sterilization [45, 46]. After sterilization,

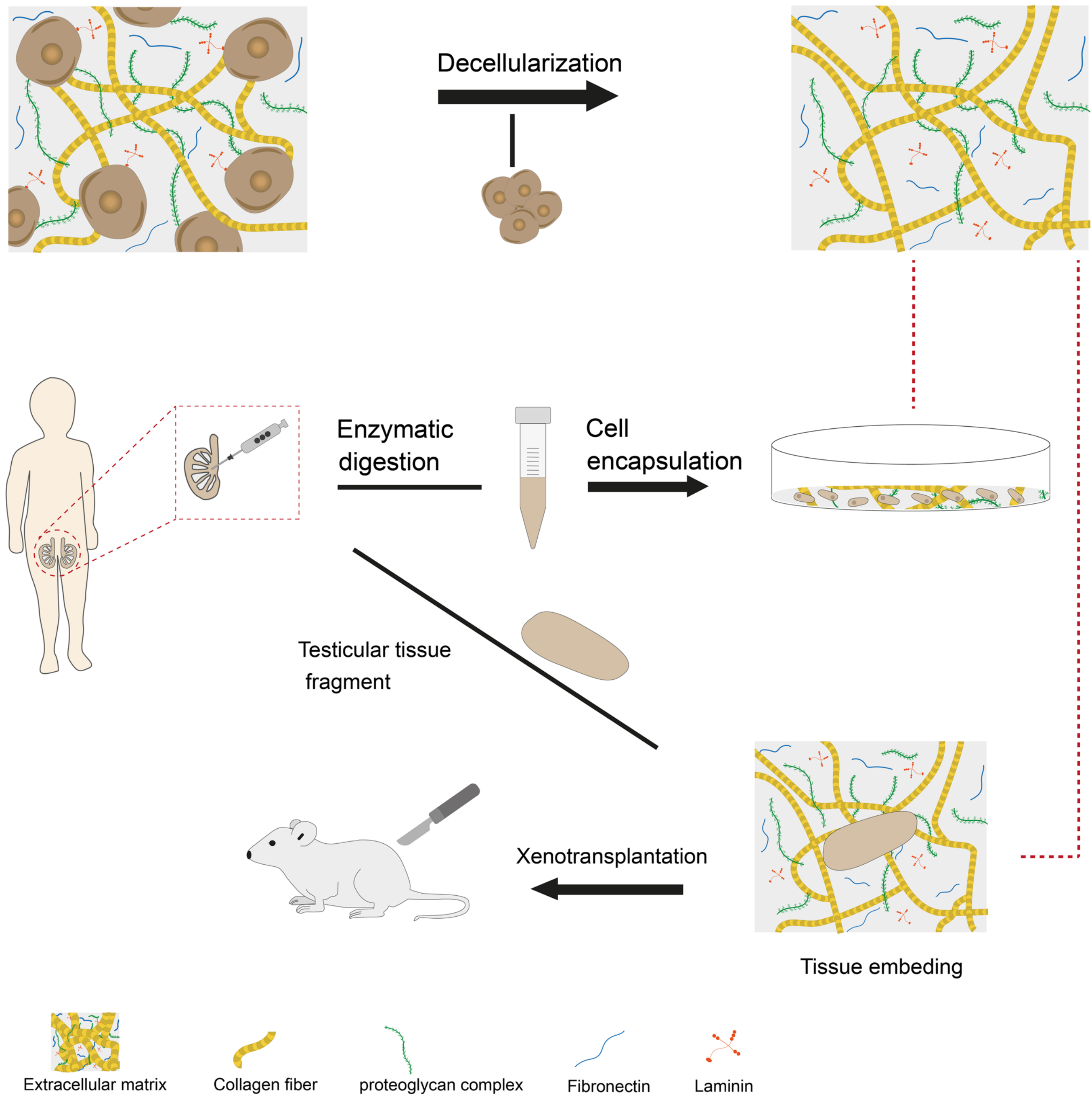

Fig. 1 The schematic diagram of the use of extracellular matrix for fertility preservation in adolescent boys suffering from cancer. The ECM resulting from tissue cellularization can be used in two ways: 1 encapsulation of testicular cells in the decellularized extracellular matrix and 2 xenotransplantations of testicular tissue fragments embedded in the decellularized extracellular matrix

the dECM is lyophilized for further application and then ground through a mixer mill. One of the methods of post-processing of the dECM is gel formation. Gel formation of the dECM makes it is easily injected into the injury site and the matrix is formed based on the shape of the lesion [47]. In addition, it can include tissue-specific cells into the matrix in the laboratory and form three-dimensional structures, or it can be implanted inside the body [48]. To obtain the gel, the lyophilized and ground dECM is digested with an enzymatic agent, most often pepsin in an acidic condition (approximately Ph ~ 2) with continuous agitation from 24 to 48 h. Then, the pH and salt concentration of the solution is adjusted to physiological state, which is an aqueous solution at 4 °C, but it becomes gel at 37 °C [49].

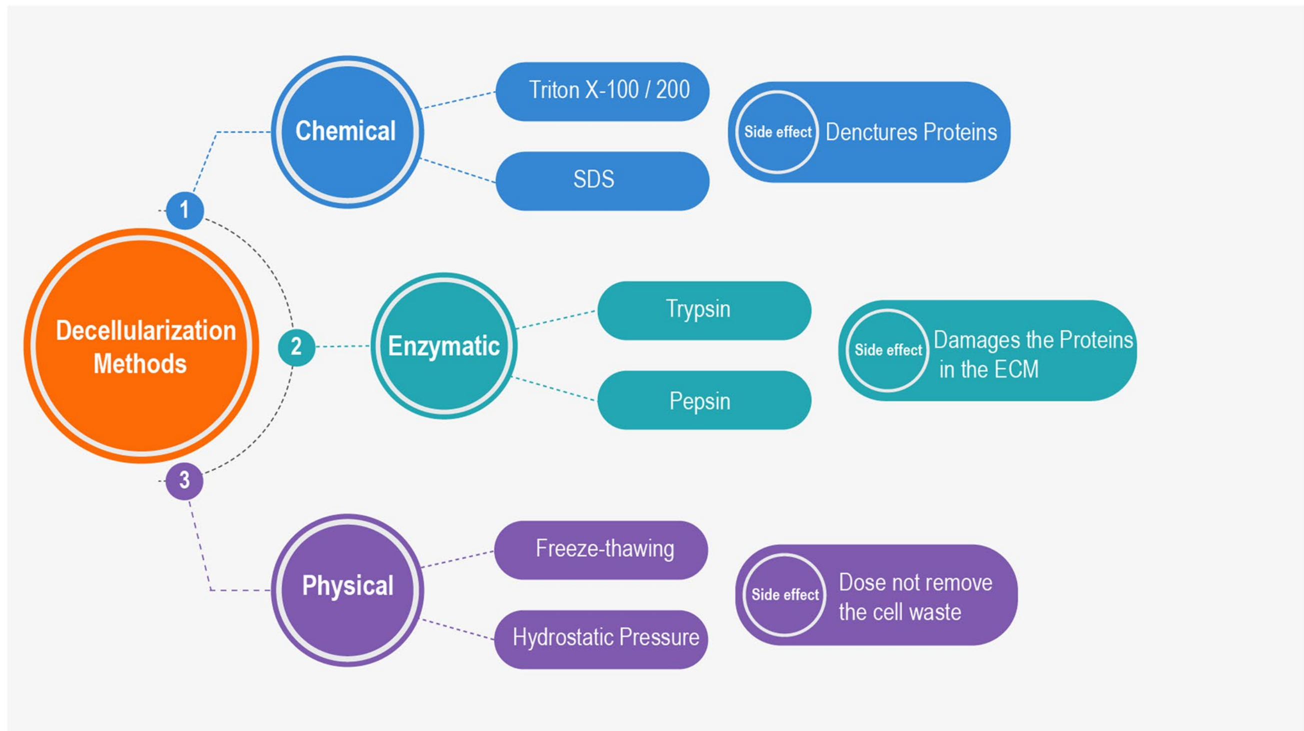

Fig. 2 The schematic diagram of the methods used for tissue decellularization

Application of the dECM-Derived Testis Tissues: In Vitro Spermatogenesis

One of the goals of researchers working in male infertility is to produce mature sperm from the clinic from testicular cell suspensions cultured in vitro, which can be applicable in assisted reproductive techniques (reviewed in [50]). Today, due to the progress of medical treatments such as bone marrow transplantation for malignancy, hereditary and metabolic diseases, and infertility complications resulting from these treatments, methods of maintaining fertility using in vitro sperm maturation have received more and more attention [6, 51]. Tissue culture and two-dimensional culture of testicular cell suspension have been considered the primary methods used to produce in vitro mature sperm [52]. The two-dimensional culture contributed to the understanding of cellular and molecular mechanisms controlling the differentiation and proliferation of spermatogonia, but this method had low efficiency in spermatozoon cell production [2]. Unlike two-dimensional culture, testicular tissue culture was most successful in producing sperm, eventually producing a live mouse offspring using assisted reproductive techniques [27, 53–55]. The tissue culture method showed that the unique arrangement of testicular cells is necessary to promote spermatogenesis in vitro; hence studies shifted to three-dimensional cultures and approaches such as scaffold-free and scaffold-based cultures to configure testicular cells in vitro were considered [2, 56]. Scaffold-free approaches using methods such as hanging drops and cellular aggregates showed less meiotic progression than scaffold-based approaches [57]. Hydrogel-based scaffolds have been used to reorganize testicular cells. Hydrogels are substances that can absorb large amounts of water, a feature that greatly facilitates the transport of nutrients, gasses, and cellular wastes [58]. In addition, they can provide mechanical support for the cells embedded in them [59]. Today, hydrogels are divided into synthetic and natural (Table 1). Synthetic hydrogels form a network with high mechanical strength but instead have less biological activity[60]. Natural hydrogels can have a protein or polysaccharide structure [61]. Natural hydrogels with a polysaccharide structure provide a more stable mechanical network over time for cultured cells but biologically lack binding ligands to react with the cells [62]. Studies have shown that testicular cells in a polysaccharide-based natural hydrogel have produced many haploid cells from SSCs [21]. However, it should be noted that new products such as RGD peptide-binding alginate have been developed today that have enhanced the biological properties of alginate by binding the cell to RGD-ligand [63]. Therefore, the use of modified alginate to support spermatogenesis can be an exciting research goal. Hydrogels with protein structures such as collagen, hyaluronic acid, fibrin, and decellularized native tissue-derived ECM have been used in in vitro spermatogenesis studies to create three-dimensional cultures

Table 1 Classification of hydrogels into two categories: synthetic and natural

Type of hydrogel	Examples	Advantages	Disadvantages
Synthetic hydrogels	Polyvinyl alcohol (PVA), polyethylene glycol (PEG), and poly-2-hydroxyethyl methacrylate (pHEMA)	High stability, the best supportive network	Biologically inactive and lack of matrix-cell interaction
Polysaccharide-based natural hydrogels	Alginate, agarose, and chitosan	High stability, biocompatible, and suitable mechanical properties	Lack of ligands for cell attachment and matrix-cell interaction
Protein-based natural hydrogels	Collagen, hyaluronic acid, fibrin, and decellularized native tissue-derived ECM	High biological activity, increase of matrix-cell interaction, and support of cellular reorganization	Sensitive to degeneration and enzymatic digestion and low mechanical support and stability

of testicular cells and have better results than three-dimensional studies [64].

These types of hydrogels are biologically active and can interact with cells through a surface receptor and play an essential role in the spatial arrangement of the cells, but because of their protein structure, they are gradually affected by enzymes secreted by cells. They are digested or deformed gradually, resulting in a change in the original network structure, finally leading to a weak mechanical structure [20, 65]. Therefore, for long-term culture, using this type of hydrogels cannot be a good option. Today, for the stability of polymer filaments of the protein-based hydrogels, different chemical cross-linkers such as glutaraldehyde [66], EDC(1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide)-NHS(N-hydroxysuccinimide) [67], and genipin [68] have been used, which create a covalent bond between the filaments and can help their stability and prevent their enzymatic digestion. Decellularized tissue-derived hydrogels are protein-based natural hydrogels that contain all the extracellular matrix components, such as hyaluronic acid, fibrin, laminin, collagen, and glycosaminoglycans [48]. Due to having a variety of extracellular proteins, these types of hydrogels are closer to the physiological state [69]. Organoids today refer to the use of extracellular matrices such as hydrogels to form three-dimensional structures in vitro, which can mimic many of the properties of the considered tissue [70]. In addition to having extracellular components, the tissue-derived matrix contains growth factors and tissue-specific cytokines that can control cell differentiation and proliferation [71, 72]. Today, testicular organoids have been used in studies as a tool to study the controlling mechanisms of germ cell niches and their role in infertility, the cytotoxic effects of drugs, and to produce fertilization-competent spermatozoa in vitro [64]. The testicular organoids are a combination of Sertoli, germ, and Leydig cells embedded in an extracellular matrix such as matrigel or testicular tissue-derived extracellular matrix to arrange the cells into structures such as seminiferous tubules [73]. It is thought that the extracellular matrix of testicular tissue can be effective in rearranging testicular cell suspension. In this regard, the first study using culture of adult human testicular cells on fragments of the decellularized testicular tissue was done and found that the scaffolds can help establish and maintain the spermatogonia niche. However, seminiferous tubules-like structures s were not formed [74]. Another study using cultured pig testicular suspension on decellularized testicular slices found that these scaffolds could not reorganize testicular cells into tubule-like structures [71]. Reorganization of the immature rat testicular cells to a seminiferous tube-like arrangement was achieved using matrigel hydrogel [75], while another study found that such structures were not in the suspension of adult human testicular cells embedded in a testicular tissue-derived hydrogel [75]. Therefore, the determining factor in forming a true testicular organoid seems to be the maturation status of testicular cells, not the type of extracellular matrix. In order to determine the impact of the maturation status of testicular cells on the efficiency of testicular organoid formation, the studies have been conducted using immature testicular cells and showed that immature testicular cells of humans, monkeys, and mice can form tubular-like structures [73]. Another factor that affects the formation of testicular organoids is the number of cells used for each organoid. The higher the cell concentration, the more testicular cord-like architectures appear [42, 57, 75, 76]. In addition to these factors, the effect of scaffold or matrix to support organoid formation is discussed [64]. Table 2 lists the studies that used natural or testicular matrix-derived hydrogels to form testicular organoids. Given the advantages of tissue-derived hydrogels, there are still very few studies comparing the effect of this type of hydrogel with other hydrogels such as matrigel on organoid formation. Therefore, according to the above, today, the testicular organoid is widely used in studies to investigate the pathogenesis and morphogenesis of testicular tissue, drug toxicity, and also as a tool to restore fertility (Table 2). In this regard, one of the components of organoids is scaffolding, which is used for this purpose from natural hydrogels such as collagen, matrigel, or hydrogels derived from testicular tissue [64]. Testicular tissue-derived

Table 2 Various applications of testicular organoid. ECM–Hydrogel: testicular tissue-driven extracellular matrix

Application	Hydrogel	Species	Result
Studies of drug toxicity	ECM–hydrogel	Human	Organoid resistance to chemotherapeutic drugs were higher than 2D cultures [72]
	ECM–hydrogel	Human	Zika virus (ZIKV) was capable to infect organoid [77]
	Matrigel	Monkey, mouse, and human	Level of autophagy increased in the organoids exposed to environmental toxins [73]
Studies of testicular physiology and pathophysiology	Matrigel	Rat	IL-1α and TNFα inhibited organoid formation [75]
	Matrigel	Porcine	Inhibition of primary cilia resulted in impaired organoid formation [78]
	ECM–hydrogel	Human	Integration of fluorescent proteins (e.g., GFP or mCherry) in cell types to study the cellular organization [72]
	Matrigel	Murine	The testicular endothelial cells plays an important role in the SSC niche.[79]
Restore fertility	ECM–hydrogel	Porcine	Assembling Sertoli cells (SCs) and germ cells (GCs) into seminiferous tubule-like structures [42]
	Methylcellulose	Mouse	Production of the morphologic spermatozoa [2]
	Soft agarose	Rhesus monkey	Producing the haploid germ cells [80]
	Decellularized	Human	Development of meiotic and postmeiotic stages from spermatogonial cells [81]
	ECM scaffold	Mouse	Production of the morphologic spermatozoa [2, 82, 83]
	Matrigel	Human	Producing the haploid germ cells [84]
		Rat	Producing the haploid germ cells [20, 85]

hydrogels can be prioritized due to their biological advantages; accordingly, it seems that the use of this type of hydrogel will be more common in future studies.

Application of the dECM-Derived Testis Tissues: Xenotransplantation of ITTs

Several studies have shown that complete spermatogenesis is obtained using grafting autologous transplantation and xenotransplantation of ITTs in other species, and even offsprings have been achieved by these techniques [86–90]. Additionally, the success of the autotransplantation technique of frozen-thawed ovarian cortex has shown that it is a clinically efficient protocol since about 100 live births have been reported with this method [8]. However, to date, no complete spermatogenesis has been observed using xenotransplantation of human ITTs to nude mice. The failure of such a method has been attributed to the severe reduction in the number of germ cells and the poor integrity of the seminiferous tubules [10, 91]. Various factors have been mentioned in connection with massive germ cells loss following transplantation of human ITTs to nude mice, including hypoxia injuries due to the lack of blood supply around the grafted tissues [12], the phylogenetic differences between species [92], and significant difference between host environment and that of donor tissue in terms of both endocrine and paracrine factors [93]. In a review of the literature, we find that methods such as tissue encapsulation in biomaterials [14, 94], growth factors administration such as vascular endothelial growth factor [14, 95, 96], and adding anti-apoptotic agents [13, 97, 98] to accelerate blood supply and maintain the survival of germ cells have yielded promising results. Biomaterials such as collagen, fibrin, and alginate, acting as a scaffold, improve the migration of host endothelial and fibroblast cells to the grafted area [14, 99]. The results of these studies suggest that tissue-derived extracellular matrix with having substances such as laminin and fibronectin (essential components for building new blood vessels) is effective in creating a vascular substrate that leads to reduction of germ cell loss due to hypoxia. On the other hand, the dECM provides inhibitory signals of apoptosis by providing interaction with the grafted tissue cells. Despite these advantages, no studies have been reported on the use of the dECM in xenotransplantation of ITTs. This knowledge gap could be addressed in future studies.

Conclusion

Today, in US centers, nearly 74% of parents of boys under the age of 12, diagnosed with an oncological disease, want to receive the services of fertility preservation. Accordingly, freezing of ITTs is performed as a strategy to maintain fertility before starting cancer treatment in many infertility centers in Europe and the US. The existence of such testicular tissues banks has put a lot of pressure on researchers and clinicians in infertility centers to meet the expectations of parents who have been received fertility preservation procedures. The strategies we itemized for fertility restoration need to be updated by the new sciences especially tissue engineering. The testis-derived dECM has several benefits

such as growth factor, fibrous structure and cell attachment can regulate organization, differentiation, and survival of the testicular cells. The dECM can also be combined with other biomaterials or drugs to optimize techniques of in vitro spermatogenesis and xenotransplantation. In addition, promising results can be achieved in future studies when proteins existing in the extracellular matrix due to their active groups on their surface are conjugated with nanoparticles or nanocapsules containing certain drugs or factors.

Abbreviations ITT: Immature testicular tissues; dECM: Decellularized extracellular matrix; SSCs: Spermatogonial stem cells; ECM: Extracellular matrix; GAGs: Glycosaminoglycans; DTM: Decellularized testicular matrix

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Author Contribution Keykavos Gholami performed the literature review and wrote the manuscript. Somayeh Solhjoo participated in the review of the literature. Seyed Mohammad Kazem Aghamir was responsible for the critical review of the manuscript.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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