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Advanced bioengineering of male germ stem cells to preserve fertility

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Abstract
In modern life, several factors such as genetics, exposure to toxins, and aging have resulted in significant levels of male infertility, estimated to be approximately 18% worldwide. In response, substantial progress has been made to improve in vitro fertilization treatments (e.g. microsurgical testicular sperm extraction (m-TESE), intra-cytoplasmic sperm injection (ICSI), and round spermatid injection (ROSI)). Mimicking the structure of testicular natural extracellular matrices (ECM) outside of the body is one clear route toward complete in vitro spermatogenesis and male fertility preservation. Here, a new wave of technological innovations is underway applying regenerative medicine strategies to cell-tissue culture on natural or synthetic scaffolds supplemented with bioactive factors. The emergence of advanced bioengineered systems suggests new hope for male fertility preservation through development of functional male germ cells. To date, few studies aimed at in vitro spermatogenesis have resulted in relevant numbers of mature gametes. However, a substantial body of knowledge on conditions that are required to maintain and mature male germ cells in vitro is now in place. This review focuses on advanced bioengineering methods such as microfluidic systems, bio-fabricated scaffolds, and 3D organ culture applied to the germline for fertility preservation through in vitro spermatogenesis.

Keywords
Male germ cells, male reproductive tissue engineering, in vitro spermatogenesis, advanced tissue engineering, male reproductive system, biomaterials, stem cells

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Introduction

Male fertility preservation is considered as an important topic in reproductive health. The development of approaches for the maintenance of farming livestock, for fertility protection in men and the conservation of scarce species have been experimentally addressed in various model systems. The current state-of-the-art for male germline or fertility conservation is through spermatogonial stem cells (SSCs) or testicular tissue cryopreservation, SSCs transplantation or in vitro spermatogenesis.[1,2] Motivated by the large number of patients struggling with infertility as the long-term side effects of oncological therapy, many studies have focused on understanding the cellular pathways involved in human male germ cell differentiation. Two new main experimental strategies have been developed, that is, I. SSCs transplantation or testicular tissue grafting into host animals[3,4] and II. SSCs

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or testicular tissue culture for the sake of male germ cell expansion and/or differentiation.[5–7] These techniques can provide forthcoming opportunities for genome conservation and fertility treatment of non-cancerous infertile men as well as adult, juvenile, and pre-pubertal cancer patients.

The underlying mechanisms of male germ cell differentiation, resulting in mature haploid spermatozoa within a structurally well-organized tissue, have been a key research topic for many decades. The bi-functional properties of the testis as a gonadal tissue and a glandular tissue means the in vitro sperm generation from male germline stem cells is still considered challenging since the entire process of spermatogenesis must be carried out within a cell culture dish.

The mammalian testicular tissue is generally divided into two distinct regions, the seminiferous tubules, and interstitial tissue.[8] It has been shown that progressive segmentation of presumptive testis tubular structures initiates from an amorphous primordium into cords (infantile/immature testis cord), and then enlargement of the testis cords occurs developing mature seminiferous tubules. Although seminiferous tubules comprise diverse testis cell types and components in young men namely SSCs, Sertoli cells, basement membrane (deposited by Sertoli cells), and peritubular myoid (PTM) cells, they become branched and the SSCs differentiate into fertile spermatozoa through spermatogenesis in adult males.[9] The Sertoli cells also experience dramatic transformation in both function and morphology at the beginning of puberty. These supporting cells produce certain types of proteins, growth factors, steroids, cytokines, and tubular fluid at different stages of development and finally form the blood-testis barrier (BTB) thereby fulfill a critical role in both the testis development and spermatogenesis.[10] Another kind of cell called Leydig cells which are also found in the testicular interstitium and secrete the steroid hormone (testosterone) in the presence of the luteinizing hormone (LH) initiating the masculinization of the male fetus and preserving postpubertal spermatogenesis.[11]

Spermatogenesis is a complex process in the mammalian testis which initiates with proliferation and differentiation of diploid spermatogonial stem cells, followed by meiosis of spermatocytes to form round and finally elongated spermatids.[12,13] To complement current knowledge from clinical studies with a description of relevant gene mutations, there is a requirement for in vitro models to shed light on the mechanisms involved in sperm generation. Fundamental new knowledge and understanding that can be gained from in vitro spermatogenesis are crucial to allow transgenic manipulation of male germ cells, and in addition in vitro systems can support the artificial maturation of immature germ cells obtained from infertile male patients.

Species-specific spermatogenic differences have remained a major obstacle toward the recapitulation of spermatogenesis in vitro.[14] Two morphologically recognizable spermatogonial subtypes are found in primates. The Adark spermatogonia are considered as the testicular stem cell (the regenerative reserve) with high-proliferative activity, however, the A_{pale} acts as progenitor cells (the functional reserve) which can divide mitotically to produce both A_{pale} and differentiating B spermatogonia. The latter is followed by further mitotic and meiotic divisions after puberty to give rise to primary and secondary spermatocytes and finally functional spermatozoa.[15,16] However, in contrast to primates, a non-progenitor buffered system exist in rodents where all seven types of A spermatogonia (A_{single}, A_{pair}, $A_{aligned}$, A1, A2, A3, and A4) result clonally and directly from a single testicular stem cell type (the A_{single} spermatogonia). The further differentiation of A spermatogonia through incomplete mitosis form intermediate and B spermatogonia which have cytoplasmic bridges. The subsequent maturation of B spermatogonia leads to primary and secondary spermatocytes, spermatids, and spermatozoa commitment, respectively[17] (Figure 1). It is noteworthy that these physiological differences in testicular tissue could be related to the different lifespan of small rodents (short) compared to primates (long) with the aim of compensating the numbers of offspring.[15]

In vitro investigations have aimed at mature and functional spermatozoa generation, and the first study of in vitro spermatogenesis was carried out with testicular organ cultures of neonatal rodents. Germ cells could maintain their normal 3D arrangement and microenvironmental composition, however, these studies demonstrated the arrest of spermatogenesis and no progression beyond the meiotic stages.[18,19] Conversely, it has been shown that it is possible to continue the in vitro differentiation of developmentally hindered round spermatids into mature spermatozoa in patients with round spermatid arrest.[20] Although in vitro mature spermatids are likely to have low fertilization ability, in the cases of successful fertilization, they are able to form normal blastocysts.[21] It should be noted that successful in vitro differentiation of human male germ cells from earlier developmental stages, starting from cultured SSCs with differentiation into mature spermatozoa, has been reported recently by Yuan et al.[22] for the first time.

Recent studies have shown that cells often exhibit unphysiological characteristics such as poor cell differentiation, less cell-to-cell communications as well as different gene and protein expression profiles in comparison to in vivo models when cultured on a two-dimensional (2D) surface as a monolayer.[23] Characterization of stem cell proliferation and differentiation has revealed acute differences in cell function and behavior between 2D and 3D microenvironments, which suggests that models of hierarchical biology in 3D structures may be required to efficiently mimic natural tissue.[24] Although conventional culture systems have been widely used to grow germ cells for assisted

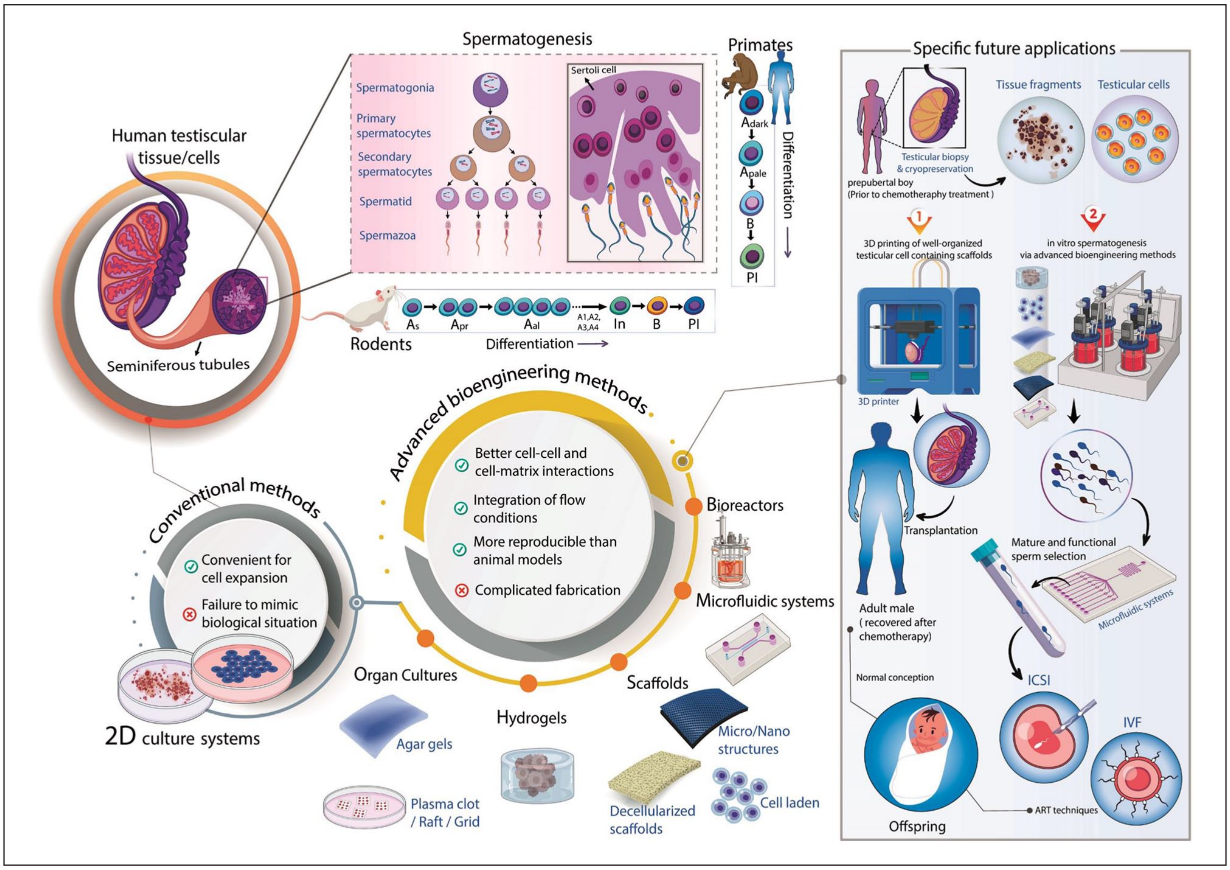

Figure 1. Schematic representation of species-specific spermatogenic cycle differences between rodents and primates (Aal: Aaligned; Ap: Apair; As: Asingle; B: Type B spermatogonia; In: intermediate spermatogonia; Pl: preleptotene spermatocytes). The current and specific future applications of advanced technologies in male fertility preservation and in vitro spermatogenesis, utilizing organ culture techniques, hydrogels, scaffolds, microfluidic systems, bioprinters, and bioreactors are illustrated and compared to the conventional culture systems. Some items created with BioRender.com.

reproductive techniques (ART), the complex physiological, functional, and spatial arrangements of testicular tissue cannot be remodeled and finally, the complete process of spermatogenesis is not able to be replicated in such 2D systems.[25] The recapitulation of physiology and morphology of the mammalian testis microenvironment outside of the body requires one more dimension compared to the 2D culture systems.[26,27] Comprised of natural and synthetic biomaterials along with testicular cells, the 3D approaches support the relevant cell-cell and cell-matrix signaling involved in spermatogenesis.[28,29] The three-dimensional culture models for testicular tissue/cell culture potentially can provide all essential parameters of this organ for both in vitro spermatogenesis or male fertility preservation.

The development of reliable and robust in vitro bioengineered testicular models introduces options for replacement of animal models in order to fulfill the 3Rs ethical principles (reduction, refinement, and replacement) and provide well-organized systems to be alternatives for scarce immature or mature human testicular tissue samples. Besides the medical ethical concerns, there are several logistic, religious, or cultural hurdles which impede male reproduction research utilizing conventional tissue/cell culture methods.[30–32] To overcome this challenge, innovative technologies and broad interdisciplinary knowledge from related areas could be applied to induce in vitro spermatogenesis or improve pregnancy outcomes (which were hindered by male infertility causes) in clinical settings in the future. Considering the existing scientific and technical developments in research settings and the potential for generating viable sperm in vitro, the next decade will bring both foreseen and unexpected opportunities and ethical challenges to traditional ideas of human and animal reproduction. Dialog with and involvement of patient groups, the general public, politicians, and regulatory bodies at an early stage is needed. It is important that these technologies are applied within a fair and open human societal framework and aid in opposing social disparities rather than adding to them. There is a clear need for ethicists and legal experts to be involved at each stage of future development and implementation.

The technologies described in this review will be the key to future solutions making use of 3D culture, advanced scaffolds, and microfluidics to provide in vitro spermatogenesis and male fertility preservation. These technologies can provide better mimics of the species-specific and age-specific arrangements of the testis and biomechanical and biochemical properties of the mammalian reproductive tract and may overcome the imperfections of in vitro 2D culture vessels (Figure 1).

Organ culture techniques in male fertility preservation

In vitro organ culture systems are considered as relevant models for the investigation of pathophysiological mechanisms which can accurately mimic the functions of an organ in various states and conditions.[33] By culturing tissue fragments or entire organ in vitro, the tissue structure can be preserved to support the natural developmental processes.[34] Organ cultures provide an opportunity to manipulate the paracrine environment and also to examine the role of each growth factor individually on the spermatogenesis process.[35]

The 3D testicular tissue culture systems are appropriate for spermatogenesis progress as they can maintain the interaction of the seminiferous tubules and interstitial area.[36] It seems that this system can be used to induce and resume spermatogenesis by in vitro SSC transplantation, in order to produce mature sperm for high-level therapeutic reproductive medicine applications.[37] Although the appropriate conditions for culture of testis tissue and testicular cells are different, the media used for organ culture are generally the same as those used for cell growth. However, such media need to be optimized by adding specific essential and effective ingredients (such as retinoic acid, luteinizing hormone, FSH, triiodothyronine, testosterone, or other sorts of vitamins, antioxidants, hormones, and growth factors) to promote in vitro spermatogenesis.[38–40]

Plasma clot, raft, and grid methods

Several methods for in vitro culture and maintenance of intact tissues have been developed since the "watch-glass method" was introduced by Fell and Robison[41,42] (Figure 3(a)–(c)). In this approach, the main goal is to provide adequate oxygen availability to explants to reduce the risk of cell death utilizing a watch glass. The plasma clot approach is one example that can be employed for the study of morphogenesis in embryonic organs or assessment of carcinogens, hormones, or vitamin functions in adult mammalian tissues.[43–45] A clot of plasma with specific active additives (embryo extract) is the key part of this method which is placed in a watch glass. In another technique called the "raft method," the fragmented tissue is placed on a floating raft of lens paper or rayon acetate mesh instead of a watch glass.[46,47] In the modified version of the plasma clot technique which is a combination of two abovementioned approaches, the explant is laid on a raft of lens paper or rayon in order to transfer the tissue easier and facilitate removal of the excess fluid. Champy showed the first result of in vitro spermatogenesis using the organ culture of rabbit testis tissue.[48] This report revealed somatic and undifferentiated germ cells survived for 7 days and in vitro male germ cell development was terminated up to the meiotic prophase. Around two decades later, Martinovitch[49] indicated that neonate mouse spermatogonia can differentiate to pachytene spermatocytes (merely based on cell morphology) on a clot consisting of equal parts fowl plasma and fowl embryo extract. This first experimental method caused preservation of the original form of numerous seminiferous tubules for 17 days cultivation, but could not support germ cells growth after the 20th day.

One stumbling block of the raft floating approach is that it did not prevent the immersion of tissues into the medium which led to the establishment of a grid system by Trowell.[50] The grid system consists of perforated steel sheets in which the tissue of interest is laid on before being placed in a culture chamber filled with fluid up to the grid. Regarding different stiffness levels of tissues, skeletal tissues are usually placed directly on the grid, but softer tissues, such as the skin or glands, first need to be laid on rafts so that can be held over the grids. Several organs of adult rats such as the testis were cultured on this system but the results revealed that most tubules had degenerated with only a few tubules surviving for up to 3 days.[18,51,52]

Agar gel method

In this method introduced first by Spratt,[53] a combination of agar, embryo extraction and horse serum is used as a medium for organ culture, furthermore, the defined media supplemented with or without serum also can be utilized with agar as alternatives options. Although agar-based media offers a suitable environment for the culture of embryonic organs, adult organs almost not stay alive on such medium.

Until now, different studies have investigated the potential features of an agarose gel layer as a supporter and inducer of spermatogenesis.[54–57] It is worth saying that this agarose-based system can be used as a support layer for testis organ culture in either fragmented or whole testis and for the culture of host testis fragments.[34,58] Some researchers have reported successful spermatogenesis induction by use of agar gel supports in 3D organ culture (Table 1). In 2011, Sato et al.[58] designed a new in vitro organ culture system onto which mouse SSCs lines are transplanted and can form colonies and differentiate up into fertile sperm. The obtained haploid cells characterized based on both cell morphology (histological, immunohistochemical examinations) and genetic markers (SYCP-1, SP56), gave rise to healthy offspring when tested using micro-insemination. The authors

Table 1. Organ culture systems for male reproductive preservation.

3D cell culture system	Cell or tissue type/cell source (transplanted)	Species	Progression stage of spermatogenesis	Results	References
Plasma clot method	New-born mice testis tissue	Mouse	Spermatocytes (pachytene phase) identified through cell morphology.	Seminiferous tubules structures were preserved up to 17 days cultivation and then start degradation	Martinovitch[49]
Agarose gel	Testis fragments (4.5–14.5 day post-partum (dpp))	Mouse	Round spermatids identified through gene marker and chromosome analysis (Gsg2-GFP and Acr-GFP).	This liquid-gas interphase method supported the growth of the seminiferous tubules in size (at 14.5 dpp)	Gohbara et al.[60]
Agarose gel	Testis fragments (12.5–15.5 days post-coitum (dpc))/testicular cells (5.5 dpp)	Mouse	Haploid cells identified through morphological, histological, immunohistochemical, and gene markers evaluations (SYCP1, Gsg2-GFP, and Acr-GFP).	GFP-GS cells injected into seminiferous tubules stayed until a few days, although cell migration toward the periphery of the tubules started after 2 h. The differentiated fertile sperms from spermatogonial stem cell lines could generate five healthy offspring via micro-insemination approach.	Sato et al.[58]
Agarose gel	Pups testes (0.5–11.5 dpp)	Mouse	Haploid cells identified through morphological, histological, and immunohistochemical evaluations (SYCP3, SYCP1, AR, Gsg2-GFP, and Acr-GFP).	In vitro spermatogenesis was supported over 2 months using this method and the obtained spermatids and sperms could give rise to fertile offspring through ROSI and ICSI.	Sato et al.[29]
Agarose gel	Testis fragments (5.5–10.5 dpp)	Mouse	Haploid cells identified through morphological, and immunohistochemical evaluations (Acr-GFP-GS).	Differentiated fertile sperm from germ-line stem (GS) cells generated healthy offspring through micro-insemination	Sato et al.[55]
Agarose gel	Thawed neonatal testis fragments (0.5–5.5 dpp)	Mouse	Round spermatids and sperms identified through morphological, and immunohistochemical evaluations (SYCP1, Gsg2-GFP, and Acr-GFP).	The sperm obtained from resumed spermatogenesis of cryopreserved immature testis tissues generated eight healthy offspring through micro-insemination.	Yokonishi et al.[61]
Agarose gel	Fetal testis fragments (12.5–19.5 dpc)	Mouse	Haploid cells identified through morphological, and histological evaluations (Acr-GFP).	The agarose gel stand supported thickening and enlargement of the seminiferous tubules. Spermatogenic progression up to the haploid cell only observed for fetal testis of 14.5 dpc or older.	Kojima et al.[62]
Agarose gel	Seminiferous tubules and testicular cells (2- to 6-day-old)	Mouse	Spermatozoa identified through morphological, histological, immunohistochemical, and gene markers evaluations (SYCP3, Plzf, Itga6, and Acr)	Seminiferous tubules were grown in size on a modified soft agar culture system while maintaining their specific arrangements similar to that in vivo. Appropriate Sertoli and germ cells interactions facilitated by this method could improve in vitro spermatogenesis to the morphologically mature spermatozoa.	Gholami et al.[57]
Agarose gel and PDMS-ceiling chip	Testis fragments (1 dpp)	Mouse	NA	Spreading explants on agarose gel stand by applying an external pressure through PDMS-ceiling chip led to significant tissue size increment and also prevented testis tissue necrosis and central degeneration.	Kojima et al.[63]
Agarose gel	Testis fragments (4.5–6.5 dpp)	Mouse	Haploid cells identified through histological, immunohistochemical examinations (SYCP1, Tra98, Sra8, γH2AX, and Acr-GFP)	This agar-based organ culture method could promote spermatogonial cells differentiation up to meiotic divisions in reference to the type of supplemented chemically defined medium	Sanjo et al.[39]
Agarose gel	Testis fragments (young cat and mouse (5dpp)	Cat, Mouse	NA	Cat testis fragments cultured on agar gel blocks did not initiate germ cell differentiation unlike results obtained in mouse controls but several germ cells were survived after 6 weeks in vitro culture.	Silva et al.[64]
Agarose gel	Testis fragments (4–6 week-old)/spermatogonial stem cells (obstructive azoospermia)	Mouse/ human	NA	This organ culture system could support homing of human SSCs in vitro transplanted in recipient mouse testis until 2 weeks but did not indicate further maturation.	Mohaqiq et al.[65]
Agarose gel	Testis fragments (adult)	Fish	Spermatids or spermatozoa identified through histological, immunohistochemical examinations (SYCP3).	The agarose gel stand support medaka whole testis culture and in vitro spermatogenesis by providing a gas-liquid interface.	Kang et al.[56]
Agarose gel	Testis fragments (4 week-old)/spermatogonial stem cells (obstructive azoospermic)	Mouse/ Human	Spermatozoa identified through histomorphometric, immunohistochemical, and gene marker examinations (SYCP3, PLZF, Tekt1, ACRBP, and TP1).	In vitro spermatogenesis induction was achieved by culturing host mouse azoospermic testis fragments (transplanted by human frozen-thawed SSCs) on agarose gel	Mohaqiq et al.[34]
Agarose gel	Testis fragments (12- to 19-week fetuses)	Human	Spermatids identified through histological, immunohistochemical (SYCP1, SYCP3, PRM1, SOX9, CYP17A1, γH2AX, MLH1, DDX4, PLZF), FISH, CNV, STR analysis, and bisulfite sequencing.	Mature seminiferous epithelium was formed in human testis fragments cultured on agarose gel stands ROSI of *in vitro*-derived spermatids led to the embryo development to the blastocyst stage	Yuan et al.[22]

further suggested that the developed system could generate sperm from SSCs with the observed spermatogenic arrest caused by a microenvironmental deficiency in their original testes. Soft agar gels also provide protective effects against ischemia and were found to be beneficial for prolonged cultures. In 2013, Yokonishi et al.[59] cultured immature mouse testicular tissue on agarose gels (1.5% (w/v)) under culture conditions. Above and beyond the generation of sperms, which are identified by morphological, histological, Immunohistochemical evaluations, they pointed out other advantages of this approach as follows: (1) viable testis tissue fragments after the freeze-thaw process leading to the generation of fertile haploid cells, (2) experimental repetitions were more feasible, (3) preservation of in vitro spermatogenesis for more than 2 months.

In recent years, in vitro transplantation of SSCs to testis and organ culture of host testis for full spermatogenesis induction are considered a significant accomplishment. For instance, Sato et al.[55] offered an organ culture system that supports sperm generation from mouse germ stem cells (GSCs) when transplanted in tissue fragments. Sperm formation from GSCs takes approximately 6 weeks for mice[58] and 8 weeks for humans[34] and only mouse sperms were viable and resulted in healthy offspring.

Furthermore, a research team recently presented an innovative organ culture system as a potential model for spermatogenic regulation investigation by wholly culturing the marine medaka testes outside of the body as a result of their small size. An agarose gel stand was the key part of this system which provided a gas-liquid interface for the culture of adult Oryzias dancena (a type of fish) driven whole testes. Their result according to the morphology and genetic investigations showed that germ cell proliferation preservation and germ cell differentiation induction was achieved via the organ culture of the medaka whole testis on an agarose gel stand.[56]

As a remarkable breakthrough for male reproductive research, a very recent study carried out by Yuan et al.[22] reported robust modeling of in vitro human testicular organogenesis from the fetal genital ridge. The human gonads of aborted 12- to 19-week fetuses were separated into fragments and were laid on agarose gel stands. Functional seminiferous tubules were formed in this 3D system which was supplemented with components such as 10% KSR, BMP 4/7 (20 ng/ml), SCF (20 ng/ml), Activin A (100 ng/ml), testosterone (10 mM), FSH (200 ng/ml), and BPE (50 mg/ml), and could support both spermatogonia self-renewal and the maturation of haploid spermatids. Furthermore, the development of the resulting embryo to the blastocyst stage via ROSI proved the functionality of in vitro-derived spermatids with a fertilization rate of 12.5%.

Hydrogel applications in male fertility preservation

Hydrogels are known as a group of scaffolds providing a temporary tissue-mimicking environment for cells to become attached, efficiently proliferated, differentiated, and even regenerated. Hydrogels are 3D self-assembled hydrophilic biopolymer networks that consist of highly interconnected microscopic pores and are therefore capable of binding and absorbing a large quantity of water as well as biological fluids. Hydrogels can effectively provide a cell-compatible and mechanically stable microenvironment which can disseminate and transport vital nutrients and cell-secreted molecules and can also stimulate specific cellular responses.[66,67] Their biocompatibility, their similarity to the native extracellular matrix, and the ease of processability make them promising scaffolds for well-engineered culture environments with replicated anatomical structures and primary functions of a particular tissue.[68] It is well known that the speed of revascularization and neoangiogenesis is fundamentally dependent upon the biophysical, chemical, and mechanical properties of the scaffold. Cross-linking and/or controlling the affinity of hydrogels in an aqueous medium are simple methods for altering the porosity and the structure of hydrogels to facilitate the migration of cells.[69,70] These appealing features provide unequivocal evidence of the capability of hydrogels and the excellent opportunity for meiotic or postmeiotic differentiation of germ cells by their application. Recently, several efforts have been directed toward the use of hydrogels for testicular tissue and cell culture, and co-culture strategies have mainly focused on the differentiation of spermatogonial stem cells into haploid sperms (Figure 2). A variety of natural and synthetic polymers have been used to fabricate hydrogels; however, natural-based hydrogels are particularly interesting 3D matrices, highly appreciated for their non-toxic and biocompatible nature (Table 2). The biodegradability and bioresorbability of these biomaterials provide cells and tissues with multi-functional 3D matrices without inducing inflammation.[68,71] Based on the fact that ECM is mainly comprised of proteins and polysaccharides[67,72], two groups of natural-based polymers including proteins (such as collagen) and polysaccharides (such as chitosan and alginate) have been utilized for the spatial arrangement of testicular cells.

Collagens are the most widely investigated polymer with a biological origin since it is the most abundant structural protein of ECM. Collagen-based hydrogels have prepared a permissive environment for culturing, differentiation, and maturation of germ cells, providing a niche for re-aggregation of testicular cells isolated from either humans or animals.[73–76,90] The similarity of their structure to ECM has provided adequate access to structural proteins, biological molecules, air (oxygen), and the growth factors secreted by Sertoli cells.[73] The presence of laminins in the structure of collagen-based gels causes beneficial effects on the viability of testicular cells. Laminins are one of the significant components of the basement membrane, possessing a remarkable modulatory role in the secretion of paracrine and autocrine growth factors, proteins, and transferring from Sertoli cells, which

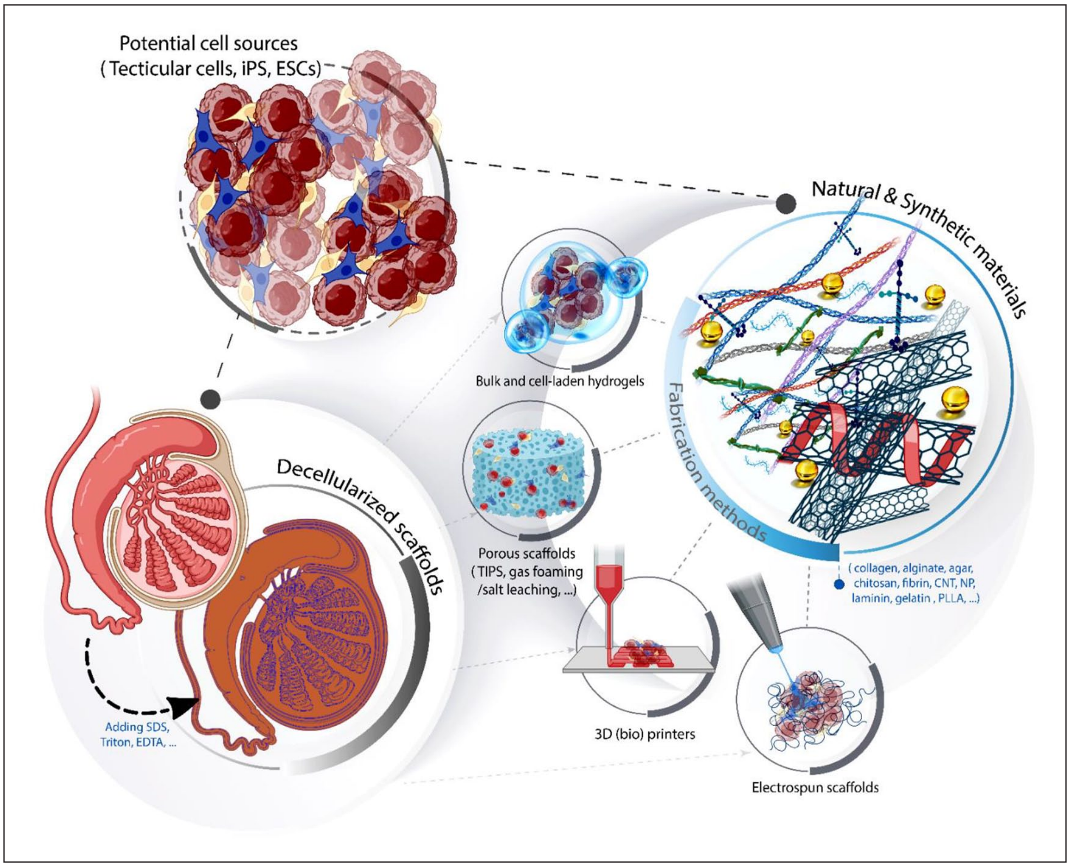

Figure 2. Schematic representation of applicable certain state-of-art technologies in male fertility preservation and in vitro spermatogenesis. Bioscaffolds in forms of hydrogels, porous, or fibrous structures can be fabricated via different approaches (electrospinning, 3D printers, thermally induced phase separation (TIPS), gas foaming/salt leaching, acellularization of tissues, and so on) using natural (collagen, alginate, agar, decellularized testis tissue-driven ECM, . . .) or synthetic (PLLA, PVA, PCL, . . .) materials. The fabricated scaffolds could be seeded with potential cell sources to accomplish their goal for male fertility preservation. Some items created with BioRender.com.

directly affect the survival and differentiation of testicular cells throughout development.[74,91–93] Direct cell-to-cell communication is one of the essential signaling routes and of extreme importance for spermatogenesis efficiency and could be well-supported by hydrogels. Using collagen gel matrices, cells could be embedded in a thick layer, providing an extracellular milieu which successfully resembles the functions of the seminiferous epithelium.[74,76] Besides these benefits, such 3D culture microenvironments protect the cells from ischemia, especially in long-term culture systems.[94]

Collagen gel solely or in combination with Matrigel has provided a great opportunity for germ cells to be in close contact and interacted actively with somatic cells and ECM. In a study carried out by Lee et al., rat testicular cells were cultured on the collagen gel (CG), or collagen + Matrigel (CGM). These matrices had the potential to re-aggregate dissociated cells and supported meiotic and post-meiotic progression and differentiation of male germ cells which were proven by analyzing DNA content and immunohistochemical examination (TP2 marker).[74] Moreover, the observation of 3β hydroxysteroid dehydrogenase-positive cells and occludin-positive cells in a cyst-like structure indicated the existence of Leydig and Sertoli cells, respectively.

Encouragingly, the cellular phenotype on collagen matrices, the cell subpopulation composition, and cell behaviors were shown to be similar to those in in vivo situations.[76] In addition to these effects, the presence of Sertoli cells in the collagen gel matrix and co-culture of these somatic cells with mouse SSCs helped the promotion of meiotic and post-meiotic differentiation through looking at mRNA expression profiles of synaptonemal complex protein-3 (SYCP3), Crem, and thyroid transcription factor-1 (TTF1).[76] This corroborates the prominent role of these cells in the propagation of germ cells in 3D culture microenvironments.

Mention must be made that Matrigel also can be employed to support in vitro male germ cell development without combination with other natural/synthetic-based

Table 2. Hydrogel-based cell culture systems for male reproductive regeneration and in vitro spermatogenesis.

3D cell culture system	Cell source	Species	Progression stage of spermatogenesis	Results	References
Collagen-based hydrogels	SSCs + Sertoli cells	Newt	Primary spermatocytes identified merely through morphological observations	Providing the air-liquid interface through placing the embedded testicular cells within a collagen matrix on a filter led to the proliferation and differentiation of spermatogonia into primary. Spermatocytes in the presence of FSH which is regulated by Sertoli cells.	Ito and Abé[73]
Collagen hydrogels/ collagen + Matrigel	Testicular cells isolated from seminiferous tubules (18 days after birth)	Rat	Zygotene spermatocytes identified through morphological, histological, immunohistochemical, and DNA content evaluations (TP2, Prm2)	These 3D culture systems based on collagen gel (CG), or collagen + Matrigel (CGM) could enhance testicular cell viability up to approximately 76% compared to the flat surface and support male germ cells differentiation by zygotene spermatocytes stage.	Lee et al.[74]
Collagen-based hydrogels	SSCs (nonobstructive azoospermia premeiotic or early meiotic maturation arrest)	Human	Round spermatids identified through morphological, histological, immunohistochemical, and DNA content evaluations (Prm2)	3D collagen gel matrix facilitates in vitro reaggregation of testicular cells and boosted the maturation of male germ cells into mature spermatids in long-term culture up to 12 days.	Lee et al.[75]
Collagen-based hydrogels	SSCs + somatic testicular cells (7 dpp)	Mouse	Meiotic and post-meiotic stages identified through morphological, immunohistochemical, and gene marker evaluations (SCP3, TTF1, Crem)	The 3D collagen-based co-culture system of somatic testicular cells (Sertoli and peritubular cells) and SSCs had favorable impacts on colony formation and induced spermatogenesis in vitro into meiotic and post-meiotic stages within 21 days culture.	Khajavi et al.[76]
Collagen-based hydrogels	Testicular cells (6 dpp)	Mouse	Primary spermatocytes identified through morphological, and immunohistochemical evaluations (DDX4, SYCP3, Ar, PLZF)	The collagen-based hydrogel by adding Knockout Serum Replacement (KSR) promoted spermatogonia differentiation into primary spermatocytes. Moreover, seminiferous tubule-like structures and blood–testis-barrier were constructed through this culture system.	Zhang et al.[77]
Soft-Agar-Culture-System (SACS) (single phase scaffold)	Spermatogonia + Sertoli cells isolated from seminiferous tubules juvenile (10-day-old) mature (30-day-old)	Mouse	Late pachytene spermatocytes identified through morphological, immunohistochemical, and gene marker evaluations (5-bromodesoxyuridine, Boule, Crem, LDH, Protamine-2, and Sp-10)	3D soft-agar-based culture system provided appropriate cell-cell contacts between germ cells and Sertoli cells and supported germ cell proliferation and differentiation up to late pachytene spermatocytes.	Stukenborg et al.[78]
Soft-Agar-Culture-System (SACS)	Testicular cell mature (8-week-old) immature (7-day-old)	Mouse	Spermatozoa identified through morphological, immunohistochemical, and gene marker evaluations (Boule, Crem, LDH, Protamine-1, Acrosin, and Sp-10)	Seeding with pre-meiotic germ cells and testicular somatic cells, the established SACS promoted germ cell development up to morphologically normal spermatozoa with intact acrosomes.	Abu Elhija et al.[79]
Agarose gel	Testicular cells isolated from neonatal testis (7 dpp)	Rat	Early pachytene spermatocytes identified through morphological, immunohistochemical, and gene markers evaluations (Kit, Zbtb16 or PLZF, Dazl, Boll, Crem, Prm1)	No meaningful differences between different culture media groups were found in the promotion of germ cell maturation; however, significant changes in the Leydig cell's functionality were detected in three-dimensional cultures. The SSCs differentiation to the pachytene stage was identified in some colonies where they were cultured into an agarose gel system.	Reda et al.[80]
Alginate and fibrin hydrogels loaded with VEGF@NPs	Testicular tissue of male NMRI mice (4–5 weeks)	Mouse	NA	Encapsulated VEGF-NPs in both alginate and fibrin hydrogels increased vascular density on day 5 for avascular testicular tissue but the highest recovery rates for spermatogonial cells were achieved with the aid of alginate hydrogels. The spermatogenesis progress was not assessed in this study.	Poels et al.[70]
Alginate hydrogel	Spermatogonial stem cells (6-day-old)	Mouse	NA	Cytotoxicity evaluation of the cell-laden hydrogel revealed alginate hydrogel could provide an appropriate microenvironment for in vitro SSCs culture owing to its antioxidant properties. The spermatogenesis progress was not assessed in this study.	Jalayeri et al.[81]
Alginate hydrogel	Spermatogonial stem cells (6-day-old)	Mouse	Spermatozoa identified through histological, immunohistochemical, and gene markers evaluations (PLZF)	Encapsulated SSCs in alginate hydrogel could be protected against damage during cryopreservation by maintaining their stemness potential. Fertility restoration in busulfan azoospermic mouse was achieved after transplantation of frozen-thawed encapsulated SSCs.	Pirnia et al.[82]

(Continued)

Table 2. (Continued)

3D cell culture system	Cell source	Species	Progression stage of spermatogenesis	Results	References
Matrigel	Testicular cells (18-day-old)	Rat	Spermatocytes identified through morphological, immunohistochemical, gene markers, and DNA content evaluations (Chk2, γ-H2AX, TH2B, TP2)	This 3D engineered Matrigel-based BTB structure showed similar organization and function to rat seminiferous epithelium, Moreover, in vitro spermatogenesis was accomplished up to haploid cells applying this system.	Legendre et al.[83]
Matrigel supplemented with KSR, RA, BMP4, SCF, and testosterone	Spermatogonial stem cells + inactivated Sertoli cells (obstructive azoospermia (OA) patients of 13–47 years old)	Human	Spermatids identified by DNA content, meiotic chromatin spread, immunocytochemical, FISH, and multiplex real-time PCR analysis, RNA sequencing, and bisulfite sequencing (γ-H2AX, TP1, TP2, SYCP1, SYCP3, Acrosin, PLZF, Prm2, Prm1, PIWIL1, PIWIL2)	Complete in vitro spermatogenesis by generating functional haploid cells was achieved by utilizing the three-dimensional-induced (3D-I) culture system	Sun et al.[84]
Three-layer gradient system (3-LGS) using Matrigel	Primary testicular cells (5–8, 20, 60 dpp)	Rat	Spermatogonia identified through histological, and immunofluorescence evaluations (PLZF, Ddx4)	Generation of testicular organoids using testicular cells and the 3-LGS indicated reconstruction of seminiferous-like structures in vitro. The 3D Matrigel-based culture system supported germ cell establishment and propagation and permitted testicular organoids development with a functional blood-testis barrier (BTB) but further germ cell differentiation was not investigated in this study. The spermatogenesis progress was not assessed in this study.	Alves-Lopes et al.[85]
Matrigel	Testicular cells (2, 8, 12, and 16 dpp)	Mouse	NA	Seminiferous tubules self-organization with BTB formation and Leydig cell differentiation were accomplished with the aid of Matrigel, however, the in vitro spermatogenesis progress was not followed up in this study.	Gao et al.[86]
Fibrin	Endometrial stem cells (hEnSCs)	Human	NA	hEnSCs were differentiated into germ cell-like cells on fibrin hydrogel but spermatogenesis progression was not investigated in this study.	Ramzgouyan et al.[87]
Tri-calcium phosphate NPs + human serum albumin	Spermatogonial cells (6-day-old)	Mouse	NA	No remarkable cytotoxicity was found with utilizing of fabricated scaffold for in vitro culture of SSCs. Spermatogenesis progression was not investigated in this study.	Yadegar et al.[88]
Chitosan-based hydrogel	Testicular tissue human (25 and 31 years of age) Rat (8- or 20-day-old)	Human and rat	Spermatozoa identified through morphological, immunohistochemical, gene markers, and FISH evaluations (TP1, TP2, Prm3)	The chitosan hydrogel-based bioreactor assisted complete ex vivo spermatogenesis from fresh or frozen rat/human segmented seminiferous tubules and elongated spermatids and spermatozoa appeared 32 and 55 days after culture, respectively.	Perrard et al.[89]

polymers.[83,84,86] Human SSCs cultured on the induced 3D system comprising Matrigel with defined media (DMEM/F12, 10% KSR, RA 2 μM, SCF 100 ng/ml, BMP4 100 ng/ml, and testosterone 10^{-6}M) are able to differentiate and generate functional haploid spermatids. The obtained round spermatids identified by DNA content, meiotic chromatin spread, immunocytochemical, FISH, and multiplex real-time PCR analysis along with RNA sequencing, and bisulfite sequencing revealed the differentiation efficiency of this culture system to be up to 17.9%.[84] In another study carried out by Fayomi et al.,[95] the implementation of Matrigel for autologous grafting of cryopreserved prepubertal rhesus testis did not exhibit any significant impacts on the graft growth, sperm recovery, and the percentage of tubules containing spermatids and sperm.

Immense attention has been directed toward either single-layer soft agar scaffolds or with a two-layered architectural arrangement in which a solid layer (for culturing supporting cells such as Sertoli cells) is placed beneath an SSCs-enriched soft gel layer.[78–80] Such arrangements more closely simulate the in vitro conditions in seminiferous tubules and appear to promote spermatogenesis by preventing the contamination of the SSC-embedded gel phase.[78,94] The provision of such a complete milieu caused enhancement of cell colony formation and clonal outgrowth into the gel phase and make these systems promising for the expansion/support of differentiation in premeiotic, meiotic, and even postmeiotic steps of spermatozoa.[79] The major advantage of co-culturing the somatic and germ cells in these arrangements is the enhancement in the extent of germ cell expansion as well as colony formation.[78,94] However, Stukenborg et al.[78] have suggested; based on mRNA expression profile and immunohistochemistry results, that the co-culture of these cells (isolated from mouse) in this type of biphasic culture media could not result in post-meiotic differentiation and this stage is only available in scaffolds where all the cells are co-cultured in a single compartment of agar gel. The presence of gonadotrophins in the culture medium and supplementation of the soft agar scaffolds with this hormone enabled the meiotic mouse germ cell colonies to be maintained continuously in the culture medium and consequently allowed the formation of late post-meiotic round and elongating spermatids identified through immunohistochemical and morphological analysis and the nuclear DNA content assessments.[94]

Alginate is another available biomaterial with high applicability for 3D cell culture as well as cell immobilization and cryopreservation.[96] Alginate possesses such unique characteristics as the ability to formulate hydrogels with 98%–99% aqueous media at physiological conditions. Attractive features of this system include the ease of de-gelling process and retrieval of cells, transparency enabling for optical and fluorescence examination, their desirable porous network, and their limited inherent cell adhesion making them of interest for cell encapsulation and cell cultivation applications.[81,82,97–101] Alginate hydrogel properties such as desirable nutrient release, oxygen diffusivity, hydrophilicity, and antioxidant activity give them the potential to enhance cell survival and support their further proliferation. Jalayeri et al. investigated the biocompatibility of alginate, encapsulating mouse spermatogonial stem cells. These hydrogels have demonstrated the decrease of apoptosis-related gene expression including Caspase3, BAX, P53, Bcl2, and FAS with no membrane disruption of spermatogonial cells, showing the non-toxic composition of these scaffolds.[81]

Alginate is also capable of attenuating the toxicity induced by freezing during cryopreservation.[102] Poels et al. encapsulated mouse testicular tissue fragments into scaffolds made of VEGF-loaded NPs/alginate and VEGF-loaded NPs/fibrin hydrogels. The follow-up histological and immunohistochemical analysis of scaffolds engrafted into male NMRI mice testis indicated the improvement in spermatogonial recovery of cryopreserved tissue engraftments and vascular density.[70] The pluripotency capacity maintenance of cells during storage is a critical issue that deserves to be taken into consideration during development of cell-therapeutic protocols.[103] Alginate-based hydrogels are one of the best currently available choices for cryopreservation of transplantable SSCs and embryonic cells both to preserve their pluripotency, and for encapsulation and protection of these cells to enhance survival during freeze-thaw cycles and a promising microenvironment for the maintenance of animal spermatozoa motility during cryopreservation without compromising their functional integrity.[82,98,99] Interestingly, encapsulation of semen and bovine spermatozoa in alginate, solely, or in combination with other ions such as calcium, could cause prolongation of the preservation and allowed their release.[104–106]

Pirnia et al. used the alginate hydrogel system for encapsulation of mouse spermatogonial stem cells during the cryopreservation process. The paper provides an in-depth comparison between the stemness status, colonization potential, and viability percentage of SSCs before and after freeze-thaw cycles. The average diameter of the alginate beads was 3 mm and the encapsulation of these cells in hydrogel beads caused elevation of two markers levels for SSCs (Lin28a and Sall4 stemness genes), while the expression of other stemness markers such as Oct4, Nanog, and PLZF decreased, significantly. These results also demonstrate that no differentiation could be observed from freeze-thaw cycles themselves. The restoration of spermatogenesis was also successfully achieved, with characterization relying on histological staining and gene marker assessment after cryopreservation at cell-culture substrates.[82]

It is worth noting that the differentiation processes of SSCs occur in the basal layer. Cells dwelling in this layer develop interactions through integrins (as a class of cell surface receptor) with the basal layer matrix, in which proteins with Arg-Gly-Asp (RGD) peptide sequence play a prominent role in regulating their binding.[107,108] Thus, the inclusion of RGD peptides in the structure of hydrogels is routinely used to simulate the interactions in basement membranes and has the potential to engineer and regulate the proliferation and differentiation of SSCs. Alginate, with the ability to be conjugated with oligopeptides, successfully prepared a hydrogel matrix for function modulations of a broad array of stem cells, especially SSCs.[108]

In addition to the aforementioned achievements on gel matrices for 3D culturing, ex vivo culturing SSCs has also been investigated. Hydrogel-based 3D microenvironments, in part, could allow spermatogenetic processes by providing a milieu for testicular cells similar to in vivo conditions. To achieve complete human and rat spermatogenesis, hydrogel-based bioreactors (formed from chitosan) have been introduced.[89] These bioreactors were comprised of a hollow cylinder of chitosan and were utilized for the prolonged culture of testicular tissue (specifically the seminiferous tubule) of either rat or human subjects. Some important observations (according to the morphology and gene markers such as Tp1, Tp2, Prm3, Cx43) were reported ex vivo culture (until 60 days) of these tissues including completion of spermatogenesis with the appearance of morphologically mature spermatozoa with an efficiency similar to that in tubule segments. These and other observations make this design architecture promising for clinical application to provide enough spermatozoa for intracytoplasmic injection of sperm in patients. To carry out a high throughput analysis of germ-to-somatic cell associations, Alves-Lopes et al. generated testicular organoids by encapsulation and cultivation of rat primary testicular cells in microscale droplets called the three-layer gradient system. This system consists of three layers, where the intrinsic core was made out of cells incorporated into Matrigel, and two external layers which were formed from pure Matrigel. This system was designed for in vitro mimicking germ-to-somatic cell communications.[85]

Bioscaffold applications in male fertility preservation

Biodegradable 3D scaffolds have emerged as potential templates for regenerative medicine for reconstitution and stimulation of different tissues.[109] Conceptually, 3D transplantable scaffolds are promising routes to prepare interconnected networks as niches for homeostasis of different tissues and supporting the maintenance, recruitment, and differentiation of isolated cells.[72] One of the most important benefits of 3D scaffolds is the provision of physical and chemical signals suited for homing, proliferation, and growth of cells. Several studies reported unambiguous and direct evidence that suitable cell-cell interactions could be obtained between spermatocytes and Sertoli cells via these microenvironments leading to the formation and self-renewal of daughter cells.[75,110] Some mechanical characteristics of scaffolds such as stiffness, pore size/porosity, and elasticity are highly dependent on the specific synthesis conditions such as the concentration of the preformed biomaterial, solvent system, or operational temperature.[112]

Several synthetic and natural-based biomaterials have been utilized for generation of these supportive culture systems (Figure 2). In addition to these biomaterials, acellular tissue matrices, obtained via decellularization techniques, have been used in preparation of transplantable scaffolds for reproductive applications[112] and each approach possesses specific advantages and applications (Table 3). Natural-based polymers are valuable due to their cytocompatibility and biologically-potent nature; many synthetic mimics can be synthesized and their mechanical and degradation properties are believed to be well-controlled.[113,114]

Decellularized scaffolds

Acellular tissue matrices, fabricated via decellularization of different tissues or organs, are capable of keeping the vital components, functional molecules, and growth factors of the ECM and their activities unchanged to a sufficient extent to provide integrity for cell growth.[133] These collagen-rich matrices with unique natural ultrastructure can be provided from xenogeneic or allogeneic tissues.[77] The close similarity in the structure of specific organs between humans and other animals such as monkeys or pigs can support a huge supply of decellularized scaffolds for tissue engineering. Removal of cellular components and debris from the tissues can overcome potential toxicity, diminishing their negative effects, and minimizing any interference with the structural integrity.[134] While the bioactivity of growth factors such as bFGF, TGF-β, and VEGF present within the scaffolds remains unaltered even after prolonged preservations.[135] A variety of physical, chemical, and even enzymatic decellularization protocols have been introduced depending upon the tissue type, tissue density, and biological properties.[116,134] These scaffolds are commonly derived from tissues by immersing them into appropriate detergents/solutions which are able to disrupt the bonds between cells and the ECM and dissolve cellular materials and cellular debris (for instance: sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), sodium hypochlorite, and Triton (X-100)).[28,116,136] Decellularized testicular matrices offer an ideal platform for growth and migration of testicular cells. Although in these matrices, cells, DNA, bioactive

Table 3. Different scaffolds applied in male fertility preservation, with a variety of morphologies, materials, and cells.

Type	Material/fabrication method	Cell type	Species	Progression stage of spermatogenesis	Results	References
Decellularized scaffolds	Decellularized testicular matrix (DTM) Reagents: Triton X-100 SDS	Testicular cells	Human	N/A	Maintaining the native three-dimensional human testicular tissue structure, DTM scaffolds showed appropriate cell attachment/infiltration and cytocompatibility.	Baert et al.[28]
	Hanging transwell inserts containing decellularized testicular matrix (DTM)/agarose Reagents: Triton X-100 SDS	Testicular cells Adult or pubertal tissue	Human	Spermatogonia identified through immunohistochemical evaluations (UCHL1, UTF1, DDX4, and FGFR3)	The developed scaffold-based culture system support spermatogonia proliferation without further differentiation.	Baert et al.[115]
	Decellularized pig immature prepubertal testicular tissue Reagents: Triton X-100 SDS Trypsin EDTA	Sertoli cells Adult	Human	N/A	Comparing certain protocols for decellularization of pig testicular tissue revealed the combination of two detergents (SDS-Triton (ST) 0.01%) for decellularization, developed a suitable environment for Sertoli cell attachment and proliferation and suggests application of such scaffolds in reproductive biology.	Vermeulen et al.[116]
	Decellularized porcine immature testicular tissue hydrogel (tECM) and collagen hydrogel Reagents: Triton X-100 SDS	Testicular cells	Pig	Spermatogonial stem cells identified through immunohistochemical evaluations (DDX4)	Germ cells population in both tECM hydrogel and collagen hydrogel significantly decreased within 45 days tECM hydrogel kept more Leydig cells compared to collagen hydrogel and offer better preservation of growth factors and functionality of Sertoli cells and Leydig cells were preserved for 45 days.	Vermeulen et al.[117]
	Human decellularized amnion membrane (DAM) scaffold Reagents: Sodium hypochlorite solution 1.25%	induced Pluripotent Stem cells (iPS)	Human	Haploid cells identified through immunohistochemical, gene markers, and DNA content evaluations (SYCP3, VASA, DAZL, PLZF, STELLA, and NANOS3)	Haploid male germ cells were efficiently developed from induced pluripotent stem cells on the fabricated 3D DAM scaffolds compared to the 2D groups.	Ganjibakhsh et al.[118]
	Decellularized ram testicular tissue Reagents: Serial combination of SDS and Triton X-100 in PBS	Testicular cells (3–5 dpp)	Mouse	Post-meiotic cells identified through immunohistochemical and gene markers evaluations (Oct4, Stra8, SYCP3, Smc1b, Prm1, and Acrv1)	Optimized decellularization conditions were found by a serial combination of SDS and Triton X-100 for 2 days. Porous testis-derived scaffolds provided an optimal microenvironment for male germ cell growth and maturation into post-meiotic cells.	Rezaei Topraggaleh et al.[119]
	Decellularized adult mouse testicular tissue Reagents: Triton X-100 SDS	Spermatogonial stem cells (6-day-old)	Mouse	Spermatocytes identified through immunohistochemical and gene markers evaluations (PLZF, SYCP3)	3D fabricated scaffolds supported injected SSCs to successfully differentiate up to the post-meiotic stage.	Majidi Gharenaz et al.[120]
	Mice decellularized testicular matrix (DTM) hydrogel Reagents: Triton X-100 SDS	Spermatogonial stem cells (6-day-old)	Mouse	Round spermatids identified through DNA content, immunocytochemical and gene markers evaluations (Prm1, SYCP3, Stra8, Crem, Acrosin)	DTM hydrogel provided a feeder-free culture system for SSCs proliferation and differentiation up to round spermatids.	Yang et al.[121]

(Continued)

Table 3. (Continued)

Type	Material/fabrication method	Cell type	Species	Progression stage of spermatogenesis	Results	References
Macro/nano-structured scaffolds	MWCNTs/SWCNTs coated coverslips Chemical vapor deposition, Arc discharge method MWCNTs (50–100 nm × 4–7 μm), SWCNT (1.2–1.5 nm × 2–5 μm)	Spermatogonial cells of the prepubertal testis	Buffalo calves	N/A	Spermatogonial cells culture (within 21 days) on glass coverslips coated with both SWCNTs and MWCNTs showed higher cell viability was achieved for SWCNTs group due to its higher protein adsorption	Rafeeqi and Kaul[122]
	Poly (D, L-lactic-co-glycolic acid) or PLGA Combination of gas-foaming and salt-leaching method	Testicular cells (18 days-old)	Rat	Elongated spermatid identified merely through morphological, and immunocytochemical observation (TP2)	Porous PLGA scaffolds supported in vitro male germ cells proliferation and differentiation toward morphologically identified elongated spermatids	Lee et al.[109]
	Polyamide Electrospinning	Spermatogonial stem-like cells Adults (10–12 weeks) and pups (6-day-old)	Mouse	N/A	Electrospun polyamide scaffolds preserved short-term in vitro culture of spermatogonial stem-like cells but did not show any significant effects on germ cell differentiation.	Shakeri et al.[123]
	Poly-L-lactic acid (PLLA) Electrospinning	Spermatogonial stem cells (3- to 6-day-old)	Mouse	N/A	Fibrous 3D PLLA scaffold provided a proper environment for neonate fresh and frozen-thawed SSCs to proliferate in vitro during 3 weeks, however, these scaffolds did not reveal any significant effects on germ cell differentiation in this study.	Eslahi et al.[124]
	Polyvinyl alcohol/human serum albumin/ gelatin Electrospinning	Testicular cells	Human	N/A	Testicular cells were settled within the electrospun composite scaffolds and shown appropriate viability percentage (78%) during 14 days of culture. The spermatogenesis progress was not assessed in this study.	Borzouie et al.[125]
	MWCNTs incorporated into PLLA nanofibers Electrospinning	Spermatogonial stem cells (3- to 5-day-old)	Mouse	Differentiating spermatogonia identified through morphological, immunocytochemical, and gene markers evaluation (PLZF, C-kit, SCYP3)	The electrically conductive PLLA/MWCNTs fibrous scaffold combined with GDNF, BMP4, and naringenin provided a 3D environment for SSCs growth and differentiation.	Ghorbani et al.[126]
	Gelatin nanofibrous scaffolds Electrospinning	Embryonic stem cells (ESCs) + Sertoli cells	Mouse	Male germ cells identified through immunocytochemical, flow cytometry, and gene markers evaluation (Gcna, Stella, Mvh, Stra8, Piwil2, and Dazl)	Co-culture of both cell types on scaffolds caused better attachment and differentiation of embryonic stem cells toward male germ cells.	Vardiani et al.[127], Vardiani et al.[128]
	Polycaprolactone/gelatin (PCL/gel) nanofibrous scaffold Electrospinning	Spermatogonial stem cells (3- to 6-day-old)	Mouse	Round spermatid identified through immunocytochemical, and gene markers evaluation (PLZF, C-kit, Ptm1, TP1)	The electrospun PCL/gel scaffolds provided an appropriate environment for SSCs to grow and differentiate into round spermatid outside of the body.	Talebi et al.[129]
	Agar/polyvinyl alcohol (PVA) Electrospinning	Testicular cells (3- to 6-day-old)	Mouse	Meiotic and post-meiotic cells identified merely based on gene markers evaluation (Id-4, Gfrα-1, SYCP-3 Tekt-1, PLZF)	Testicular cells seeded on the 3D agar/PVA fibrous scaffolds in presence of growth factors (1 μM RA and 50 ng/ml BMP4) could proliferate and differentiate into meiotic and post-meiotic cells during 4 weeks of culture.	Ziloochi Kashani et al.[130]
	Alginate-based 3D scaffolds Bioprinter with/without alginate cell-laden ($CD49f^{+}$) inside pores of the scaffold	Testicular cells Prepubertal (<7 dpp) and adult (6 months old)	Mouse	Round and elongated spermatids identified through histochemical, and immunocytochemical evaluation (Acr3-EGFP, CREM, PNA)	Although the tubular architecture was not preserved using this approach, formation of cell spheres in the pores in the weeks following cell seeding on both cell-free scaffold (CFS) and cell-laden scaffold (CLS) were detected. Besides, larger numbers of post-meiotic cells (66%) containing elongated spermatids were achieved employing CFS.	Baert et al.[131]

(Continued)

Table 3. (Continued)

Type	Material/fabrication method	Cell type	Species	Progression stage of spermatogenesis	Results	References
	3D printed scaffold using decellularized ram testicular tissue fragments in combination with alginate–gelatin as bio-ink Decellularization/bioprinter	Spermatogonial cells (3–7 day-old)	Mouse	NA	Bioprinted scaffolds with a bio-ink made out of testicular tissue extracellular matrix/alginate/gelatin showed favorable cytocompatibility properties with a uniform surface morphology and high SSCs attachment. Although in vitro sperm production was suggested by means of this scaffold, spermatogenesis progression was not investigated in this study.	Bashiri et al.[132]

cellular proteins, and other materials with the potential to interfere with the efficacy of culture should be removed, while at the same time, the matrix should retain the main components of testicular ECM. Collagens are believed to be the most important components, especially for maintaining the ECM integrity; laminins and fibronectin along with collagens are well-established known as cell-adhesion ligands and together with glycosaminoglycans (GAGs) are vital for both attachment and migration of cells.[28,119,121] Based on these requirements, the most recent efforts on decellularization of testicular tissue have tried to optimize the protocols by which decellularized tissue can be obtained and achieve the highest levels of cell proliferation, and the most appropriate elements of the ECM structure and composition. The most effective protocols for culturing Sertoli cells have utilized a serial combination of SDS and Triton X100 (0.01%) in the decellularization step to remove cellular materials from immature porcine testicular fragments.[116] Scaffolds produced in this way showed promising potential to preserve the functionality of other vital testicular cells (Leydig cells, Peritubular myoid cells (PTMCs)), as well as SSCs.[115] Vermeulen et al. decellularized the immature testicular tissue of pigs and use it as a scaffold supporting human Sertoli cells (SCs). The biocompatible natural scaffold increased proliferation and functionality of cultured Sertoli cells. In this study, the expression of GATA4 and vimentin by SCs cultured on scaffolds was maintained until the end of the culture.[116] In the most recent attempt, organized testicular organoids were generated in decellularized extracellular matrix-based hydrogels to restore the fertility of males. The prepared system had an appropriate storage modulus (the capacity for energy storage in elastic deformation of the material) for the porcine testicular organoid culture and sufficiently simulated testicular ECM composition. The prepared scaffold could form seminiferous tubule-like structures and showed proper preservation of growth factors within organoids and potent regenerative capacity.[117]

Micro/nano-structured scaffolds

Different micro- and nano-structured bio-scaffolds from biocompatible natural and synthetic polymers (i.e. NPs, electrospun fibers, carbon nanotubes) have been successfully fabricated aiming to provide a niche for support, attachment, and differentiation of spermatogenic cells and for treatment of impaired spermatogenesis. Among the synthetic polymers, poly (D, L-lactic-co-glycolic acid; PLGA) and poly(L-lactic acid; PLLA) have been frequently used for production of porous scaffolds which are compatible with tissue and cell culture.[124,126,137] The thermoplastic nature of these polymers facilitates the formation of 3D scaffolds by various fabrication methods. PLGA-based scaffolds are tissue-friendly giving no evidence of malignancy or adverse effects on tissue or cell growth,[109] likely because of their biocompatible and biodegradable nature. The degradation rate of typically used biodegradable polymers ranges from weeks to several years.[112] However, efforts have been made to modulate the composition of these biomaterials to optimize the biodegradability time profile, porosity, and maximize cell adherence. The biodegradability of these scaffolds was found to be higher at high proportions of glycolic acids to lactic acids, while the other two parameters were not dependent on the composition.[109,138] These scaffolds successfully enhanced the survival of cells and induced spermatocytes toward formation of elongated spermatids. The large surface area of PLGA makes these scaffolds supportive and adherent, allowing stable attachment and spreading of cells, while their macroporous and well-interconnected structure allows nutrients and oxygen to be readily transported via the pores. PLLA nanofibrous scaffolds in combination with glial cell line-derived neurotrophic factor (GDNF 10 ng/ml) have been applied to maintain the clonogenic and differentiation potential of mouse SSCs at high levels referring to morphological assessments and spermatogonial genes expression analysis.[124] GDNF is believed to be a key factor for balancing self-renewal and differentiation of SSCs and promoting the survival of these cells.[139,140] Cell seeding on such scaffolds fabricated by electrospinning approaches caused a significant increase in the formation of cell clusters and a decrease in cell cluster size, respectively. The subsequent transplantation of these cultured cells into a mouse busulfan azoospermic model evidently revealed the homing of these cells to the basal membrane of tubules.[124]

Interestingly, using biocompatible polymers in combination with carbon nanotubes, a favorable supportive architecture can be obtained for growth, renewal, and differentiation of different cells.[137] Carbon nanotubes have exhibited an exquisite capability for the adsorption of serum proteins and promote adherence, spreading, and growth of cells.[122] Unlike most other nano-scaffolds, the cytotoxic effect of carbon nanotubes has remained a primary concern.[141] However, the direct binding of ECM proteins onto the nanotube surface potentially increases the biocompatibility of scaffolds and facilitates the adherence of cultured cells to the surface.[122] Regardless of their possible cytotoxic effect (which could be overcome by absorbing serum proteins prior to cell culture[142] or purifying scaffolds after removal of toxic metal traces[143]), carbon nanotubes can provide bio-inert supportive substrates for spermatogonial maintenance, preservation, and growth of SSCs in vitro even for long-term applications. The cell growth and survival persistence for the spermatogonial cells isolated from buffalo calves testis were observed even after a prolonged culturing period (21 days).[122] Recently, it was revealed that carbon nanotubes incorporated into electrospun nanofibers could affect the spermatogonial stem cells' fate determination. Incorporation of multi-wall carbon nanotubes (MWCNTs) in PLLA nanofibers improves the conductivity and mechanical strength of nanofibrous structures and more importantly can enhance the propagation and differentiation of mouse SSCs which were detected by histological, morphological evaluation, and associated gene markers invetigation.[126]

Electrospun 3D scaffolds are known to be highly favorable for cell seeding, because they are thought to be more morphologically and structurally similar to ECM, compared to those synthesized by other approaches.[123,127,128,144] The effect of surface topography of these scaffolds on expansion of testicular cells has thus far not been reported. Fibrillar electrospun scaffolds have been reported to provide appropriate interactions mimicking the ECM and promoting appropriate migration and morphological alterations.[144,145] Such nano-surfaces have improved the paracrine secretions from Sertoli cells, modulated the expression of genes participating in ECM formation, promoted the functionality of signaling molecules, and maintained the stemness of spermatogonial stem-like cells.[125,128,129,144]

In 2020, Kashani et al. found that PVA in combination with agar (agar/PVA electrospun nanofibers) can potentially promote differentiation of spermatogonial stem-like cells without showing significant loss throughout the initial stage of cell culture. Unlike agar-based hydrogels, the prepared electrospun scaffolds can offer a 3D culture system for spermatogonial stem-like cells, in which the viability of spermatogenetic cells is independent of Sertoli cells. This testicular-like niche was found to be potent for differentiation of mouse spermatogonial stem-like cells into meiotic and post-meiotic cells (judged by measuring the expression mRNA levels of corresponding markers), thus can be taken into account as a promising tool for male fertility preservation.[130] In another approach, Yadegar et al. fabricated 3D human serum albumin (HSA) scaffolds by incorporation of tri-calcium phosphate nanoparticles (TCP NPs—50–100 nm) and evaluated the viability of mouse spermatogonial cells on this scaffold. Their results showed that the increase of TCP concentration in HSA/TCP NPs scaffold did not alter the cytotoxic effects of the scaffold on mouse SSCs but longer incubation times caused higher cell death.[88]

Reproducing complex testicular compartments at the microscale can be achieved by a new sophisticated approach called "3D bioprinting." Layer-by-layer deposition and patterning of biological materials via 3D bioprinters can facilitate the in vitro establishment of testicular organizations at a higher resolution.[132,146] 3D cell-free/cell-containing scaffolds could be fabricated by utilizing a combination of synthetic or natural polymers to bear a resemblance to the mechanical and biological properties of testis tissue. Baert et al. recently printed cell-free (CF) and cell-laden (CL) alginate-based scaffolds to explore their impacts on in vitro spermatogenesis. Testicular cells (TC) of prepubertal mice were seeded on CF scaffolds (single-cell compartment) while CL scaffolds (double cell compartment) contained juvenile mice driven $CD49f^+$ interstitial cells. Although the native testis structure was not recreated, employing these scaffolds, some post-meiotic cells including round and elongated spermatids were observed (as revealed by specific histological and immunohistochemical staining) on this new culture system (66% of TC/CFS, 33% of $CD49f^+$/CLS).[131]

Microfluidic systems for male reproductive regeneration

There exist certain ethical and experimental limitations in the availability of enough human-related resources, and in the capability for long-time maintenance of tissues/organs outside the body making human reproduction in vitro research a challenging issue. In this regard, novel micro/nanofabrication techniques such as microfluidics have the potential to significantly enhance the efficacy of common techniques in wet labs and clinics for male reproductive regeneration. Microfluidics is defined as the technology of designing, modeling, and fabrication of devices for handling, manipulation, and analysis of small amount of fluids.[147] In the last decade, these systems attracted a tremendous amount of attendance in biomedical applications such as drug discovery and development,[148,149] diagnostics,[150] biosensors,[151] tissue engineering,[152,153] and regenerative medicine.[154,155] The polydimethylsiloxane (PDMS)-made microfluidic devices can be designed and fabricated in different patterns for various applications. These transparent gas-permeable systems allow monitoring using various microscopy techniques, microelectromechanical systems, and sensors and can be connected to the different programmable valves and pumps.

Microscale fluidic devices are introducing new generations of technologies for the research, diagnosis, and therapeutic applications in male and female reproductive disorders.[156,157] Separation and imaging of gonad cells, investigation of the basic biology of sexual stem cells, and proliferation and differentiation of spermatogonial cells are some applications of these systems in reproductive biology and medicine.[158]

The complex physiological and tubular organizations of primate testis along with the intricate endocrine regulation hinders in vitro spermatogenesis.[159] Microfluidic systems can help reproductive researchers to remove some of the barriers such as testicular cells death, limited access to primary testis cells, and lack of novel tools to mimic complexity and functionality of native tissue to successful testis tissue engineering.[160] A close relationship between the testis function and fluid dynamics within the testis has been proved which impacts both testis structure and fluid dynamics.[161,162] Sertoli cells play a key role in the secretion of fluid inside the seminiferous tubules which then flows toward the rete testis while the steroid and protein concentrations and ionic components are changed.[163] Fluid flow in microfluidic systems has specific microscopic behaviors that can mimic the fluid dynamic properties of testicular tissue microenvironments for recapitulation of functional testicular organogenesis and spermatogenesis outside of the body.

The dynamic condition and behavior of fluids in different tissues are entirely different from traditional culture flasks.[164] In vivo, blood, intercellular fluids, and lymph provide support in all tissues by exchanging gases, hormones, signals, immunologic agents, and proteins based on fluid dynamic principles at the microscale. In this complicated fluid network, especially inside tissue structures, molecules are usually exchanged via diffusion, not by temperature or pressure-induced flows. By contrast, cells in culture flasks are in contact with huge volumes of cell culture medium where the mixing mechanism is not merely diffusion, taking the conditions away from the biologically relevant ones. Here, microfluidics as a technology has the potential to contribute control of microscale and mimetic flows.[165] Accurately controlling and monitoring the fluid behavior in microfluidic channels assists scientists to develop novel microfluidic devices for the isolation of motile sperm cells from non-motile ones.[166] It is anticipated that microfluidic devices can do sperm-gender isolation due to the intrinsic behavior of sperm cells in microscale.

The culture of limited primary testis cells from patients is the other barrier in male reproductive system regeneration that can be solved using open microfluidic cell culture systems. These systems provide biologists with microenvironments that contain channels with air-liquid interfaces and reduce the risk of cell death and loss of cells due to the handling of the material during experiments. Importantly, this equipment as an advanced version of organ culture allows the study of small liquid volumes and culture of low cell numbers which are well-matched for use with rare primary cells such as germ cells. Komeya et al. cultured neonatal mouse testis tissue fragments in a simple microfluidic device. The fabricated microfluidic system was in a simple pattern and was able to maintain spermatogenesis and endocrine function of tissues for 6 months. Their device separated testis tissues and flowing medium using a thin porous membrane while the culture medium flowed in channels with the same conditions of a capillary vessel. This device also enhanced the induction of spermatogenesis compared to conventional interphase methods[167] (Figure 3(d)). The pumping of medium toward cells directly inside microfluidic systems can be harmful to the cells or tissue. To remove the mentioned challenge, the same group in 2017, reported successful induction and maintenance of mouse spermatogenesis for 3 months using a hydrostatic pressure and a resistance circuit. This pumpless system enabled a slow, longer-lasting medium flow for the nutrition of Acr-GFP transgenic mice testis.[168]

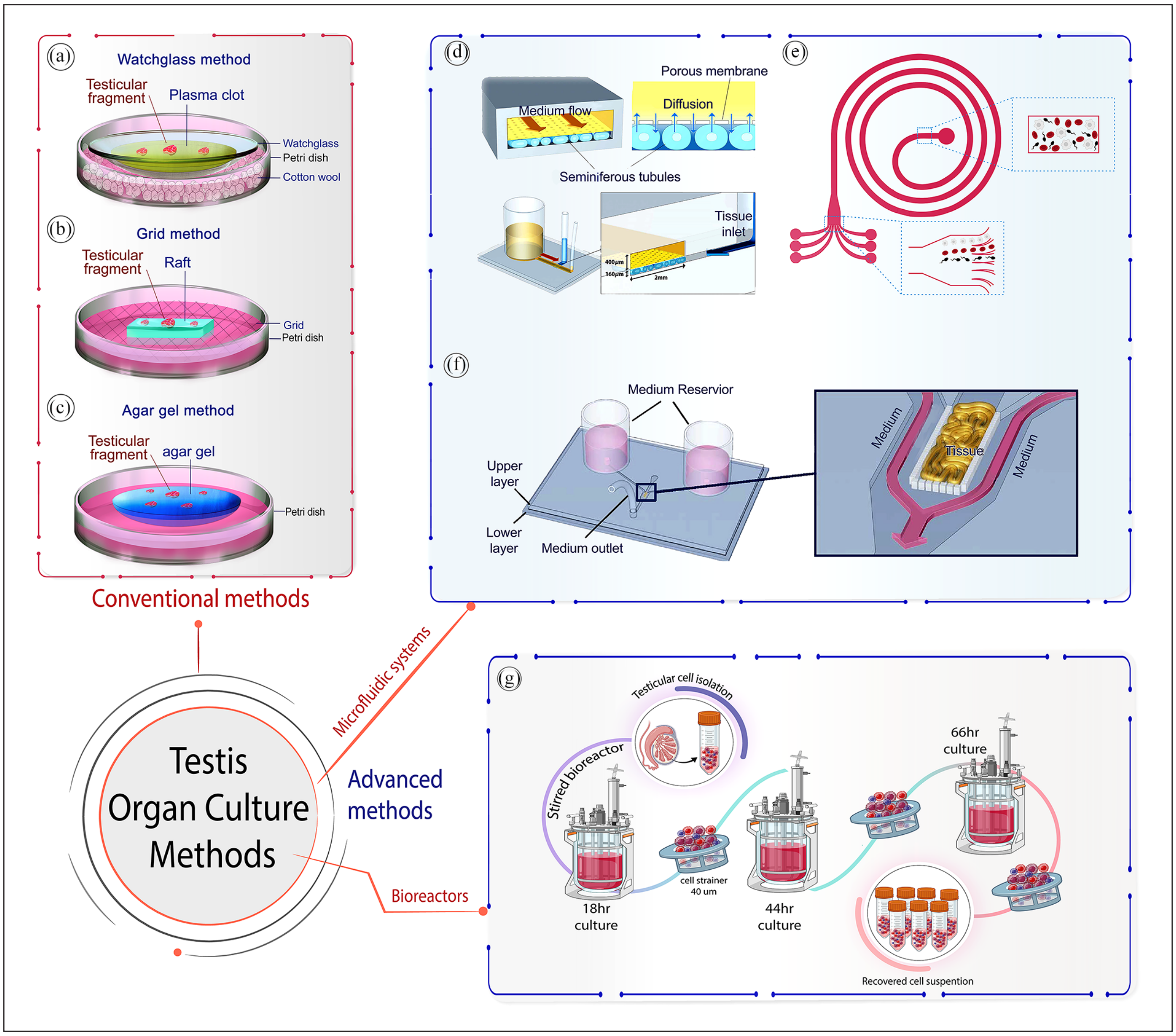

Figure 3. (a–c) Schematic illustration of conventional testis organ culture methods including watch glass (a), grid (b), and agar gel methods (c). (d–g) Schematic illustration of advanced testis organ culture approaches. (d) Long-term ex vivo maintenance of testis tissues inside an advanced pumpless microfluidic device by separated parts of medium flow and tissue[167,168]. (e) A schematic demonstrating motile sperm selection using microfluidic systems (inertial microfluidics). (f) A monolayer microfluidic device for testis tissue culture consisted of a glass substrate and a PDMS overlayer. The running medium fellows through two separate medium reservoir tanks which are connected to channels on both sides of the tissue chamber, and then combine to form an outlet (Source: Adapted with permission from Yamanaka et al.[169] Copyright 2018, Elsevier). (g) Representing bioreactor applications in male fertility preservation. The 1-week-old pig testes-driven cell suspensions were placed in stirred suspension culture and assigned to the enrichment method. The cell suspensions were filtered through a 40 µm strainer after 18, 44, and 66 h to remove larger cell aggregates and poured back into culture until the next time point until the enriched germ cell suspension is obtained (Source: Adapted with permission from Dores et al.[170] Copyright 2015, John Wiley and Sons). All images were reproduced with permission and some items created with BioRender.com.

The low efficiency and limited duration of in vitro spermatogenesis during common in vitro studies are big challenges in testis regeneration. The microfluidic technology can provide the researchers in this area with desirable culture conditions that mimic the testis tissue microenvironment. Yamanaka et al. designed and fabricated a monolayer microfluidic device as a testis organ culture system. This device induced mouse spermatogenesis successfully and maintained it for 15 weeks which is a significantly longer period than the conventional culture methods. This system is designed in a way that tissue can obtain nutrients from the medium in adjacent microfluidic channels and oxygen through the bulk PDMS (Figure 3(f)). In that work, testis tissue of Acr-GFP transgenic mouse was cultured and morphological changes of the acrosome during spermatogenesis were observed.[169]

Another major challenge of in vitro organ culture is the induction of central degeneration (necrosis) which is associated with inadequate permeation of oxygen and nutrients to the inner parts of the testis tissue. This issue significantly decreases the function and growth of cultured tissues. To overcome this issue, Kojima et al. seeded the neonatal mouse testis on an agarose gel molded into a disk shape by placing a ceiling of a microfluidic chip. The PDMS on the surface of the hydrogel is highly oxygen permeable and supports oxygen transport for the tissue layer and could prevent central necrosis and increase the growth of cells during 7 days.[63] In follow-up work in 2019, Komeya et al. placed the immature mouse testis tissues on agarose gel blocks and forced them to be spread as a monolayer using a microfluidic ceiling system. They observed that the presence of the PDMS microfluidic device chip elevated the initiation and maintenance of spermatogenesis, following by increasing the number of meiotic germ cells, and enhancing the spermatogenesis up to round/elongating spermatids which were confirmed by immunohistochemical evaluation[171] (Table 4).

Bioreactor application in male fertility preservation

Bioreactors are a class of instruments which use mechanical elements to influence biological processes such as cell culture but at a large scale. Different kinds of bioreactors have been introduced, including stirred suspension bioreactors (SSB) which were introduced about 70 years ago for cell culture in controlled environments. Deores et al.[170] developed a novel stirred suspension bioreactor for stem cell enrichment from undifferentiated spermatogonial cells using the adhesive properties of Sertoli cells from a mixed cell population prepared from pre-pubertal porcine testes in 2015 (Figure 3(g)). Thereafter, the stirred suspension bioreactors have been used for the enrichment of undifferentiated germ cells (obtained from 1-week-old pigs) using the adherent properties of somatic cells[172] (Table 4).

Future outlook

Spermatogenesis in vivo is a sensitive and complex biological process regulating by the endocrine system, and the microenvironment in the testis of different species is tuned for maximum productivity. Offering functional solutions to address male infertility treatment is a challenging yet crucial task for modern reproductive biology. Microfluidics and nanotechnology can pave the way toward the next generation of devices for infertility treatment due to their intrinsic advantages. These devices are functional with a low sample volume, for example, semen samples; thus, they can be ideal candidates for human reproduction research areas. So far, various devices have been proposed for sperm separation and selection and separation of motile and viable sperms from non-motile ones. Some of these devices are only based on the separation of sperms cells from other cells like debris, RBCs, or WBCs. Others consider the separation of motile sperm cells with high DNA integrity from other components. However, the overall yields in these devices are low, and some alternatives should be found to address this issue. Moreover, the combination of motility, thermal gradient, and PH variation of the carrier fluid in the selection of sperm cells can perfectly mimic the ideal situation of natural sperm selection. Advances in additive manufacturing also hold the promise of developing novel microfluidic channels similar to the 3D structure of female reproductive systems to better understand the mechanism of sperm selection in the human body. The addition of some active forces, including acoustic force, to these devices can increase the separation resolution of sperm cells; however, the degree to which these forces affect the sperm cells and their DNA must be critically evaluated.

It would be anticipated that home-based semen testing will improve significantly due to the enormous advancement of point of care devices during the COVID-19 pandemic. Since men usually are not in favor of laboratory-based semen experiments and analyses, the development of paper strips for semen analysis, similar to those for pregnancy tests or glucose meter, is recommended. The current commercially available semen tests measure sperm motility. Some other important factors such as DNA fragmentation index or sperm morphology analysis must also be added to the output results of these devices. Home-based semen analyzers are either qualitative or quantitative. The qualitative ones are based on visual detection; thus, they are prone to human error. On the other hand, quantitative ones require exact measurement, which is not preferable for non-expert users. Therefore, the interface of these analyzers must be improved.

New options for fertility preservation are emerging; among these, sperm cryopreservation is a viable option. However, sperm cryopreservation often results in increased DNA fragmentation index, damage of mitochondria, and reduced motility post-thaw. Therefore, it is anticipated that novel methods of sperm cryopreservation be developed to address all these issues mentioned above and increase their efficiency. Creating droplets of frozen sperm cells with high motility, viability with intact DNA fragmentation index can ideally be the next generation of sperm parcels ready for defrosting and use.

Beyond spermatogenesis and male fertility preservation, there will be an increased focus on technologies for reproductive tissue replacement or repair to address the significant challenge of providing endocrine function for future generations. The use of 3D culture systems to replace 2D techniques has demonstrated significant potential for the treatment of male infertility. Such 3D microenvironments are promising since they can mimic ECM

Table 4. Microfluidic-based and bioreactor culture systems for in vitro spermatogenesis.

Type	Materials and culture methods	Cell/tissue source	Species	Progression stage of spermatogenesis	Results	References
Microfluidic systems	PDMS/PCL/PDMS Long-term organ culture	Neonatal testis tissue (0.5–5.5 dpp)	Mouse	Spermatids and sperms identified through histological, immunohistochemical, and gene markers evaluations (SYCP3, GFP and Acr-GFP)	Circulation of culture medium flow through the tissue surface within microfluidic chambers led to long-term architectural maintenance of testis tissues. Tissue kept producing testosterone for 6 months and complete spermatogenesis by appearing haploid cells were achieved via this method. The generated spermatids and sperms could give rise to fertile offspring through ROSI and ICSI.	Komeya et al.[167]
	PDMS/PCL/PDMS Pumpless device for long-term organ culture	Neonatal testis tissue (0.5–4.5 dpp)	Mouse	NA	Fabricating of a pumpless microfluidic device by means of hydrostatic pressure and a resistance circuit instead of a power-pump resulted in slow, longer-lasting medium flow, and supported testis tissues culture for 3 months. The spermatogonial population was maintained during three months of culture but further maturation was not indicated.	Komeya et al.[168]
	PDMS/glass Monolayer device for testis organ culture	Testis tissue (0.5–5.5 dpp)	Mouse	Round and elongating spermatid detected merely based on GFP-acrosome shape (Acr-GFP)	Visualization of the cultured tissue using the monolayer microfluidic device was more precise and clearer with an inverted microscope. Tissue cultured in this microfluidic system obtained nutrients from both sides of the tissue chamber while received oxygen vertically through PDMS. Although Acr-GFP expression continued over 15 weeks, spermatogenesis in the device was intermittent.	Yamanaka et al.[169]
	PDMS/agarose gel Organ culture	Neonatal testis tissue	Mouse	NA	Tow- dimensional culture of testis tissue molded on agarose gel stand by placing a ceiling of PDMS chip shaped tissue into a flat layer so that every part of the tissue can obtain an adequate supply of oxygen. Supplemented culture media with FSH and insulin supported normal neonatal testis tissue growth over 7 days.	Kojima et al.[63]
	PDMS/agarose gel Organ culture	Testis tissue (0.5–9.5 dpp)	Mouse	Round or elongating spermatids identified through immunohistochemical examination (Acr-GFP)	Testis tissue was spread out on an agarose gel stand and a PDMS ceiling chip and thereby receiving sufficient source of oxygen and nutrients. Inducing spermatogenic differentiation up to round/elongating spermatids were achieved using 2D testis organ culture system.	Komeya et al.[171]
Bioreactor	Stirring system Cell culture	Undifferentiated germ cells (1-week-old)	Pig	NA	Stirred suspension bioreactors promoted the enrichment of undifferentiated germ cells (9-fold enrichment) by capitalizing adherent properties of somatic cells. Spermatogenesis progression did not examine in this study.	Sakib et al.[172]

conditions and provide a suitable environment for cell proliferation and differentiation. Nevertheless, the absence of circulatory systems in 3D microenvironments is considered one of the main drawbacks of these systems. Recently, organ-on-a-chip models have progressed significantly. The development of a novel organ-on-a-chip model can meet these demands by creating a dynamic situation, that is, circulation of gas and nutrients in the system in a controlled manner similar to those in the respective organ or tissue. Hence, the use of these systems might enable the potential of spermatogonial cells for successful maturation and sperm generation in the testicular tissue.

A specific example of a future application of advanced bioengineering of male germ cells is highlighted in Figure 1. Chemotherapy-induced male infertility for prepubertal boys could be addressed using state-of-the-art in vitro environments for the growth and maturation of testicular cells, isolated, and cryopreserved from patients prior to chemotherapy. Later on demand and selection of functional sperms from a pool of in vitro generated sperms by microfluidic systems for use to fertilize eggs by means of different ART techniques could provide one route to give healthy offspring. A second route could be the fabrication of cellular-based or cellular-free testis scaffolds with the same size and structure of the normal and functional tissue using 3D bioprinters for the case of prepubertal boys undergoing chemotherapy (or for other conditions of male infertility) restoring testis tissue function as adult males (Figure 1).

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Author's note

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