The Limitations of Testicular Organoids: Are They Truly as Promising as We Believe?

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Abstract

Organoid systems have revolutionized various facets of biological research by offering a three-dimensional (3D), physiologically relevant *in vitro* model to study complex organ systems. Over recent years, testicular organoids have been publicised as promising platforms for reproductive studies, disease modelling, drug screening, and fertility preservation. However, the full potential of these systems has yet to be realised due to inherent limitations. This paper offers a comprehensive analysis of the current challenges associated with testicular organoid models. Firstly, we address the inability of current organoid systems to fully replicate the intricate spatial organization and cellular diversity of the *in vivo* testis. Secondly, we scrutinise the fidelity of germ cell maturation within the organoids, highlighting incomplete spermatogenesis and epigenetic inconsistencies. Thirdly, we consider into the technical challenges faced during organoid culture, including nutrient diffusion limits, lack of vasculature, and the need for specialized growth factors. Finally, we discuss the ethical considerations surrounding the use of organoids for human reproduction research. Addressing these limitations in combination with integrating complementary approaches, will be essential if we are to advance our understanding of testicular biology and develop novel strategies for addressing reproductive health issues in males.

Key words: Spermatogenesis, testicular organoids, tissue culture, sperm, in vitro spermatogenesis

Conflict of interest

Authors confirm they have no conflict of interest regarding this review

Introduction

Organoids are three-dimensional (3D) organ-like cellular *in vitro* structures generated from induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs) or primary tissue with the ability to self-renew and differentiate to resemble a specific organ (Kanbar *et al.* 2021). In the past decade, research aiming to establish *in vitro* gametogenesis has made substantial progress in an attempt to help understand and restore fertility. Recently, interest in testicular organoids (TOs) has been gaining traction as TOs show great promise in the realm of reproductive biology. These organoids hold immense potential for various applications across scientific, clinical, and pharmaceutical/industrial domains (Figure 1). However, despite several attempts, there is yet to be a reproducible TO model that has successfully recapitulated spermatogenesis *in vitro* to yield viable sperm. The testis, unlike some other organs, presents a high degree of complexity and heterogeneity due to the presence of a multitude of cell types and cell-to-cell interactions (Guo *et al.* 2021), all of which need to be considered for *in vitro* gametogenesis. Additionally, the involvement of germ cells adds another layer of complexity, as replicating an environment conducive to the meiotic process, transitioning from a diploid to a haploid state, and understanding potential transgenerational effects pose significant challenges when considering TOs as potential clinical solutions (Yuan *et al.* 2020). This review will briefly discuss the benefits of TOs and the current progress before addressing why TOs are so difficult to generate and their limitations. This review is not aimed to denounce the possibility of generating TOs but moreover, by shedding light on these limitations, it aims to guide future research towards refining and potentially overcoming these challenges; paving the way for more physiologically accurate and clinically relevant TO systems.

{Insert Figure 1}

Benefits of Testicular Organoids

The testis is comprised of a complex microenvironment that involves numerous cell-to-cell interactions in order to create an ideal spermatogonial stem cell (SSC) niche that is crucial for the production of gametes. The complex testis microenvironment has yet to be entirely understood; thus, there are several gaps in knowledge on the inner workings of the SSC niche and spermatogenesis. One way to develop a better understand of spermatogenesis is by culturing testicular tissue *in vitro*. Successful testicular tissue culture and grafting has been carried out in animals and even produced viable sperm (Fayomi *et al.* 2019; Ntemou *et al.* 2019), however, it is yet to be carried out in humans as it maintains the risk of reintroducing cancer cells amongst other technical issue such as long term culture and cell apoptosis (Portela *et al.* 2019a). Testicular tissue culture can be carried out in a variety of different ways as seen in Figure 2, this ranges includes two-dimensional (2D) culture of dissociated tissue, culture of tissue fragments, 3D cultures and the testis-on-a-chip. Currently, most research aimed to understand the SSC niche has been carried out using animals models, for example, in the murine and porcine testes (Naughton *et al.* 2006a; Agrimson *et al.* 2016; Park *et al.* 2016; Yokonishi *et al.* 2020); however, animal models can never truly recapitulate the microenvironment of that of a human, be it due to developmental, genetic, or morphological differences (Portela *et al.* 2019b; Cunha *et al.* 2023). 2D cultures allow for some cellular interactions and structural conditions, but their cellular morphology can vary from that in tissues. The effectiveness of 2D cultures for supporting spermatogenesis *in vitro* is generally limited. Therefore, testicular organoids provide the opportunity to recreate and study the SSC niche and spermatogenesis within an *in vitro* environment whilst using human derived cells and have several advantages over the culture of tissue fragments, including more control over cell to cell interactions and large scale production (Kanbar *et al.* 2021). The 3D organoid models can act to help shed light on understanding the basic biological functions within a testis and help uncover the intricate processes that are required to produce haploid gametes. Furthermore, the organoids can be adjusted to model and study diseases and toxicology (Strange *et al.* 2018; Sakib *et al.* 2022; Yang *et al.* 2023). Hence, testicular organoids will have a wide scientific and academic impact to help uncover the basis of sperm production and post-natal testicular development.

{Insert Figure 2}

TOs also hold exciting relevance within a clinical context. Prepubertal boys only begin to produce sperm at puberty, thus, their options to preserve fertility prior to and post cancer treatment remain sparse. Recently, experimental studies on testicular tissue grafting carried out in animal models has shown that spermatogenesis may be restored for prepubertal males (Fayomi *et al.* 2019). However, testicular tissue grafting poses the risk of reintroducing cancerous cells. The *in vitro* culture of tissue in the form of organoids allows for more controlled cell selection and culture, thus, reducing the chances of reintroducing cancer cells (Vlachogiannis *et al.* 2018). Therefore, TOs may one day provide paediatric male cancer patients with a safer, less invasive, clinical option to preserve their fertility. Fertility concerns create major mental repercussions for cancer survivors (Gilleland Marchak *et al.* 2018; Sandheinrich *et al.* 2018), hence, the ability to generate viable sperm will therein not solely provide clinical but also societal benefits, as the proposed treatment may aid in preventing the added concern of infertility for many survivors of male childhood cancer. Apart from this, testicular organoids may be beneficial to adult patients with non-obstructive azoospermia (NOA) as these patients only produce SSCs, (Robinson *et al.* 2022), so long as the NOA is not due a germ cell progression genetic defect. Furthermore, this treatment may help evade the ethical or religious dilemmas regarding adoption, assisted reproductive technology or the use of donor sperm, that is faced by several cultures worldwide (Sallam and Sallam 2016).

Finally, TOs may represent a useful tool for industrial purposes for companies involved in pharmaceutical drug production. TOs act as good model systems to test against drugs and toxicity and to see how specific drugs may impact particular cells and their interactions within the testis. One group tested their organoids against chemotherapy drugs cisplatin, doxorubicin, busulfan, and etoposide, and showed that the TO cultures were able to maintain much higher IC50 values when compared to 2D culture (Pendergraft *et al.* 2017a). TOs allow for more precise modelling when compared to animal models as they can be derived from human tissue/iPSCs. TOs may also provide improved and more tailored modes of medicine as tissue samples can be collected from patients and personalised dose plans may be created in a patient-specific manner as the organoids are derived from the patient's own cells. Thus, functional TOs may be used for a variety of reasons to reap numerous benefits.

Current Progress

Organoids have begun to grow in popularity as 3D *in vitro* culture systems and have been created to mimic various organs, including the pancreas (Boj *et al.* 2015), brain (Quadrato *et al.* 2017; Mansour *et al.* 2018), intestine (Sato *et al.* 2009) (Sato et al., 2009) and have even exhibited the potential to generate embryonic organoids (Turner *et al.* 2017; Oldak *et al.* 2023). To allow for controlled growth and formation, organoids are typically cultured with differentiation factor-containing culture medium in combination with a supportive extra cellular matrix (ECM) such as Matrigel. A handful of groups have reported the successful culture of testicular organoids from single testicular cells derived from a variety of mammalian species (Table 1). Baert *et al*. (2017) were the first to generate testicular organoids from human adults and prepubertal tissue using a decellularized adult testicular ECM. However, these organoids showed no morphological similarities to a mammalian testis, although they were able to produce essential testicular hormones such as testosterone, and inhibin B, and could sustain germ cells for nearly four weeks. Alves Lopes *et al*. (2017) used a different approach with prepubertal rat cells, by applying a three-layer Matrigel gradient as an ECM-scaffold. These authors observed tubule formation and organization from germ cells and Sertoli cells and managed to sustain germ cells for almost 21 days. Furthermore, the Sertoli cells formed a functional blood testes barrier. In addition, his model was able to exhibit similar responsivity to retinoic acid (RA) as in an *in vivo* prepubertal testes environment (Endo *et al*., 2017). Pendergraft *et al*. (2017) generated organoids using human adult testicular cells *via* a hanging drop system that was morphologically different from the *in vivo* testis architecture but supported the transition of haploid germ cell. These authors used these organoids as a model to test against cytotoxic compounds and even went on to model infection from the Zika virus against the organoids (Strange *et al.* 2018); each of which mirrored expected results. Since then, there have been several more attempts to generate TOs using various species and approaches as described in Table 1. Attempts have also been made generate TOs using pluripotent stem cells cultured with testicular cells (Pryzhokova & Jordan, 2020). These cells were cultured within bioreactors and had fluorescent transgenes introduced using CRISPR-Cas9 gene-editing technology. The cells were able to exhibit proliferation and exhibited Sertoli, Leydig, endothelial, myoid cell and macrophage markers.

Recently, a new type of organ culture system has emerged that is built upon protocols from previous organoid research, including that of TOs. This technique is known as the organ-on-a-chip platform, which used a microfluidic engineered chip that harbors miniature tissue growth. Baert *et al* (2020) showed that when multiorgan chips containing human liver and testicular cells were cultured, the testicular organoids produced higher levels of inhibin B but lower levels of testosterone when compared with standalone testicular chip cultures, thus, suggesting that the liver organoids may have metabolized the steroids in

co-culture. A more recent study generated a testis-on-a-chip platform that was able to maintain cells for up to 7 weeks of culture and showed markers of various stages of spermatogenesis including meiosis II (AbuMadighem *et al*., 2022).

Despite these advancements, several hurdles remain on the path to perfecting TOs. Achieving full spermatogenesis within these organoids, understanding the nuances of the testicular microenvironment, and addressing potential transgenerational effects remain active areas of investigation.

Limitations

Incomplete Replication of the Testis Structure *in Vivo*

As previously mentioned, the testis consists of several cells that interact with one another in a highly regulated and complex process (Guo *et al.* 2020a). Our understanding of these processes remain limited as testicular cells communicate via the sharing of material occurs in very intricate ways that are delicately timed and selected for (Mäkelä and Hobbs 2019). Although most TO research has identified 3D clusters containing several cells types including Sertoli, Leydig, Peritubular Myoid cells and Stem cells (Rezaei Topraggaleh *et al.* 2019; Vermeulen *et al.* 2019b; Cham *et al.* 2021b). The proportion of cells and spatial arrangements seem to lack consistency (Table 1). The interplay between these cells is crucial to allow for spermatogenesis and testicular function *via* the generation of a refined gene network (Guo *et al.* 2020a; Chen *et al.* 2021), thus an improper and inconsistent ratio of cellular diversity proves a looming hurdle for this research. Overcoming this hurdle may contribute consistent self-renewal and long-term differentiation of the stem cells.

One must also consider the syncytial nature of germ cells within an in vivo testicular niche and the importance this syncytium has in germ cell division and differentiation (Iwamori *et al.* 2020). Although often overlooked in many organoid studies; understanding how a loss of intercellular bridges, either due to tissue digestion or passaging/splitting organoids, may affect germ cell progression is crucial. In an in vivo environment, it has been shown that losing said intracellular bridges could lead to a germ cell pachtyene block in progression (Rezende-Melo *et al.* 2020). Therefore, the widespread failure of TO culture to progress past meiosis may be partially or entirely contributed to the lack of maintenance of the syncytial nature of germ cells during the early stages of spermatogenesis. Additionally, should TO culture

surpass this challenge and somehow complete meiosis - if these intracellular bridges of germ cells are not maintained- then X- and Y-bearing spermatids could be mutually deficient for transcripts specific to the other sex chromosome. Intracellular bridges have been thought to be important for chromosome dosage compensation amongst haploid germ cells via mRNA and organelle transportation between spermatids (Iwamori *et al.* 2020). Therefore, should improper maintenance of intracellular bridges occur, one may predict that Y-bearing spermatids are unlikely to be viable and X-bearing spermatids will remain with a lack of key late-differentiation genes. This further raises a potential ethical issue: if only viable X-bearing spermatids can be produced, all organoid-based assisted reproductive technology (ART) would inherently involve sex selection of offspring.

Apart from the necessity to retain the correct testicular cell types and their ratios, the spatial organisation of these cells is pivotal in deciding the fate of the spermatozoa (Chung *et al.* 2020; Chen *et al.* 2021). In an *in vivo* environment, the cells are intricately arranged in a highly specific fashion within seminiferous tubules which play a crucial role in spermatogenesis due to their convoluted arrangements and unique compartments (Chen *et al.* 2021). Incorrect spatial arrangement of the seminiferous tubules can affect the intricate balance of hormone regulation and cell-cell communication which leads to impaired spermatogenesis (Jensen *et al.* 2022). Only a couple of groups have reported seminiferous tubule- like rearrangement of cells within TOs (Alves-Lopes *et al.* 2017; Vermeulen *et al.* 2019b; Edmonds and Woodruff 2020; Richer *et al.* 2021). Some groups have stated that this may be age-dependent, with immature tissue self-assembling more easily than adult tissue (Alves-Lopes *et al.* 2017; Edmonds and Woodruff 2020). Other groups have observed an inside-out arrangement of cells, showing the germ cells and Sertoli cells located on the outer ends of TOs while the Leydig and endothelial cells were located on the inside (Sakib *et al.* 2019b). The group who were first to generate TOs with accurate SSC architecture from larger mammalian species used porcine tissue (Vermeulen *et al.* 2019b). These authors identified correctly organised Germ and Sertoli cells within tubule-like structures with Leydig and Peritubular cells located outside the basement membrane surround the tubules. However, these researchers struggled to maintain germ cell numbers for long periods of culture and were unable to progress differentiation past the spermatocyte stage, indicating that the model lacked either correct culture conditions or cell-cell interactions conducive for spermatogenesis.

Within the TOs, testicular cells typically congregate into spherical-like-structures. This 3D structure can pose a problem that may affect functionality and viability. As an organoid increases in size, it becomes increasingly difficult for nutrients to reach the centre of the structure since the cells are tightly bound in

layers (Pendergraft *et al.* 2017a). Furthermore, hypoxia can occur due to the lack of oxygen delivered to the centre of the organoids, thus, leading to increased necrotic tissue (Sakib *et al.* 2022). This may not only damage and disrupt the development of the TOs but can also lead to the increased release of reactive oxygen species (ROS) that have been found to severely damage sperm cells and possibly have detrimental effects of fertilisation and embryogenesis (Dorostghoal *et al.* 2017; Vallet-Buisan *et al.* 2023). One way to combat increased central necrotic tissue is by splitting or breaking-up the organoids; previous studies showed that this can reduce the chances of internal cell death (Giandomenico *et al.* 2019; Qian *et al.* 2020) . However, it may still pose the risk of introducing mechanical stresses on the cells, which in turn is another limitation of organoids. Organoids, have already been found to show increased expression levels of cellular stress marker genes; for example, markers of metabolic stress, the unfolded protein response of the endoplasmic reticulum, and electron transport dysfunction (Pollen *et al.* 2019; Xiang *et al.* 2019; Bhaduri *et al.* 2020). Cellular stresses may interfere with the development of TOs; thus, understanding how to minimise cell stressors within culture is an important factor to consider when generating TOs.

Another issue that arises when generating TOs is the lack of vasculature. The absence of a vascular system in organoids can limit their size and longevity, as the cells lack important vasculature-related supplements such as nutrient delivery, metabolite elimination, endothelial cell signalling, and the formation of the blood-testis barrier (Mruk and Cheng 2015; Song *et al.* 2022). There have been several attempts to generate vasculature for organoids derived from other tissue; for example, neural organoids were cultured with the addition of ectopically-grafted endothelial-cells derived from different tissue types to attempt to create vasculature (Shi *et al.* 2020). The organoids expressed genes related to blood vessel morphogenesis and when transplanted into mice, they formed blood vessels in the grafts that aided in the survival of the organoids. Another research group attempted to induce the promotion of endothelial genes in the ESCs of human cortical organoids. These researchers were able to identify the presence of vasculature-like structures that enhanced organoid maturation (Cakir *et al.* 2019). Only one study has reported vasculature-like structures within TOs (Cham *et al.* 2021b). These authors generated immature porcine-derived TOs cultured in an air-liquid interface and found the presence of single layer endothelial-cell tubes that resembled immature vasculature. If a protocol to generate a functional vasculature system were to be established, then this would be highly beneficial as it would facilitate the maintenance of the SSC niches and spermatogenesis within TOs.

Fidelity of Germ Cell Maturation

One of the main objectives of generating TOs is to recapitulate spermatogenesis. As mentioned previously, spermatogenesis is a highly regulated process that occurs within the seminiferous tubules. Many research groups have expressed the incentive to generate TOs for pre-pubertal boys undergoing cancer treatment such as chemotherapy (Pendergraft *et al.* 2017a; Vermeulen *et al.* 2019a; Saulnier *et al.* 2021). These patients are usually left infertile post-treatment due to the gonadotoxicity of the drugs in combination with the inability of pre-pubertal boys to produce sperm that can be cryopreserved prior to treatment. Hence, the challenge lies not only in emulating the complex testis architecture, but also in priming the organoid niche to allow the SSCs to differentiate from their pre-pubertal state into one that can accommodate spermatogenesis. There have been a handful of promising studies that show the initial stages of SSC differentiation from TOs (Table 1). One research group noted a few human germ cells that changed their states from diploid to haploid (Pendergraft *et al.* 2017a). Another group reported some germ cell differentiation until the spermatid stage in TOs derived from ram cells (Rezaei Topraggaleh *et al.* 2019). Baert *et al*. (2017) identified human post-meiotic cells after 4 weeks of culture, some of which even displayed elongation (Baert *et al.* 2017). Complete spermatogenesis has only been achieved from *ex vivo* and *in vitro* tissue culture of pre-pubertal mouse testis sections (Sato *et al.* 2011; Arkoun *et al.* 2015; Delessard *et al.* 2022); this elicits inquiries as to whether this achievement stems solely from insufficient exploration within primate/domestic animal studies or if it pertains to inherent disparities in testicular development and the process of spermatogenesis between rodents and other mammalian species (Fayomi and Orwig 2018). Should the latter proposition hold true, it prompts a critical evaluation of whether the extensively utilized mouse model indeed represents the optimal choice for the study of spermatogenesis. There is yet to be a report on the actual recapitulation of spermatogenesis or even the commencement of later stages of spermatogenesis and maturation of spermatozoa from TOs. In order, to achieve this, a better understanding of the specificity of the hormones, protein and RNA markers in domestic animals and humans, as well as essential developmental time points from birth to post puberty is required. This is essential to fill much needed gaps in the field.

{Insert Figure 3}

Another challenge is accounting for the morphological and chemical changes that occur between different developmental stages. The SSC niche changes drastically between the pre-pubertal and pubertal stages of development (Figure 3). This is because the SSC niche is changing to prepare for puberty (Voigt

et al. 2023); therefore, the germ cells exist in very different conditions (Table 2). Accounting for these differences when generating TOs poses an added challenge. In human, the developmental progression of SSC niches from prepuberty to peripuberty and through post-puberty stages remains inadequately understood, which mainly due to the lack of human prepubertal testicular tissues for research. Without a comprehensive understanding of this process, the establishment of an *in vitro* 3D culture system for human testicular cells to accurately mimic and study it will remain challenging. Therefore, investigations are needed to fill this research gap. Much of the research on TOs has been carried out using pre-pubertal testicular tissue (Sakib *et al.* 2019b; Cham *et al.* 2021b; Yang *et al.* 2022); this may be because younger SSCs may possess more plasticity. Alvez-Lopez *et al*. (2017) showed that the spatial arrangement of rat organoids was age-dependent; 5-8 days-post-partum (*dpp*) rat cells were found to have faster migrations rates but formed less compact spherical-tubular structures when compared to the colonies formed from older rat cell of 20 days-post-partum and 60 days-post-partum. Edmonds & Woodruff (2020) also showed that when murine TO generation was compared amongst different age groups, the pre-pubertal age group (5 *dpp*) exhibited rapid self-assembly and growth, which the pubertal age group (12 *dpp*) did exhibit, delayed self-assembly, and the adult age group exhibited no self-assembly at all. Interestingly, the adult group was rescued when cultured with 5 *ddp* cells, thus, demonstrating the age-dependency of TOs (Edmonds and Woodruff 2020). Understanding the intricate differences between the various stages of development will shed light on why it is easier to generate TOs from younger tissue. Additionally, since the pre-pubertal stage is longer in humans when compared to most mammals - from infancy until 12 years of age - understanding and staging the developmental time points within the pre-pubertal period *in vivo* will provide crucial information to help develop TOs derived from varying ages.

Another concern about *in vitro* germ cell maturation is the rise of epigenetic inconsistencies. Germ cells undergo critical epigenetic modifications during development and maturation (Guo *et al.* 2021) which may in-fact effect the ability of the sperm cell to fertilise an egg (Vallet-Buisan *et al.* 2023). Discrepancies in these modifications within organoids can impact their authenticity and potential applications in disease modelling in addition to therapeutic interventions. Research suggests that organoid-derived cells might not exhibit the same epigenetic marks as their *in vivo* counterparts (Thalheim *et al.* 2021), thus, raising concerns about their physiological relevance. Changes such as DNA methylation, histone modifications and non-coding RNA expression can impact both the testicular germ cell and somatic cells which have been found to have long-lasting, transgenerational effects (Guerrero-Bosagna *et al.* 2013).

Technical Challenges associated with Culture

TOs are typically cultured within a scaffolding system that mimics an extracellular matrix such as Matrigel which is submerged in culture medium. The culture medium is designed to provide nutrients to the converging cells. Additionally, the culture medium can be supplemented with external factors that help the self-renewal and differentiation of the germ cells, as well as to facilitate somatic cell maintenance. Growth factors are useful tools in the maintenance and development of the organoids. Spermatogenesis requires a specific balance between hormones and growth factors (Table 3) some of which are not yet clearly understood or easily sourced. In the prepubertal phase; the levels of Follicle Simulating Hormone (FSH), Luteinising Hormone (LH), and testosterone (T) concentrations exist in low amounts. However, as puberty commences, Anti-Müllerian Hormone (AMH) levels gradually decline, while the serum levels of inhibin B, FSH, LH, and T increase; this leads to testicular growth. The onset of male puberty is marked by activation of the hypothalamic-pituitary-gonadal axis (HPGA), thus resulting in significant alterations in testicular physiology. These changes encompass an increase in testicular size, hormonal and molecular adjustments, and the initiation of spermatogenesis (Plant 2015; Koskenniemi *et al.* 2017). The process of spermatogenesis begins with a differentiating division of the SSCs and spermatogonia, followed by meiosis of spermatocytes and, finally, the formation of round spermatids. Glial cell line-derived neurotrophic factor (GDNF)(Di Persio *et al.* 2021), Fibroblast growth factors (FGF) (Takashima *et al.* 2015; Masaki *et al.* 2018) and retinoic acid (RA) (Kirsanov *et al.* 2023) are important growth factors regulating SSC development. In the developing testis, retinoic acid (RA) signalling exhibits a cyclical pattern, where its levels periodically rise and fall to regulate the precise timing of germ cell differentiation and entry into meiosis. This cyclical nature ensures synchronized development of spermatogonia and maintains the orderly progression of spermatogenesis (Gewiss *et al.* 2020). Therefore, in an *in vitro* environment, one must consider if it is indeed possible to mimic the clockwork-like nature of such a crucial hormone and this may be carried out.

As shown in Table 1, many groups have used varying approaches to generating testicular organoids with the aid of various growth factors. However, it is still not clearly understood how these specific growth factors truly effect the organoids. Some groups reported that TOs produced their own hormones, such as T (Cortez *et al.* 2022; Yang *et al.* 2022); therefore, if the TOs produce their own hormones would the addition of external growth factors be useful or even possibly detrimental? Researchers must ask, would minor deviations amongst the type of growth factors used, and their concentrations, significantly impact organoid health and development? It is also important to consider, if TOs are to be used in a clinical

setting, whether there would be substantial variations in hormone requirements for the development of TOs among various age groups or even among different individuals.

One of the most significant questions amongst researchers regarding the fate of TOs seems to be the importance of the scaffolding systems in which they are cultured. Organoids are typically cultured in a scaffolding system to maintain their 3D-like structure and provide supporting materials, the scaffold acts to mimic an in vivo ECM. Various groups have used different scaffolding systems (Table 1), including the hanging-drop method, 3D bio-printing, 3-layer Matrigel systems (Table 4). It is still not fully understood which system is ideal for TO culture. In contrast, one group showed that when murine TOs were compared when cultured in scaffold free *vs* Matrigel-based culture conditions, there was no difference in organoid growth and arrangement, rather, both groups showed multicellular assembly into tubular and interstitial compartments (Edmonds and Woodruff 2020). However, when Cham et al. (2021) compared various scaffolding vs non-scaffolding approaches for TO generation they concluded that scaffold-free derived TOs have more limitations when compared to their scaffold-based counter-parts. This is due to the increased amount of cells/volume of tissue required to produce correctly formed TOs and the lack of nutrients provided to support cell growth/maintenance, which is otherwise naturally supported by a 3D ECM due to the high porosity of the medium, then leading to increased necrosis and hypoxia (Cham *et al.* 2021a). Therefore, although TOs have been cultured in various supporting conditions, more research is required to understand exactly which scaffolding system provides the best support for the cells and to what extent they decide the ultimate fate of the cells.

Furthermore, testicular organoids suffer from limited scalability and reproducibility. As previously mentioned, there is a significant amount of variability within the existing research carried out on TOs. Variability in the quality and functionality of organoids generated from different batches of cells hinders standardized experimentation and comparative studies. There is yet to be a standardised method for testicular tissue culture with regard to culture medium, growth factors, and scaffolding. This inconsistency also poses challenges for large-scale drug screening and personalized medicine approaches.

Ethical Considerations

Finally, if TOs were to achieve complete spermatogenesis, the potential to use these sperm cells for reproduction raises ethical, social, and legal questions. While these models offer exciting opportunities

for research, the potential use of TOs for gamete production and assisted reproductive technologies raises ethical concerns related to germ cell manipulation, germ line transmission, and unintended transgenerational consequences. As little is known about both the epigenetic and genetic consequences of *in vitro* gametogenesis, one must consider how similar the generated sperm cell to its *in vivo* counterparts and if these difference may affect the development of the resulting offspring. It is also important to consider the effects and possible use or mistreatment of genetic manipulation that may be carried out prior to the completion of spermatogenesis. Understanding these nuances is critical as germ cells not only affect the next generation but future generations as well (Zheng *et al.* 2021). Additionally, as with many stem cell-related technologies, the source of cells can be a contentious issue, if the cells are not derived directly from patient tissue. Human embryonic stem cells, for instance, come with significant ethical baggage (Lovell-Badge *et al.* 2021). Striking a balance between research advancements and responsible ethical restrictions is crucial.

Conclusion

In conclusion, testicular organoids have opened new exciting avenues for investigating male reproductive biology, but several limitations impede their full utilization. In this review we have point out benefits, current progress, some limitations of TOs as well as several research gaps in this field including reproducibility, accuracy of testis reconstitution, inconstant culture systems and ethical concerns. Addressing these challenges will require concerted efforts from the scientific community to develop more robust and representative models, thus, paving the way for advancements in male reproductive health research and potential applications in personalized medicine and assisted reproductive technologies. Despite these limitations, the field of testicular organoid research holds significant promise, and overcoming these challenges is vital to unlock their full potential. Advancements in cell reprogramming techniques, tissue engineering, and microfluidics may offer solutions to enhance the complexity, functionality, and reproducibility of testicular organoids. Additionally, interdisciplinary collaboration between reproductive biologists, tissue engineers, and ethicists can foster the responsible and informed development of testicular organoids.

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Data availability statement

Data sharing is not applicable as no new data were generated or analysed during this study.

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Figure Legends

Figure 1. Development of testicular organoids and possible clinical applications. (A) Hypothetical *in vitro* spermatogonial stem cell (SSC) niche based on a testicular organoid. Dissociated adult human testicular cells can form basic organoids with tubular structures, SSCs from prepubertal testicular tissue can be added to these artificial niches for proliferation and differentiation with the support from mature somatic cells. (B) Potential clinical applications of testicular organoids. Testicular biopsies from individual patients are digested using an enzyme digestion solution and are maintained by different culture methods to form tubule-like structure testicular organoids, organoids without tubule-like morphology, and testis-specific morphology organoids in a microwell. In these culture systems, testicular cells proliferate or differentiate based on culture techniques. The amounts and combinations of multiple cells can be manipulated by researchers depending on their needs. These organoids can potentially be used for *in vitro* disease models, drug tests, and *in vitro* spermatogenesis. Figure created by author using BioRender.com

Figure 2. Engineering techniques for the culture of *in vitro* testicular tissue. *In vitro* testicular cell culture starts with organ culture, two-dimensional (2D) culture followed by three-dimensional (3D) culture and bioreactors. *In vitro* testicular organoids and testis-on-a-chip are recent engineering

techniques that provide cells a microenvironment mimicking *in vivo* conditions for SSC. Figure created by Authors using BioRender.

Figure 3. Schematic representation of a cross-section of the seminiferous epithelium and germ cell niche in human prepubertal testis (A) and adult testis (B). In prepubertal testis, Sertoli cells and spermatogonia are present at the basal surface together with peritubular myoid cells forming the SSC niche. Spermatocytes and spermatids begin to appear during puberty. Mature Sertoli cells in the pubertal testis are larger than immature Sertoli cells in prepubertal testis. Figure created by Authors using BioRender.

Table 1. 3D organoids used for *in vitro* testicular cell culture. *MEM – Minimum Essential Medium, AlbuMAX – bovine serum albumin (BSA) purified with chromatography, DMEM/F12 – Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12, FBS – fetal bovine serum HSA – human serum albumin, KSR – Knockout Serum Replacement, SCs-Sertoli cells, GCs- Germ cells, dpp- days post-partum, SSCs- spermatogonial stem cells, PTMs- Peritubular Myoid Cells, Leydig cells-LCs · DTM-decellularized testicular matrix, CG – collagen gel, CGM – collagen + Matrigel, FSH – follicle stimulating hormone, PGLA – poly D,L-lactic-co-glycolic acid, hCG- human chorionic gonadotrophin, SCF – stem cell factor, GDNF – glial cell derived neurotrophic factor, FGF – fibroblast growth factor, EGF- Embryonic growth factor.* Table created by Authors

Donor age and species	**Cell types or Full tissue sections**	**Culture medium+ protein/serum**	**Growth factor**	**Culture model**	**Maximum culture length**	**Reconstructed structure or cluster formation**	**SSC identification and SSC niche (cell morphology, structure, function, result…) Progression of spermatogenesis**	**Methods and Marker**	**Reference**
18 dpp rats	Dissociated testicular tissue	DMEM/F12 +10%FBS	10^{-7} M testosterone	3D: collagen gel (CG), or collagen + Matrigel (CGM) contains laminin (56%), collagen IV (31%), entactin (8%)	22 days	cyst-like structures	increase in the haploid cell population in the CGs and CGM post-meiotic differentiation of spermatogenetic cells	IHC, flow cytometry; occludin (SCs), TP2, and PRM2 (for post-meiotic GCs)	(Lee *et al.* 2006)
10 dpp and mature (30 dpp CD-1 mice	Digested testicular tissue and enriched GCs and SCs	DMEM/HAM F12	-	Soft-Agar-Culture-System (SACS): 0.7% (w/v) agar and 1.0% (w/v) agar	21 days	Colonies	Higher proliferation, round spermatids were observed when all testis cells seed	IHC, flow cytometry; Gfralpha-1(SSC) Crem (post-meitotic GCs) BrdU; (proliferation marker)	(Stukenborg *et al.* 2008)
18 dpp peripubertal rats	Isolated GCs and SCs	DMEM/F12 + 10% FBS	Retinol (3.3.10−7 m), rFSH(100 mIU), testosterone(10^{-7} M)	3D; 4.3 mg/mL or 2.5 mg/mL Matrigel with or without type I collagen	22 days	cluster organization	Round spermatids	IHC, RT-qPCR; Vimentin (SCs), claudin-11 (tight junction of SCs), α*SMA* (peritubular cell), Chk2	(Legendre *et al.* 2010)

								(spermatogonia), γ-H2AX (spermatocytes)	
18 and 30 dpp rats	Digested testicular tissue	DMEM/F12 + 10% FBS	retinoic acid (3.3×10^{-7} M), retinol (3.3×10^{-7} M), rFSH (100 mIU), testosterone(10^{-7} M), phytohemagglutinin(100 µg/ml)	poly(D,L-lactic-co-glycolic acid) (PLGA) co-polymers	18 days	initial colonization	Differentiation of spermatocytes into spermatids.	ICC, IHC: TP2(haploid spermatids)	(Lee *et al.* 2011)
0.5–5.5 dpp neonatal mice	Isolation of GCs and digested tissue	α-MEM + 10%KSR	human rEGF (20 ng/ml), human bFGF (10 ng/ml), human rGDNF (10 ng/ml)	Cellular pellets cultured using air-liquid interface technique	14 days	irregular and maze-like reconstruction of tubular structure	reconstructed tubules support germ cell differentiate pachytene spermatocytes .	IHC: Sox9 (SCs), 3β-HSD (LCs), Tra98 (GCs), SYCP1(GCs), H2AX(differentiated leptotene, zygotene, and late pachytene spermatocytes)	(Yokonishi *et al.* 2013)
7 dpp prepubertal rats	Digested tissue enriched for GCs, SCs, PTMs and LCs	DMEM without serum	with or without hCG (5 IU/I) and human rFSH (5 IU/I)	PDMS nanotubes	14 days	cord-like structure formation	Not investigated	IHC: α*SMA*	(Lee *et al.* 2011)
Prepubertal rats	Digested testicular tissue	DMEM high glucose + GlutaMAX	with or without hCG (5 IU/I) and human rFSH (5 IU/I)	Collagen sponges	35 days	Clusters containing undifferentiated spermatogonia were formed.	No spermatogenes is progression reported.	Live-cell imaging system, flow cytometry: BrdU (proliferation); IHC: Vasa (GCs), Lin28 and Pgp9.5 (spermatogonia), alpha smooth muscle actin (ASMA) and vimentin (SCs)	(Reuter *et al.* 2014)
5–8-, 20- and 60 dpp rats	Digested testicular tissue	αMEM + 10% KSR	retinoic acid (1 µM),	Matrigel, three-layer gradient system	21 days	Seminiferous tubule-like structures organoid	Maintenance of SSC proliferation	ICC: Sox9, α-Sm, vimentin, Zo1, occluding; Ddx4(GCs), Plzf GCs), Pcna GCs). ,	(Alves-Lopes *et al.* 2017)
15-year-old and adult human	Digested testicular tissue	KnockOut DMEM + 10% CTS	with or without hCG (5 IU/L) and rFSH (5 IU/L)	Decellularized testicular matrix (DTM)	28 days	Formation of testicular organoids occurs with or without scaffold support in both adult and prepubertal testicular cells.	No progression of spermatogenes is, but spermatogonia were maintained and proliferated in culture.	iF: STAR and 3βHSD for LCs, Sox9 and ZO1 for SCs, UCHL1, UTF1, FGFR3 for spermatogonia	(Baert *et al.* 2017)
Adult (ages 56–61) human	Isolated SSCs, LCs, SCs	StemPro-34	retinoic acid (2 µM), FSH (2.5 x 10−5 IU), human rSCF (100 ng/ml)	human testis ECM in hanging drop culture	23 days	Compact, tight organoids with GCs being in the center while somatic cells tend to be in the periphery of the structure.	Diploid to haploid GCs.	IF and RT-qPCR: PRM1 and Acrosin (post meiotic GCs), PLZF, UCHL1, and THY1 for early GCs; GATA4, CLU, and SOX9 for SCs; STAR, TSPO, and CYP11A1 for LCs.	(Pendergraft *et al.* 2017b)
1-week-old piglets; P8-P10 mice; 2-year-old rhesus macaque; 6-month-old and 5-year-old human	Digested testicular tissue	DMEM/F12	EGF (20 ng/ml), retinoic acid (1 µM)	Testicular organoids in microwell culture	5 days + 48h of retinoic acid stimulation.	Generation of porcine testicular organoids with inverted cell organization (GS and SC located outside while peritubular	No progression of spermatogenes is reported.	IHC: GATA for SC, α-SMA for peritubular myoid cells, UCHL1 for spermatogonia, Cytochrome P450 for LCs, CD31 for endothelial cells	(Sakib *et al.* 2019a)

						myoid cells and Leydig cells at the core of organoids). Testicular cells containing 50% GCs failed to form organoids.			
3-5 dpp mice	Digested testicular tissue	DMEM + 10% FBS	10^{-6} M retinoic acid, hCG (5 IU l^{-1}), FSH (5 IU l^{-1}),	Scaffold derived from decellularised testicular fragments of 2 to 3 year-old rams	30 days	Sertoli cells and Leydig cells were found in the marginal zones of the organoids. The germs cells at different stages of differentiation were randomly distributed in the Organoids.	Expression of post-meiotic germ cell marker PRM1 after 30 days	IHC: vimentin, 3β HSD, SSEA1, DAZL, SCP3, TNP1, fibronectin, Ki67, laminin RT-qPCR: OCT4, STRA8, SYCP8, SMC1B, PRM1, ACRV1	(Rezaei Topraggaleh *et al.* 2019)
Prepubertal pigs	Digested testicular tissue	DMEM/F12 + 10% KSR	retinol (1 µM) FSH (35 IU/l,) hCG (2 IU/l)	Collagen hydrogel	45 days	Reassembled tubule-like structures with SCs and GCs in the middle while LCs and peritubular cells localized outside.	Generation of functional testicular organoids, no spermatogenes is progression; the number of germ cells was decreasing in culture.	IHC: AMH AND sox9 for SCs, CREM spermatids, CYP19A1 for LC S DDX4 for GCs, SCP3 for spermatocytes.	(Vermeulen *et al.* 2019a)
Prepubertal(<7 *dpp*) and adult(6 months old) mice	Digested testicular tissue and enriched SSCs and SCs	αMEM + 10% KSR	-	Cell-free or cell-laden scaffolds bioprinting with nanocellulose-alginate hydrogel	Up to 48 days	No restoration of the tubular architecture	Progression of spermatogenes is up to the elongated spermatid stage; cell reorganisation into tubular structures was not observed.	IF: SOX9(SCs) and MVH (spermatogonia), CREM or PNA (post-meiotic GCs)	(Baert *et al.* 2019)
Pre-pubertal 1-week old pigs	Digested testicular tissue and enriched SSCs and SCs	DMEM/F12 + 10% FBS	-	Testicular organoids in microwell culture. Normal or knockdown for ODF2 and IFT88 Sertoli cells culture	6 days	Control organoids formed distinct basement membranes	No progression of spermatogenes is reported.	RT-pPCR: GLI1, HPRT1, ODF2, IFT88, ODF2 CELI	(Goldsmith *et al.* 2020)
5 dpp, 12 dpp, 21 dpp and adult (8-16 weeks) mice, Human Peri-pubertal (10-19 years) and Adult (40-50 years)	Digested testicular tissue	αMEM + 10% KSR	FSH (20 mIU ml^{-1}),hCG (4.5 IU ml^{-1})	Culture of testicular cells in agarose blocks(ECM-free). And culuted in a 1:1Corning Matrigel Basement Membrane Matrix	90 days	Self-assembly with immature testicular cells, long term endocrine function with gonadotrophic responsi Mature cells are able to self-assembly when co-cultured with immature testicular cells.veness,	No meiotic differentiation was observed	Histology and IF: SOX9, αSMA , DDX4, βCatenin, 3β HSD, SALL4, SCP3, COL IV, Laminin, ZO1,	(Edmonds and Woodruff 2020)
6 dpp prepubertal mice; culture of isolated SSCs	Digested testicular tissue	DMEM + 10% FBS	testosterone (10 µM), FSH (100 ng/ml), retinoic acid (100 ng/ml)	laminin, Matrigel, DTM-derived hydrogel	7 days	-	DTM is able to maintain SSCs' stemness and promote SSC differentiation into round spermatids without SCs	IHC and flow cytometry: CD49f, integrin β6, and CD326 SSCs; RT-qPCR: Gfra1, c-Ret-1, c-Ret-2, Plzf, and Oct4 for SSCs	(Yang *et al.*, 2020)
3-6 dpp neonatal mice	Isolated SSCs	DMEM + 5% FBS	bFGF (10 ng/ml), GDNF (10 ng/ml)	Agar and polyvinyl alcohol (PVA) nanofibers	14 days	Formation of spermatogonia l stem-like cell colonies	Differentiation of SSCs to meiotic and postmeiotic germ cells.	RT-qPCR: ID-4 and GFRα-1 (pre-meiotic), SYCP-3(meiotic), Tektin 1, TEKT-1(post-meiotic);	(Ziloochi Kashani *et al.* 2020)

								flow cytometry：plzf.	
1 week-old pigs	Digested testicular tissue	DMEM + 10% KSR	-	Cultured on small blocks of 1.5% agarose gel bedding in an air-liquid interface	4 weeks	Developed testis tubular structures and basic vasculature	Gonocytes	IHC; UCHL1, GATA-4, αSMA, CYP17A1, vWF	(Cham *et al.* 2021b)
4-5 dpp mice	Digested testicular tissue and isolated GCs	αMEM + 10% KSR	10^{-6} M retinol	Chimeric (with Germ stem cell line cells) or not chimeric cells cultured on agarose scaffolds in an air medium interface	6 weeks	Compartmentalized internally with one or more tubule-like structures with lumen,	Spermatocytes until the zygotene phase of meiosis	SOX9, ACTA2, yH2AX, 3β HSD, DDX4, ZO1, CREM, laminin	(Richer *et al.* 2021)
7 – 10 month old bulls	Isolated LCs, SCs and PTMs	DMEM + 10% FBS	100 ng/mL of BMP4, 10 ng/mL of FGF2, 15 ng/mL of GDNF	Cultured in ultra-low attachment dishes	28 days	-	No germ cells used	IF; COL1, STAR, WT1 Rt-qPCR; WT1, STAR, αSMA	(Cortez *et al.* 2022)
7 dpp mice	Digested testicular tissue	DMEM + 10% FBS	50 ng ml^{-1} epidermal growth factor, 1 ng ml^{-1} luteinizing hormone (LH) ,1 ng ml^{-1} FSH	Hanging drop culture and rotation in Matrigel Matrix	28 days	Testicular organoids formed a functional blood testis barrier, inside-out architecture	No meiotic differentiation was reported	RT-qPCR: markers for SC (Ar, Cldn11, Defb36, Dhh, Tyro3, Mro, Gata4, Wt1, Sox9, Tubb3), GCs (Ybx3, Crem, Dcn, Aldh1a1, Tmsb4x, Maged1, Clu, Aard, Tpt1, Gstm1), LCs (Cst12, Hsd3b1, Lhcgr, Sox4, Cyp17a1, Cst9, Errfi1, Por, Lbh, Pdgfra), and PTM cells (Acta2, Lif, Des, Igf1, Myh11) IF; SF1, WT1, GFRA, αSMA, Ki67, PCNA, Collagen IV, ZO1, SOX9, CX-43, Caspase9, Caspase 3, Fibronectin	(Yang *et al.* 2022)
28, 32, 44-year-old human	Digested testicular tissue	DMEM + 10% FBS	2.5×10^{-5} IU FSH, 2 μM retinoic acid, 100 ng/mL recombinant human stem cell factor	1:1 ratio of cells to Matrigel Matrix in hanging drop culture	5 weeks	No restoration of the tubular architecture reported	Expressions of Meiotic and post-meiotic markers were identified	IHC; PRM2, αSMA, LHR, vimentin, GFRA1. Rt-qPCR; PLZF, SYCP3, PRM2, Oct4, Vimentin, CYP11A1, BAX, BCL2	(Nikmahzar *et al.* 2023)
6–8-week-old mice	Digested testicular tissue	DMEM		Microporous culture plates with Matrigel	8 days	Sertoli cells, myoid epithelial cells, Leydig cells located on the outer layer of the organoids while spermatogenic cells located on the inside.	No progression of spermatogenes is reported	IF; CLDN1, OCLN, ZO1, Caspase 9, Caspase 3, DDX2, Vimentin, HSD3B1, C3, MAC, Anti-ZIKV 4G2	(Yang *et al.* 2023)
2-week-old bovine	Digested testicular tissue	αMEM + 10% KSR	FSH (10 IU/L), testosterone (10−6 M), GDNF (20 ng mL−1), FGF2 (2 ng ml−1;), and LIF (100 ng ml−1)	1:1 Matrigel Matrix	Up to 28 days	3D clusters containing germ cells	Expression of Premeiotic markers and maintenance of GCs	IF: PGP9.5, Vimentin, KI-67 Rt-qPCR: PGP9.5, PLZF, GFRa1, Stra8, C-kit, AMH and STAR	(Tang *et al.* 2024)

Table 2. Germ cell content at different testicular development stages in humans. Table created by the Author using published information (Oatley and Brinster 2012; Masliukaite *et al.* 2016; Sohni *et al.* 2019; Guo *et al.* 2020b)

Developmental period	Infant	Juvenile	Adult
Germ cell development	Prospermatogonia proliferate until 5-6 months after birth and differentiate into type A spermatogonia	Undifferentiated spermatogonia only	Complete spermatogenesis
Germ cells according to scRNA-seq results	Three populations: primordial germ cells-like (PGCL) and two prospermatogonial populations, some resemble SSCs	Undifferentiated spermatogonia proliferate and start to differentiate at the age of 10-12 years	Complete spermatogenesis
Prepubertal spermatogonial cell counts per seminiferous tubular cross section (S/T)	Decreasing trend during the first 3 years of life (from 2.5 to 1.2)	Plateau until 11 years	Sharp incline marking the onset of puberty (to a level of 7)
Spermatogonial numerical density per testicular tissue volume of 1cm^3 (S/V)	Decreasing trend during the first 3 years of life (from 30 to 19 106/cm^3)	48 106/cm^3	Approximately 100 106/cm^3

Table 3. Overview of growth factors regulating the fate of spermatogonial stem cells (SSCs) in the niche. Some factors promote proliferation and self-renewal to maintain the germ cell pool, others induce differentiation and progression of spermatogenesis. Sertoli cells produce most growth factors, confirming their importance within the SSC niche.

Growth factor	SSC effect	Producing cells	Key references
Glial cell line-derived neurotrophic factor (GDNF)	Proliferation/self-renewal	Sertoli cells	(Naughton *et al.* 2006b; Jijiwa *et al.* 2008)
Colony-stimulating factor 1 (CSF1)	Proliferation/self-renewal	Leydig cells	(Oatley *et al.* 2009)
Fibroblast growth factor (FGF)	Density and stem cell homeostasis	lymphatic endothelial cells	(Kitadate *et al.* 2019)
Fibroblast growth factor 2 (FGF2)	Proliferation/self-renewal	Sertoli cells	(Zhang *et al.* 2012)
Activin A	Differentiation	Sertoli cells	
Bone morphogenetic protein 4 (BMP4)	Differentiation	Sertoli cells	(Ogawa *et al.* 1998)
Retinoic acid (RA)	Spermatogonial differentiation and meiosis entry	Sertoli and Leydig cells	(Zheng *et al.* 1996; Amory *et al.* 2011)

Table 4. The advantages and disadvantages of scaffolding biomaterials to grow organoids. Table modified from Cham *et al*. (2021)

Scaffolding biomaterials (hydrogels)		Advantages	Disadvantages
Natural hydrogels	Agarose (SACS)	• Cytocompatible • Excellent sacrificial biomaterials • Low cell adhesion favors the formation of 3D cell spheroid	• Poor cell interaction due to the lack of binding protein

		• Versatile, widely published in different culture systems	• Poor mechanical properties
	Alginate	• Cytocompatible • Rapid crosslinking rate • The viscosity of alginate solution can be tailored to improve the printability and the stability of the scaffolds • Widely used and published bio-ink in 3D printing	• Poor cell interaction due to the lack of binding protein • Poor mechanical properties • Excessive crosslinkers (e.g., Ca2+, Ba2+) may impair cells
	Methylcellulose (MC)	• Cytocompatible • Low cell adhesion favors the formation of 3D cell spheroid • Can be a sacrificial biomaterial • The viscosity of MC solution can be tailored to improve the printability and the stability of the scaffolds	• Biologically inert and thus not ideal for cell growth • Not widely used and published. More studies are required to explore its effects and potentials in cell culture and 3D printing
	Collagen	• Cytocompatible • High level of proteins and cell receptors favors cell growth and development	• Slow crosslinking rate leads to low printability, and poor scaffold fidelity/stability • Poor mechanical properties
	Matrigel	• Cytocompatible • High biomimicry to native ECM, which promotes cell adhesion/differentiation	• Complex and undefined biological components • Derived from malignant source (EHS tumor) • Slow crosslinking rate and low printability • Poor mechanical properties
Synthetic hydrogels	Poly-dimethylsiloxane (PDMS)	Good mechanical properties, printability, well-defined components, consistent qualities, and less expensive due to the large and efficient production	Low biocompatibility causes poor cell adhesion/development
	Poly-L-lactic acid (PLLA)		
	poly- D, L-lactic-co-glycolic acid (PLGA)		

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