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REGENERATIVE MEDICINE (A ATALA, SECTION EDITOR)

In Vitro Spermatogenesis: How Far from Clinical Application?

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Abstract Male infertility affects 7 % of the male population, and 10 % of infertile men are azoospermic. In these instances, using microsurgical testicular sperm extraction (m-TESE) and intra-cytoplasmic sperm injection (ICSI) helps a significant number of patients. However, in vitro differentiation of diploid germ cells to mature haploid germ cell has the potential to benefit many others, including pediatric cancer survivors who have previously cryopreserved their immature testicular tissue prior to starting gonadotoxic cancer treatment as well as men with spermatogenic arrest. This systematic review evaluates and summarizes half a century of researchers' efforts towards achieving in vitro spermatogenesis in mammalian species. A myriad of experimental assays and approaches has been developed using whole testis tissue or separated single cells from testis in two- or three-dimensional cell culture systems (2D versus 3D). Recent advances in the mammalian in vitro spermatogenesis, particularly in murine and nonhuman primate systems, hold promise towards translating the availability of in vitro spermatogenesis models in the human clinical setting in the near future.

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Introduction

Male infertility is a major health problem in our society, currently affecting millions of people worldwide. Approximately 15 % of couples aiming to have children suffer from fertility impairments; of these, half are due to male infertility [1]. During the last few decades, several technical revolutions have consistently improved the chance of achieving healthy offspring for men with impaired fertility. Microsurgical testicular sperm extraction (m-TESE), intra-cytoplasmic sperm injection (ICSI), and round spermatid injection (ROSI) have enabled azoospermic men with at least round spermatids present in their testis to complete fertilization and achieve live deliveries [2]. Unfortunately, none of the above mentioned techniques meets the needs of either azoospermia patients without spermatids in their testis or pre-pubertal patients whose germ cells are depleted before achieving germ cell differentiation, such as pediatric cancer survivors following chemotherapy. Effective therapeutic options for these patients are currently limited but will likely increase as research into this growing field continues. Most recently, research undertakings in the field of regenerative medicine using cell-tissue culture, biomaterials, and bioactive factors are opening new avenues to in vitro germ cell differentiation [3], which could eventually lead to new therapeutic approaches to male infertility.

This article aims to systematically review the evolution of spermatogenesis in vitro techniques starting from the most basic initial studies all the way up to the most recent advances. Our focus here is on mammalian models using only testicular

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germ cells as starting point and no other sources such as embryonic stem cell or induced pluripotent stem cells.

Methods

The electronic database MEDLINE was systematically searched via PubMed using the following terms:

- In vitro techniques [Mesh]: methods to study reactions or processes taking place in an artificial environment outside the living organism.
- Spermatogenesis [Mesh]: the process of germ cell development in the male from the primordial germ cells, through spermatogonia; spermatocytes; spermatids; to the mature haploid sperm.
- Spermiogenesis [all fields]: allusion to the cell differentiation after meiosis. The use of "all fields" was justified by the lack of specific Mesh word.
- Regenerative medicine [all fields]: this term brings together the fact of reproducing physiologic processes in vitro with a therapeutic approach. In this case, a Mesh word was available but being a quite newly introduced term, not many articles were indexed under Mesh term.

Articles published in languages other than English were excluded. The final search was performed in Oct. 30th, 2015, and 756 articles were reached. The process of selection, exclusion, and inclusion is summarized in Fig. 1.

Results

The analysis of the selected bibliography revealed different approaches to achieve in vitro spermatogenesis using testicular tissue. Some groups explanted whole pieces of testis for culture conserving their connective tissue and their 3D structure (Table 1). Others isolated and purified different cell types and then differentially cultured them to recreate the spermatogenic process (Table 2). Differences in the developmental stage of used testicular tissue for culture were present: while some groups used pre-pubertal tissue to insure the original cells in culture had an early stage of differentiation, others have preferred to use adult samples containing germ cells in all stages of differentiation. Other variations occurred as well. For example, the temperature of culture has been considered a critical point in different studies. Most of the following studies utilized similar culture media. We decided to categorize the studies in two main groups: (1) organ culture (Table 1) and (2) isolated cell culture (Table 2). In each group, we reviewed studies in chronological order and placed them in subgroups of different species. Details of studies including culture type, temperature, and duration, latest achieved stage of differentiation, fertility assessment of differentiated germ cells, and live offspring outcome are summarized in Tables 1 and 2.

Organ Culture

Viability of Tissue Over the Culture

The first challenge in the process of achieving in vitro spermatogenesis was the capability to maintain viable testicular tissue during culture. In 1959, Trowell described a culture system where small bunches of tubules from rat testis were placed in a cavity slide in a modified of Eagle's minimum essential media (MEM). Samples were kept in a clean space at 37 °C with 5 % CO_2 air and media, but all cultures were nonviable by day 6 [4]. During the 1960s and 1970s, several studies attempted to apply new organ culture methods to assess spermatogenesis in vitro (Table 1). Steinberger and colleagues (1964) reported a successful culture of rat testicular tissue at various ages (new born to 37 days old) modifying Trowel's method. They supplemented the culture media with different concentrations of follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG), and/or vitamins A, C, and E. They observed that this culture system supported the tissue for up to 4 weeks without major architectural alteration. However, no clear germ cell differentiation was achieved [5, 6].

Radiolabeled Thymidine

Especially when mature testes were used for culture, an important concern was distinguishing between induced spermatogenesis in vitro, maintenance of an in vivo-induced spermatogenesis process, or even just survival of spermatogenic cells in different stages of differentiation. To overcome this challenge, radiolabeled thymidine method was used by Matte and Sasaki (1971) when they cultured human testis tissue from a 62-year-old patient undergoing orchiectomy in media with 20 % fetal bovine serum (FBS). After 32 days of culture, many heavily radiolabeled young spermatids were found in the floating cell population in the supernatant. However, they could not conclude whether many late spermatids after 35 days of culture were just surviving cells or came from in vitro differentiation. [7]. Two other groups tried to reproduce Matte and Sasaki's result. However, neither Ghatnekar et al. (1974) who carried out a similar study using media enriched with 15 % FBS, 10 % coconut milk, FSH, testosterone, and/or LH [8] nor Curtis et al. (1981) who used a system containing 10 % FBS-enriched media [9] were able to achieve differentiation later than the pachytene spermatocyte level. The only critical difference between Matte and Sasaki's work and the other two studies was that Ghatnekar and Curtis used very

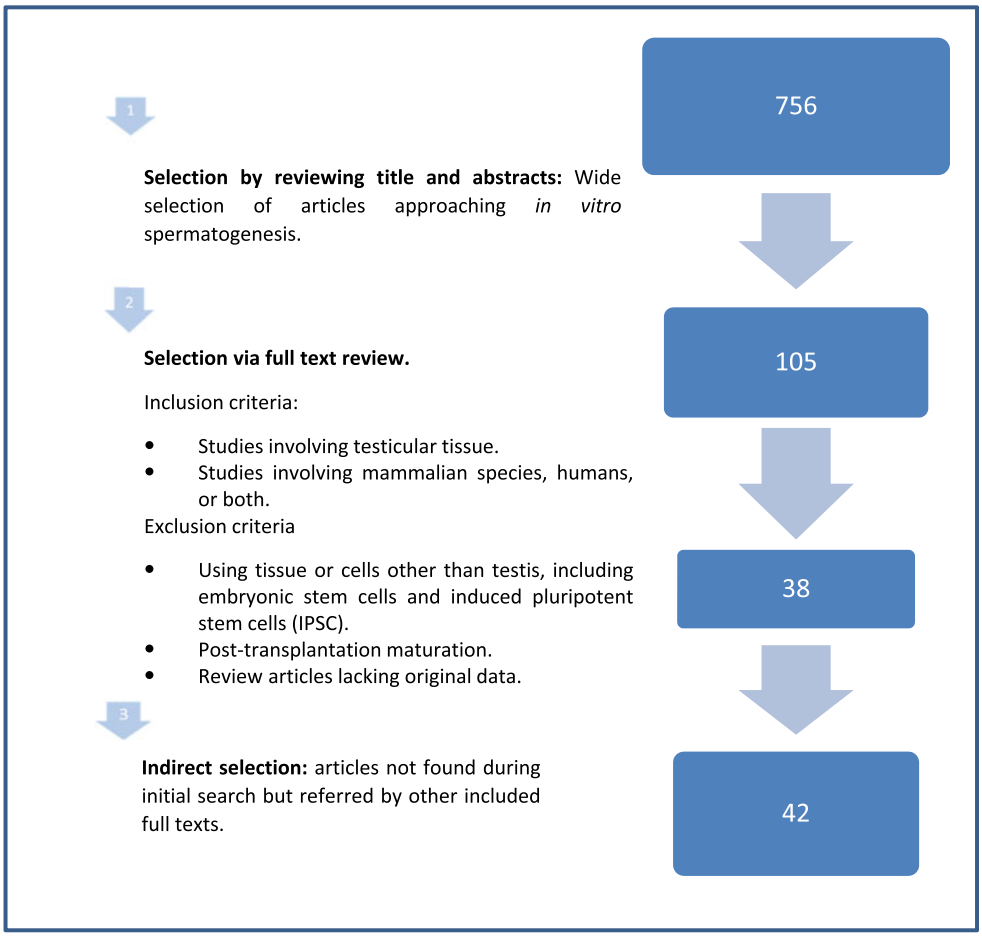

Fig. 1 Article selection flow chart

small quantities of biopsied tissue and cultured for a shorter period (21 and 14 days instead of 32 days).

Role of Fetal Bovine Serum and Its Effects on Differentiation

Aizawa and Nishimune (1979) also included radiolabeled thymidine in culture media to indicate DNA of the cells in S phase at the moment of culture which could be tracked later by autoradiography. To prevent contamination of the culture with differentiated germ cells, they utilized testes from cryptorchid mice, where the only type of germ cell present was type A spermatogonia, a diploid undifferentiated germ cell. During the first 3 days in culture in MEM media enriched with 10 % FBS, only mitotic activity was observed. Later differentiation was detected on day 15 when pachytene spermatocyte was visualized in both optic microscopy and autoradiography [10]. To evaluate the effect of FBS on differentiation, they found no indication of differentiation in negative controls (serum-free media). Interestingly, they found no significant differences on spermatogenesis stage when testis tissues were transferred to a serum-free media after 1-day culture in FBS-enriched media compared to the initial condition (culture constantly in enriched FBS medium). This suggested that FBS is a critical factor during the initial phase of in vitro spermatogenesis [10]. Once it was established that unspecific factors present in serum could induce at least the initial stages of spermatogenesis in vitro, more specific tests were designed to determine which factors were involved in this process. Haneji et al. (1983) showed germ cell differentiation to the type B spermatogonia stage from cryptorchidic mice testis using serum-free media enriched with type III fetuin protein and FSH alone or in combination. The ratio of differentiated germ cells obtained from this combination was even slightly better (up to 8 %) than positive control (10 % FBS media) [11]. These results were validated by incorporation of thymidine to DNA.

In the mid-1980s, Toppari et al. proposed new methods to characterize the spermatogenic stage in cultured rat testis. The presence of in vitro-differentiated spermatids was demonstrated by immunostaining (CRB8 and CRB11) and electronic microscopy. Additionally, DNA-labeled flow cytometry was used to study population dynamics (1C, 2C, and 4C) during culture. These data combined with the prior mentioned thymidine labeling demonstrated the in vitro transition from spermatogonia to round spermatid in rat testis cultivated in a serum-free medium and differentiated with retinoic acid

Table 1 In vitro spermatogenesis studies using whole testis tissue

Study	Species	Tissue source	Maturity level	Culture type (2D/3D)	Temperature (°C)	Duration of culture (day)	Latest achieved stage	Fertility assessment	Offspring
Aizawa and Nishimune (1979)	Mouse	Cryptorchidism	Adult	2D	32.5	15	Spermatocyte	No	N/A
Haneji et al. (1983)		Cryptorchidism	Adult	2D	32.5	9	B spermatogonia	No	N/A
Haneji et al. (1986)		Cryptorchidism	Adult	2D	32.5	9	B spermatogonia	No	N/A
Tajime et al. (1995)		Cryptorchidism	Adult	2D	32.5	9	B spermatogonia	No	N/A
Suzukiet al (2003)		Wild type	Pre-pubertal/ adult	2D	32.5	14	Round spermatid	ROSI	No
Ghobara et al. (2010)		Transgenic	Pre/peripubertal	3D	34	17–33	Round spermatid	No	N/A
Sato et al. (2011)		Transgenic	Pre-pubertal	3D	34	42	Sperm	ICSI	Yes
Sato et al. (2012)		c-kit ligand mutant	Pre-pubertal	3D	34	42	Sperm	ICSI	Yes
Yokonishi et al. (2014)		Transgenic	Pre-pubertal	3D	34	46	Sperm	ICSI	Yes
Toppari et al. (1984, 1985, and 1986)	Rat	Wild type	Adult	2D	32	3–7	Round spermatid	No	N/A
Hue et al. (1998)		Wild type	Pre/peripubertal	2D	32	40	Round spermatid	No	N/A
Staub et al. (2000)		Wild type	Pre/peripubertal	2D	32	21	Round spermatid	No	N/A
Matte and Sasaki (1971)	Human	Orchiectomy (prostate cancer)	Adult	2D	30	32	Early spermatid	No	N/A
Ghatnekar et al. (1974)		Nonobstructive or obstructive azoospermia, post-vasectomy, normal (biopsy during hydrocelectomy or prostate biopsy)	Adult	2D	36	14	Spermatocyte	No	N/A
Curtis et al. (1981)		Nonobstructive or obstructive azoospermia, post-vasectomy	Adult	2D	31	21	Spermatocyte	No	N/A
Tesarik et al. (1998)		Obstructive azoospermia	Adult	2D	30	2	Flagellated elongating spermatid	No	N/A
Tesarik et al. (1999)		NOA	Adult	2D	30	2		ICSI	Yes

NOA nonobstructive azoospermia, *N/A* not applicable

Table 2 In vitro spermatogenesis studies using isolated cells from testis tissue

Study	Species	Cell source	Maturity level	Culture type (2D/3D)	Temperature (°C)	Duration of culture (day)	Latest achieved stage	Fertility assessment	Offspring
Rassoulzadegan et al. (1993)	Mouse	Wild type	Peripubertal/adult	2D	32	12	Round spermatid	No	N/A
Hofman et al. (1994)		Immortalized	Adult	3D	37	180	Elongated spermatid	No	N/A
Movahedin et al. (2004)		Wild type	Adult	3D	37	4	Elongated spermatid	No	N/A
Stukenborg et al. (2008)		Wild type	Pre/peripubertal	3D	35	21	Round spermatid	No	N/A
Hasegawa et al. (2010)		Wild type	Adult	3D	32	2	Elongated spermatid	No	N/A
Minaee Zanganeh et al. (2012)		Wild type	Adult	2D	32	7	Elongated spermatid	ICSI	No
Abu Elijah et al. (2012)		Wild type	Pre-pubertal	3D	37	30	Sperm	No	N/A
Wang et al. (2014)		Wild type	Pre-pubertal	2D	37	8	Spermatid	No	N/A
Khajavi et al. (2014)		Wild type	Pre-pubertal	3D	37	21	Spermatid	No	N/A
Nagao et al. (1988)	Rat	Wild type	Pre-pubertal	2D	32.5	14	Round spermatid	No	N/A
Virgier et al. (2004)		Wild type	Early pubertal/adult	2D	33	14	Round spermatid	No	N/A
Lee et al. (2011)		Wild type	Pre-pubertal	3D	32	18	Elongated spermatid	No	N/A
Izadyar et al. (2002)	Bull	Wild type	Pre-pubertal	2D	37	60	Flagellated elongating spermatid	No	N/A
Xie et al. (2010)	Buffalo	Wild type	Pre-pubertal	2D	37	41	Spermatid	No	N/A
Huleihel et al. (2015)	Rhesus monkey	Wild type	Juvenile	3D	37	28-56	Round spermatid	No	N/A
Sousa et al. (2002)	Human	NOA	Adult	3D	32	10-15	Spermatid	ICSI	No
Tanaka et al. (2003)		NOA	Adult	2D	32.5	5	Round spermatid	ROSI/ICSI	No
Lee et al. (2005)		NOA	Adult	2D	32	42	Round spermatid	ROSI/ICSI	No
Riboldi et al. (2012)		NOA	Adult	2D	37	5	Round spermatid	ROSI/ICSI	N/A

NOA nonobstructive azoospermia, *N/A* not applicable

(RA), testosterone, FSH, and/or insulin. However, haploid cell (1C) nuclei never polarized in vitro and died before reaching the elongated stage. Moreover, the data suggested that the main target of FSH was the 2C (spermatogonia) population, promoting survival or mitosis of these cells rather than direct meiosis induction [12–15]. Similar results were obtained by Haneji et al. (1986) while different concentrations of retinol (vitamin A) and Bu2cAMP (to mimic FSH stimulation activity) were added to the culture medium. Even though both presented mutual synergies, it seemed that retinol promoted more efficient differentiation of germ cells while BuAMPc and FSH enhanced the mitotic activity [16]. To continue investigating the different components of FBS capable of inducing spermatogenesis in vitro, Tajima et al. (1995) cultured cryptorchidic mice testis in a serum-free medium enriched with IGF-1 or TNF-α for 9 days. The result was 40 and 25 % of differentiation into B spermatogonia, respectively, compared to FBS-enriched media as a positive control. These results suggested that even if IGF-1 or TNF-α probably had a role-enhancing differentiation, neither was sufficient to supplant the role of FBS [17].

Sertoli and Spermatogenic Cell Interactions

To find a new culture method to overcome the challenges in the field, in the late 1990s, another approach was postulated. The organ dissociation by enzymatic digestion was performed carefully to keep the interactions between Sertoli cells and spermatogenic cells. Hue et al. (1998) cultured rat seminiferous tubules in this way in bicameral chambers. Culture medium was enriched with FSH, testosterone, retinol, and 0.2 % FBS. The results showed 55 % of 1C population by day 33

and 70 % by day 40 assessed by DNA flow cytometry. These findings were also confirmed by synchronic decreased PCR expression ratios of p19/TP1 and TH2B/TP2 (the ration of pachytene characteristic gene and round spermatid characteristic gene) and morphologic recognition of bromodeoxyuridine (BrdU)-labeled round spermatids [18]. The same group in 2000 demonstrated the presence of every stage of spermatogenic cells through the round spermatid stage. They also found a progressive increase in transferrin concentration in bicameral culture system which was usually six to eight times higher in the apical compared to the basal compartments. These findings suggested a barrier effect of Sertoli cells modifying the final concentration of transferrin. The proportion of round spermatids formed in vitro in 21 days of culture was smaller when compared to in vivo findings in 40–45-day-old rat; however, it was close to that observed in 30–35-day-old rat. This meant that during pubertal maturation in vivo, the meiotic efficiency increases, but it was not observed in this in vitro system [19]. Based on previous studies in mammals, Tesarik et al. (1998) tried to achieve spermatogenesis in vitro using remaining testicular tissue from biopsies of azoospermic patients who underwent testicular sperm extraction for assisted reproduction. Both undisintegrated (samples from 12 patients) and disintegrated by enzymatic digestion (samples from six patients) tissue were used and demonstrated that intact Sertoli and germ cell clusters were not necessarily required for spermatogenesis. Enriching the culture media by adding recombinant FSH (rFSH) increased significantly the presence of round spermatids after 24 h incubation. In both culture conditions, they reported high rate of abnormal spermatids compared with fresh samples. It was interesting that cells in culture bypassed the 20 days in vivo process in only 24 h in vitro incubation. This may explain the high rate of abnormal in vitro spermatid formation reported *in this study* [20]. To evaluate the fertilization ability and safety of in vitro-formed long spermatids, the same group used ICSI. The results were one successful delivery of healthy twins out of five men with pre-culture maturation arrest at the primary spermatocyte level and successful delivery in four patients with pre-culture arrest of post-meiotic differentiation at the round spermatid level [21].

Cryopreservation did Not Inhibit Progression of Spermatogenesis In Vitro

In order to establish a therapeutic application of spermatogenesis in vitro in the future, it was essential to find out if cryopreserved samples would present additional challenges to initiate in vitro differentiation process from spermatogonial cells. Suzuki et al. (2003) performed a study consisting on mice testis tissue from four different conditions and compared the fertilization-development rates by injecting spermatogenic cells (secondary spermatocyte/round spermatid) to the oocytes: (1) fresh testis tissue from mature mouse, (2) frozen and thawed testis tissue from mature mouse and (3) cultured testis tissue from immature mouse, and (4) cultured frozen-thawed testis tissue from immature mice. They cultured immature testes for 2 weeks in 10 % FBS media but without any other hormonal supplements. After 12 days in culture, a significant increase of secondary spermatocytes and a few round spermatids were found. The fertilization and development ratio was comparable in all conditions (fertilization rates: 74, 98, 78, and 78 %, respectively; 8-cell stage embryo rate: 7, 4, 2, and 5 %, respectively). These showed that cryopreservation did not appear to inhibit progression of spermatogenesis in vitro [22].

3D Tissue Culture

Soon after these promising results, a new system using an agarose gel stand has been used to improve the procedure. Ghobara et al. (2010) cultured immature mouse testis into an agarose gel soaked in three different mediums: (1) α-MEM, (2) DMEM, and (3) Stempro-34 SFM; each medium had 10 % FBS. The mice used in this study had transgenic modification including green fluorescent protein (GFP) expression under the Acr or Gsg2, whose germ cells express GFP at the mid and end stages of meiosis onward. The differentiation of germ cells was successful up to the round spermatid stage via confirmation by Acr or Gsg2 expression, cell size (around 11 μm in diameter), and Hoechst nuclear staining. The best average success rate of GFP expression was achieved in α-MEM condition, although no statistical significance was achieved. The maximum percentage of spermatids was 29.8 % of all GFP-positive cells. However, no further differentiation beyond round spermatids (spermiogenesis) was observed [23]. Sato and colleagues (2011) presented an organ culture model using similar agarose gel system and the same transgenic Acr or Gsg2-GFP mice but with knockout serum replacement (KSR)-enriched media. The results showed a significant increase in the Acr-GFP+ cell population from 0.86 % (FBS) to 7.11 % (KSR). The population of haploid cells (1C) also increased from 0.6 % (FBS) to 1.73 % (KSR). In addition to round spermatids, they could create elongated spermatid and even flagellated sperm in some cases. To test the fertilization ability of the germ cells produced in vitro (testes tissue 23 days in culture from 3.5-day-old mice), 23 round spermatids underwent ROSI giving rise to seven fertile pups, and 35 flagellated sperms (from testis tissue 42 days in culture from 2.5-day-old mice) underwent ICSI giving rise to five fertile descendants. Moreover, the use of cryopreserved samples in this study instead of fresh testis did not manifest differences in terms of survival, differentiation, and fertility, showing promising potential for future clinical application [24••].

Subsequently, the same group reported successful in vitro spermatogenesis in genetically defective mice in 2012. Testis

tissues of mice suffering from c-Kit ligand (KitL) mutation were cultured in their previously described system. Initially, recombinant KITL (rKITL) was added to the media in high doses (500 ng/ml), and the number of spermatogonia apparently increased but no spermatogonic cells after pachytene stage were found. Later, the combination of KitL and colony-stimulating factor-1 (CSF1) produced a significant increase in spermatogonia numbers and induced spermatogenesis, giving rise to elongated spermatids and flagellated sperm. Using haploid cells from 49-day cultured testes for either ROSI or ICSI resulted in one fertile female offspring. This study raised the potential of treating the genetically defective infertile male without any genetic manipulations in the future [25••].

Epigenetic Stability and Safety

Yokonishi et al. (2014) designed a specific study using two different cryopreservation methods (slow freezing and vitrification) and four different cryoprotectants following Sato's in vitro spermatogenesis method. The rates of fertilization, implantation, and delivery were not significantly different when using fresh tissue. The offspring were fertile, and the methylation states of 11 different regions (H19, IG, Rasgrf1, Igfr2r, Lit1, Meg1, Peg1, Peg3, Peg10, Peg13, and Snrpn) were particularly equal in either ROSI- or ICSI-derived offspring and controls, which confirmed the stability of in vitro-produced gametes. This supported the fidelity and safety of the sperms produced from cryopreserved tissue under in vitro conditions [26]. However, a recent study was published by Hogg et al. (2015), the effect of KSR on culture conditions compared with using FBS. Though no differences on the morphology and architecture of the testes were observed, it was shown that KSR significantly inhibited male germ cell entry into mitotic arrest, a key milestone in male germline development. In FBS conditions, cell cycle parameters in germ, and Sertoli cells closely resembled normal development. Some epigenetic instabilities were also found when comparing transcription levels of H3K9, Ehmt2, and H3K27 from samples in culture with KSR and FBS, raising concerns about the KSR safety for spermatogenesis in vitro. This suggested the requirement for more investigations using KSR in the field before going forward to clinical applications [27].

Isolated Cell Culture

Following initial trials using organ culture methods, other groups started enzymatically dissociating and culturing testicular cells instead of organ culture (Table 2). Nagao et al. (1989) dispersed pre-pubertal (14 days old) rat testicular tissue with trypsin 0.25 % and cultured the cells as a monolayer. After 2 days of culture, the monolayer was rinsed with PBS, and the germ cells were gently pipetted to be separate from the rest of cell types. By adding either epinephrine or noradrenaline (NA), the cells survived up to 14 days in culture. After day 10 of culture, there were evidences of meiotic activity based on observation of cell morphologic characteristics of round spermatid and DNA flow cytometric peak on the 1C (haploid) population, which increased from 0 to 7 % during the culture period. These findings were in concordance with the in vivo system where the rats present their first round spermatids on day 24 post-partum. However, the fact that round spermatid was the most differentiated stage found in culture suggested that there must be other necessary factors to induce complete spermatogenesis in vitro [28].

Rassoulzadegan et al. (1993) established the 15P-1 mouse cell line, an immortalized line with similar characteristics to Sertoli cells, including phagocytic activity and expression of Steel and WT1genes. 15P-1 cells were co-cultured with germ cells from transgenic mice of different ages that expressed B-galactosidase under control of protamine-1 (PRM1) promoter. The medium was also exposed to thymidine for the first 48 h of culture in order to be able to differentiate the mitotic and spermatogenic activity induced in vitro and in vivo. When the germ cells in culture came from 10- or 18-day-old immature mice, the expression of B-galactosidase increased significantly at day 12 in culture on 10 % FBS media, indicating that germ cells were expressing the characteristic differentiation PRM1 gene. This finding was also supported by morphological appearance of round spermatids, positively marked with radiolabeled thymine and flow cytometry for DNA, showing a growing haploid population increasing from less than 1 to 13 %. On the other hand, culturing germ cells from adult mice showed successful in vitro differentiation until the flagellated sperm stage. The different outcome between these two groups (culturing immature versus mature germ cell) suggested that this culture system has limited capability to induce complete spermiogenesis on immature testis cells [29].

Temperature Differences

In a different way, Hofman et al. (1994) isolated two germ cell lines from 6-week-old mice and immortalized them with LTAg oncogene. Culturing both cell lines in different temperatures (32, 37, and 39 °C) showed a temperature-dependent p53 activity. At 39 °C, the cells rapidly proliferated during the 6 months of the study without undergoing differentiation. At 37 °C, the cells proliferated and differentiated steadily, with a constant 28 % haploid cell population in equilibrium for 6 months. The haploid cells were positively stained with acrosomal marker MSA-63 and showed ultrastructural characteristics compatible with early spermatid stage. At 32 °C, the cells weakly proliferated and prominently differentiated, producing early spermatids by passage four but losing viability by passage 10. Immortalization put this model far from

clinical application; however, Hofman's system showed an interesting basic model for research [30].

In the early 2000s, Izadyar et al. (2002) isolated 5-month-age bull spermatogonial stem cells (SSCs) for in vitro proliferation, finding some levels of spontaneous differentiation. The grade of purity achieved was close to 85 % of type A spermatogonia, and Sertoli cells were the main non germ cell population detected by vimentin immunostaining. Culturing in MEM with 2.5 % FBS kept germ cells viable for 150 days. By using higher concentrations of FBS, the proliferation and differentiation were higher but the effect on somatic cells was greater than spermatogonia therefore disrupting the balance. Two weeks after culture, the system was evaluated by immune-staining of BrdU and DBA, marker of type A spermatogonia, showing that 20 % of these spermatogonial cells in culture were positive for BrdU, indicating the presence of these cells in the S phase of the cell cycle. After 2 months, different levels of spermatogenic differentiation were found in culture based on their morphology, including elongated flagellated structures with some heterochromatin. In concordance, positive immunostaining and western blot for SCP3 (sinaptonemal complex), ODF-2 (sperm tail), and positive post-meiotic markers including VADC-2 and acrosin were observed in reverse transcription polymerase chain reaction (RT-PCR). Acrosin expression was detected only after 110 days in culture showing an increased spermatogenic cycle duration in comparison with bull's testes in vivo (62 days). The main aim of this study was establishing a propagation system for type A spermatogonia; however, spontaneous differentiation of germ cells happened and there was still challenge to achieve complete spermiogenesis in vitro [31].

Nonobstructive Azoospermia and In Vitro Differentiation

In a clinically oriented point of view, Sousa et al. (2002) combined isolated germ and Sertoli cells in culture using human testicular tissue biopsies from 61 nonobstructive azoospermia (NOA) patients who had normal karyotype [32]. After enzymatic dissociation of biopsies, in each culture about 30–80 Sertoli cells, 10–30 spermatogonia and 200 primary spermatocytes and round spermatid were seeded. Cells were cultured in media supplemented with rFSH with or without additional testosterone. The results varied depending on the subtypes of NOA; however, in general, the best results were achieved when rFSH and testosterone were used together. After 2–3 weeks of culture, 22.7 % of cells were differentiated into normal late spermatids. Performing ICSI by using normal elongating and elongated spermatids elicited a 30.5 % fertilization rate and 42.9 % blastocyst formation. Fluorescent in situ hybridization (FISH) of DNA analysis showed a high rate of sex chromosomal abnormalities in all embryos post fecundation. For ethical reasons, none of the embryos created using in vitro-matured germ cells was transferred. This culture system still required optimization; however, as mentioned earlier, Tesarik (1999) [21] had already achieved one normal delivery following a similar procedure using testicular tissue instead of culturing separated cells.

To overcome the maturation arrest in NOA biopsies by applying in vitro differentiation, Tanaka et al. (2003) co-cultured human spermatogenic cells from NOA patients together with Vero cells, a well-studied feeder cell type from African green monkey kidney [33]. Different medium conditions were also tested using various combinations of FSH, testosterone, human serum, boar rete testicular fluid which may contain important local factors for germ cell differentiation, and human synthetic oviduct fluid which has been already used for embryo culture. Based on both Giemsa staining and FISH analysis, the maximum yield of differentiation from primary spermatocyte to round spermatid was around 10 %. The best condition was achieved with co-culture on 50 % boar rete testicular fluid or human synthetic oviduct fluid plus 10 % human serum. When the in vitro-generated round spermatids were injected into mouse oocytes, 84 % of them got two-Pro-Nuclei (2PN) stage embryos. This study demonstrated the beneficial effect that feeder cells can produce on spermatogenesis in vitro models; however, the use of heterozoic cells is an important safety issue [33].

Role of Growth Factors and Feeder Cells

At this point, there were two main strategies in order to establish spermatogenesis in vitro: (1) media supplementation with hormones or growth factors and (2) culture on feeder. To elucidate which was more convenient in mammals, Movahedin et al. (2004) [34] compared the viability and differentiation of fresh versus frozen-thawed spermatids from mice adult testis in different conditions: control (only DMEM with 10 % FBS), hormone (adding rFSH and testosterone), co-culture (cultured on Vero cells), and co-culture plus hormone [34]. After 24 h of in vitro culture, by using either fresh or cryopreserved tissue, the number of round spermatids decreased and the number of elongating and elongated spermatids increased in all experimental conditions. The spermatogenic cells survived until 96 h in culture, although the survival rates decreased. More than 60 % of spermatogenic cells survived after freeze-thaw, which is the acceptable rate for potential clinical indication.

Virgier et al. (2004) used the bicameral chamber culture system to co-culture isolated middle/late pachytene spermatocyte and Sertoli cells from 20-day-old rat (early pubertal) for 2 weeks in the absence or presence of FSH and/or testosterone [35]. During the culture, the total number of cells, the number of somatic cells, and the number of 2C population was similar in all the conditions. The minimum and maximum percentages of 1C population were found in control (FSH^-/$Testosterone^-$) and FSH^+/$Testosterone^+$ condition 16.8 versus 27.7 %, respectively. These findings were in concordance with

the results of messenger RNA (mRNA) expression of TP1 (meiotic marker). To ascertain the effect of FSH and testosterone, they cultured isolated round spermatids from 32-day-old rat (adult) testis in similar conditions for 24 h. The use of any of the hormone treatments produced two- to threefold higher contents of TP1 mRNA. This study indicated that FSH and testosterone had positive overlapping effects on the meiotic divisions and the post-meiotic expressions [35].

Encapsulation

In 2005, Lee et al. brought a new perspective to the field [36]. They isolated enzymatically digested testicular cell form seminiferous tubules of NOA patients. After 2–4 weeks of 2D culture, colony cells, displaying alkaline phosphatase activity and expressed OCT-4 and ITGß1, were encapsulated with calcium alginate. Encapsulated cells were placed in a differentiating medium for up to six additional weeks. An upregulation of SCP3, TH2B, and TP1 genes after 2 weeks indicated the appearance of spermatocytes and spermatids in the culture. The presence of 4C (spermatocyte) and 1C (spermatid) cells was confirmed by FISH chromosomal analysis. Injecting the spermatids into human oocytes (from patients' spouses) resulted in a low fertilization rate, i.e., maximum 23 % 2PN with no successful embryo implantation after 10 embryo transfers [36].

Magnetic-Activated Cell Sorting

Returning to animal models, Stukenborg et al. (2008) enriched isolated germ cells from 10-day-old (immature) mice testes by using magnetic-activated cell sorting (MACS) for GFRα-1 marker [37]. The enriched fraction (containing 42–54 % GFRα-1^+ cells) was used for culture in the gel phase of 3D soft-agar-culture-system (SACS). Spermatogenesis was assessed by tracking positive cells for Boule, a meiotic marker for late pachytene, and Crem, a post-meiotic marker for round spermatids. Expressions of Boule and Crem started at days 13 and 21 in culture, respectively. However, they found $Crem^+$ spermatids only when they cultured enriched fraction together with interstitial cells (including Leydig cells). This suggested an effect of testosterone as a product of Leydig cells on spermatogenesis [37].

Minaee Zanganeh et al. (2012) isolated SSCs from 4–6-week-old mice and cultured MACS-sorted $Thy1^+$ cells for 7 days in medium expanding undifferentiated spermatogonia [38]. Then they proceeded to culture expanded SSCs together with Sertoli cells or alone and with or without adding hormonal-vitamins complexes (FSH, testosterone, and vitamins A, C, and D) for another 7 days. In all conditions, post-meiotic (TP1, TP2, and Prm1) markers showed upregulation, and spermatid-like cells were observed. The highest expressions of post-meiotic genes were detected when SSCs cultured together with Sertoli cells and hormonal-vitamins complexes. Shorr and PAS staining confirmed the presence of acrosomal granules in differentiated spermatid-like cells.

Effect of Testosterone and Retinoic Acid

In a large animal model, Xie et al. (2010) co-cultured spermatogonia and Sertoli cells from 3–5-month-old (immature) buffalo testes in a 10 % FBS media supplemented with different concentrations of testosterone (0.1, 0.2, and 0.4 μmol/l) or retinol (3.3×10^{-7} mol/l) [39]. After 30 days of culture, round or elongated spermatid-like cells were observed on the feeder layers of all conditions. The highest percentage of spermatid-like cell production was noticed after 41 days in 0.1 μmol/l testosterone (5.13 %) or retinol (4.38 %) enriched mediums, significantly higher than other conditions. In addition to morphological changes, Raman spectroscopy also showed the biochemical changes in spermatid-like cells. Associated with the morphologic and biochemical changes, spermatogenesis-specific expression of PRM-2 was determined during the differentiation process. However, post-meiotic marker TP1 did not become active indicating that spermatid-like cells were not fully functional haploid spermatids [39].

In a slightly different approach, Hasegawa et al. (2010) isolated round spermatids from 8–10-week-old mature mice testis and focused on sustaining spermiogenesis in vitro by co-culturing them with Sertoli cells in 10 % FBS media supplemented with FSH and testosterone [40]. After 2 days, 16 % of the round spermatids differentiated into elongating spermatids, demonstrating to morphologic features and nuclear condensation comparable to in vivo observations. After injecting in vitro-produced elongated spermatids to mouse oocytes, 15.3 % of them were fertilized and 17 % of fertilized oocytes achieved blastocyst stage. When they injected elongated spermatids directly collected from testes, the fertilization and blastocyst formation rates were 18.5 and 31.7 %, respectively. This study showed that the initial part of spermiogenesis in mice could be supported in vitro, comparable to the in vivo condition [40].

Trend to 3D Culture of Isolated Cells

Advantages of using biodegradable biocompatible scaffolds were subsequently applied by Lee et al. (2011) for improving in vitro spermatogenesis [41]. Isolated spermatogonia with residual somatic cells from an 18-day-old rat (immature) were seeded on a polylactic-co-glycolic acid (PLGA)-based 3D scaffold soaked in 10 % FBS media with FSH and testosterone supplements. One hour after seeding, 40–65 % was successfully attached on the scaffold and its evolution was compared with a similar population of cells seeded on a monolayer plastic culture and organ culture. After 9 and 18 days in culture, the viability and differentiation rate, assessing trypan

blue staining, morphological characteristics, and TP2 post-meiotic haploid marker, was significantly higher in the cells seeded in PLGA scaffold than in a monolayer or organ culture. After 9 and 18 days of culturing on PLGA, the "TP2/total germ cells" ratios were 70 and 80 %, respectively, which was around four times more than either monolayer or organ culture condition. The fertilization ability of these cells was not studied [41].

Abu Elhija et al. in 2012 followed the path using 3D SACS [42•] that has been established by Hulleihel and his colleagues [37]. They added SSCs and Sertoli cells isolated from 6-day-old (immature) murine testes to the soft upper layer of agar placed on top of solid lower agar layer. The evolution of differentiation was monitored by RT-PCR in different time points. Before culture, the only positive markers were pre-meiotic (OCT-4, GFR-alfa-1, c-Kit); after 2 weeks, meiotic and some post-meiotic markers turned positive (CREM-1, protamine-1); and after 30 days in culture, other post-meiotic markers (acrosin and Sp10) and low quantities of mature normal sperm (16 normal-looking sperms per 10^6 seeded cells) were detected in 11 out of 16 (68 %) independent experiments. The fact that this system produced apparently normal sperm, even in small proportion, was a great step forward for the field. However, the presence of sperm was only demonstrated after fixing the slides for histology evaluation; therefore, fertility ability of sperm was not tested [42•].

Since Tesarik et al. (1999) claimed to achieve healthy delivery of a baby from in vitro maturation of pre-meiotic germ cells [21], several studies have tried to reproduce those findings and establish a clinical protocol. Riboldi et al. (2012) suggested using KSR instead of FBS on the media to promote differentiation of CD49f-positive sorted spermatogonial cells [43]. These spermatogonial cells were isolated from biopsy tissue obtained on 11 NOA patients and co-cultured with Sertoli on 20 % KSR media supplemented with FSH, testosterone, RA, and GDNF. Gene expression was monitored by RT-PCR showing upregulation of SCP3 and CREST after 5 days in culture. At the same time, a haploid population was detected by FISH in only one of the 11 NOA patients (9 %). Hence, this study proposed a culture condition to produce haploid cells from spermatogonial cells of NOA patient; however, the efficiency was low [43]. As mentioned before, concerns have been raised over the use of KSR for in vitro spermatogenesis due to its possible alteration of methylation states (Hogg 2015) [27] so further investigations in this direction are required.

Returning to the rodent model, Wang et al. (2014) were able to demonstrate haploid differentiation in culture from mice germ cells without using KSR supplementation [44]. The isolated spermatogonial cells from 6-day-old mice were initially cultured in a medium containing 10 % FBS (M1) for 3 days; then, created germ cell colonies were induced to differentiate in a medium enriched with retinoic acid (RA) for 2 days (M2), and afterwards, the cells were relocated into initial media (M1) for 6–8 days to support the rest of the process. The haploid spermatids were detected via gene-specific expression of SYCP3, Th2B, or PRM1 and immunoflourescent staining for protamine-1. No information about efficacy of differentiation was reported in this study; however, it looks very low yield of differentiated cells [44].

In a recent study, Khajavi et al. (2014) were able to demonstrate the supporting role of testicular somatic cell achieving spermatogenesis in vitro using a 3D system. The MACS-sorted GFRa1 positive spermatogonia were seeded on 3D collagen matrix system with and without adding somatic cells. The cells were in culture for 21 days. RT-PCR showed more than twofold increase on expression of post-meiotic markers (Crem and TTF1) when somatic cells were added [45].

Recently, Huleihal et al. (2015) as part of a review paper described a pilot study on using their previously established 3D method (used for mouse [37, 42•]) on 13–33 month-old rhesus macaque testes [46•]. Freshly isolated seminiferous tubular cells including undifferentiated spermatogonia, Sertoli cells, and peri-tubular cells were cultured in 3D methylcellulose culture system (MCS), which contains only one layer of gel, up to 8 weeks. After 4 weeks, positive cells for CREM-1 (~30 %) or acrosin with round morphology (~27 %) appeared which confirmed the post-meiotic differentiation. However, they were not able to identify elongated spermatids.

Conclusion

In this systematic review, we have summarized half a century of researchers' efforts to achieve in vitro spermatogenesis in mammals. During the last three decades, in vitro spermatogenesis models have moved forward from initially conserving anatomical structure of the testis in culture to complete meiosis from testicular mice germ cells. Despite the fact that complete in vitro meiosis of human germ cells has not been achieved yet, for several reasons, the researchers believe that the current trends are in the right direction. First, the results in mice have already showed that in vitro spermatogenesis can lead to successful IVF. Moreover, establishment of a stable in vitro spermatogenesis system has allowed some groups to overcome serious fertility impairments like meiosis arrest linked to r-Kit-ligand. In humans, some certain data support the feasibility of combining both germ cell maturation and ICSI/ROSI in order to achieve live child delivery. However, these results have not been adequately reproduced. Maintaining the genetic stability of spermatogenesis in vitro, particularly as it is translated to human studies, will be a challenging issue.

Besides fertility restoration, in vitro spermatogenesis in animal models and human can be used to evaluate drug toxicity prior to initiation in vivo. It could also improve our

understanding of basic spermatogenesis physiology and germ cell population dynamics.

In conclusion, in vitro spermatogenesis from testicular tissue is progressing, and is a promising area of research if achieved, will be a revolution in the field of male infertility treatment.

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Compliance with Ethical Standards

Conflict of Interest Guillermo Galdon and Hooman Sadri-Ardekani each declare no potential conflicts of interest.

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- •• Of major importance

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