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The Future of Azoospermic Patients: *In vitro* Spermatogenesis

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Introduction

Male infertility becomes a worldwide problem and contributed to 50% of infertility cases [1]. The main cause of male infertility is spermatogenesis failure such as oligozoospermia and azoospermia. Azoospermia is defined as absence of spermatozoa from semen and it is present in 15% of infertile [2]. It is a major concern due to unability to have their own offspring. Azoospermia is related to incomplete spermatogenesis process which related to genetic disorder, hormonal problem or testicular failure [3]. Recreating human spermatogenesis outside of its original environment is a scientific curiosity in andrology world and a quest for male fertility treatment. It has a huge impact on our understanding on physiology and pathway of genetic in male reproduction using a well-characterized model of human spermatogenesis. Animal studies have provided us the knowledge of gonadogenesis, spermatogenesis and steroidogenesis based on the histology, immunohistochemistry, hormonal assays and phenotype of gene alterations [4]. *In vitro* spermatogenesis in azoospermic patients has long been attempted, however, it remains challenging due to limitation of culture system. In this review, we would address the limitation studies of the *in vitro* human spermatogenesis in developing sperm for future clinical application.

In vivo and *In vitro* Spermatogenesis

Spermatogenesis is a complex process that involves proliferation and differentiation of spermatogonia cells into mature motile spermatozoa within the seminiferous tubules in male testis. Basically, there are three stages of spermatogenesis; formation and migration of Primordial Germ Cells (PGC), mitotic division which increase the germ cell numbers and meiotic reduction in chromosome content. This followed by the spermiogenesis which involves the differentiation and maturation of sperm cell involving nuclear shaping and condensation, formation of acrosome, rearrangement of cell organelles, shredding of cytoplasm and formation of flagella [5]. In adult human testis, this process takes about 65-75 days [6]. The continuation of spermatogenesis depends on the Spermatogonial Stem Cells (SSCss) which has the ability to self-renewal and differentiation [7]. *In vitro* differentiation of SSCss is a difficult technique due to microenvironmental niche in the testis which requires structural support from the Sertoli cells. The Sertoli cells plays an important roles as a blood-testis barrier, secretes factors to maintain and control spermatogenesis and acts as phagocytosis in clearence of apoptotic germ cells (generation of fertile sperm).

Spermatogenesis process needs both endocrine and paracrine/autocrine mechanisms [8]. The endocrine stimulation in human spermatogenesis involves the Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and testosterone hormone. FSH stimulates the DNA synthesis in the mitotic and meiotic division where as LH will bind to the Leydig cells in the seminiferous tubules to produce testosterone hormone which also impacts on the mitosis and the successful completion of meiosis [8]. The normal level of FSH in men is between 5-15 IU/L and testosterone level is 270 to 1,070 nanograms. Paracrine mechanism plays an important role in the regulation of spermatogenesis by maintaining and coordinating the testicular cells' normal activity. The paracrine process includes Interleukin (IL), Tumor Necrosis Factor (TNF), Stem Cell Factor (SCF) etc. [9]. These paracrine mechanisms have shown to affect both the germ cell proliferation and the secretion and function of the Leydig and Sertoli cells.

In vivo studies on Spermatogonial Stem Cells (SSCs) provide an understanding of the spermatogenesis and endocrine regulation process. The successful transplantation of SSCs population for continuation of spermatogenesis process in mice testis donor was demonstrated in 1994 by Brinster and Zimmermen. This theoretical model has successfully proliferated, differentiated and restored spermatogenesis using both fresh and cryopreserved mouse SSCs [10] and has expanded into other animal models. However, only rats clouthier et al.; Brinster et al. [11,12] and hamsters [13] were able to complete spermatogenesis in recipient mice testis. The transplantation of SSCs model has become a useful tool of understanding the testicular stem cells and germ cell differentiation [14]. There would be a potential to clinically apply SSCs transplantation in human especially in male treated cancer patients. However, it remains unknown if the recipient will react in the similar process of the donor SSCs and there could be a risk of malignancy cells. Study by Sadri-Ardekani et al. [15] shown the xenotransplantation of human testicular cells of leukemic patients resulted successful propagation of Spermatogonial stem cells in recipient rats but it can be risk for tumor cells (Sadri-Ardekani et al.) [15]. A few studies on testis xenograft transplantation model has done from fetal or pre-pubertal cancer patients' testicular tissue onto immune-deficient nude mice and demonstrated survival of spermatogonia and few spermatocytes at the pachytene stage and spermatid-like cells for more than 135 days (Wyns et al. and Yu et al.) [16,17]. Human xenograft models have great promising results however; further study must be done to determine the risk of toxicity and safety use for clinical application.

Most of the *in vitro* studies tried to initiate and colonize the SSCs based on the microenvironment or niche in the seminiferous tubules. Variety species have been used for *in vitro* spermatogenesis using testicular tissues however, mostly gonocyte development and progression arrest at meiotic stage (Lo and Domes) [4]. Growth factors such as Growth Differentiation Factor (GDF), Basic Fibroblast Growth Factor (BFGF) and Leukemia Inhibitory Factor (LIF) have promoted the proliferation of mouse spermatogonial stem cell survival [18]. Study by Feng et al. shown Stem Cell Factor (SCF) are capable of undergoing differentiation into haploid spermatids from spermatogonia in mice (Feng et al.) [19]. The use of Retinoic Acid (RA) in *in vitro* culture

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shown SSCss can be differentiated into early meiotic germ cells (Song and Wilkinson) [20]. Testicular cells co-culture with Sertoli cells have shown positive differentiation into round spermatid (Virgier et.al.) [21]. Minaee Zanganeh et al. used co-cultured with Sertoli cells and in presence of hormones and vitamins in mouse SSCss resulted differentiation into spermatid (MinaeeZanganeh et al.) [22]. However, study by Iwanami et al. showed that type A spermatogonia of immature rats differentiate into round spermatid-like cells in co-cultured with Sertoli cells had generated abnormal gene expression patterns and micro-insemination did not result in offspring (Iwanami et.al.) [23]. In the zebra fish (Danio rerio) *in vitro* co-cultured with Sertoli cells have shown complete process of spermatogenesis (Sakai) [24]. Xie et al. used buffaloes (Bubalus bubalis) testicular cells co-cultured with Sertoli cells resulted spermatogonia differentiation into spermatids (Xie et al.) [25]. A three-dimensional (3D) environment of extracellular matrix components such as calcium alginate, matrigel, soft agar, methylcellulose or collagen might reflect the testicular environment compared to two-dimensional plastic surface in a culture dishor might provide options for re-aggregation of more complex tissue. Soft agar culture on mice have successfully differentiatedinto spermatozoa from germ cells (Elhija et al. and Stukenborg et al.) [26,27]. The use of collagen in this technique culture resulted differentiation of rat germ cells into post-meiotic stages (Lee et.al.) [28]. Animal studies provide a plateform to further study on optimal components to achieve *in vitro* spermatogenesis such as media conditions, culture temperatures, hormone concentration and requirement fraction of non-gonodal cells.

As for human studies, the development of spermatozoa from spermatogonial stem cells have not been successfully achieved. There are a few *in vitro* studies stated the differentiation of the spermatogonia cells until spermatid stage using various volume of rFSH and testosterone hormone supplement (Dong et al., Sousa et al. and Tesarik et al.) [29-31]. *In vitro* culture with variousgrowth factors such as epidermal growth factor (EGF), glial cell line-derivedneutrophic factor (GDNF), BFGF and LIF shown induced of pluripotent cells derivedfrom SSCs testicular tissues (32). Study by Piravar and collegues shown survival of SSCss using EGF, Leukemia Inhibitory Factor (LIF) and GDNF in long-term culture (Piravar et al. and Goharbakhsh et al.) [33,34]. The use of FSH and testosterone hormones in *in vitro* culture resulted differentiation of male halpoid cells from germ stem cell-like cells (Lee et al.) [28]. Co-culture with feeder cells such as Vero cells from monkey or Sertoli cells hasa positive development in human spermatogenesis process which involved meiosis division (Tanaka et.al., Sousa et al. and Cremades et al.) [30,35,36]. In the 3D culture using collagen gel shown differentiation of spermatocytes into spermatids in non-obstructive azoospermic patients [37]. Lee et al. have successfully developed specific gene expression forpostmeiotic haploid in non-obstructive azoospermia patients using calcium alginatecapsules in 3D culture (28).. However, ethical concern need to be considered due to the use of animal cells into human clinical application.The summary of culture techniques from human samples and animals are demonstrated in Table 1. There are a few *in vitro* Embryonic Stem Cells (hESC) studies shownthe development, epigenetic reprogramming, and modification of germline gene derived in the embryonic stem cells have resulted male haploid round spermatid stage (Toyooka et al. and Geijsen et al.) [38,39]. Induced Pluripotent Stem Cells (iPSC) have been developed to express embryonic stem cell-like properties by activate the plupotency transgenes in adult somatic cells [40]. However, abnormalities occured from the iPSC cultures such as chromosomal abnormalities, aberent DNA methylation and retention of epigenetic markers [41,42].

The oral intake of zinc, folate and antioxidants (vitamin C and E) supplements in humans have successfully maintain the normal spermatogenesis and sperm maturation as well as in DNA metabolism, synthesis, repair and transcription [43,44]. Zinc is also an important nutrient for sexual maturation and reproduction. Zinc deficiency in humans results in arrest of normal growth process and causes teratological abnormalities. Zinc plays multiple roles in sperm functions in stabilizing the cell membrane and nuclear chromatin, influences the sperm motility, capacitation and acrosome reaction [45]. There are a few studies shown that oral zinc supplement for infertile patients *in vivo* improve the sperm count, sperm motility and physical characteristic of the sperm [44,46]. *In vitro* study by Yamaguchi et al. showed that zinc is an essential trace element for the maintenance and regulation of both spermatogenesis and sperm motility in the Japanese eel [47]. Reactive Oxygen Species (ROS) produced by sperm are needed for capacitation, the acrosome reaction and fertilization. However, excessive production or reduced production lead to oxidative stress which result in DNA damage, reduced motility and defective membrane integrity [48,49]. The oxidative stress has become possible role in the pathogenesis of male infertility. Antioxidants such as vitamin E and vitamin C in *in vitro* or *in vivo* studies have shown may help maintain the balance between ROS production, reduced in sperm apoptosis and sperm DNA fragmentation and thus could improve the sperm quality [50]. So far there is no study *in vitro* using zinc or antioxidants for the development of human sperm. This could be a useful elements to further study for potential development of sperm *in vitro* spermatogenesis.

Molecular Marker in Spermatogenesis

Spermatogenesis has different genes that express stages of spermatogenic cells. The spermatogenesis is maintained by the ability of SSCss to provide a continual supply of differentiating spermatogonia. In the early of spermatogenesis, rodent and human have different categories of Spermatogonial stem cells. In the rat testis, six generations of differentiating spermatogonia are observed: A1, A2, A3, A4, intermediate (In), and B spermatogonia [51] while in humanSSCss divided into A_{dark} and A_{pale} and B spermatogonia [52]. The premeiotic molecular markers in human and rodents are quite similar such as Oct4 (POU5F1), α6-integrin (CD49f), GPR125, PLZF, GFR-α1, Thy1 (CD90), CD9 and β1-integrin (CD29). NGN3, RET, CDH1 (CD324) and Stra8 are other gene markers present in rodent SSCss. As for human, C-KIT, CD133, MAGE-A4 (melanoma antigen family A4), CHEK2, NSE (neurone-specific endolase), alkaline phosphatase and TSPY (testis specific protein Y-linked 1) are the other gene markers for SSCss [29,53-55]. In the meiotic and post-meiotic phases, human and rodents have different of gene markers. LDH and Crem-1 are markers for meiotic in rodents [26] whereas TH2B (human testis specific histone) and SCP3(synaptonemal complex protein 3) are meiotic markers for human [32]. In the post-meiotic phase, Protamine, Acrosin and SP-10 are gene markers for rodents. As for human, Transition Protein 1 (TP1) is the gene marker for post-meiotic [32]. The pre-meiotic, meiotic and post-meiotic gene markers for human and rodents are summarized in Table 2.

Method of culture	Studies from human samples		Studies from animals	
	Reference	Outcome	Reference	Outcome
Testicular culture with growth factors such as GDF, BFGF, EGF, GDNF, LIF, SCF or RA for survival of cell culture	Lim et al., [32] Piravar et al., [33], Goharbakhsh et al., [34]	Pluripotent cells induced from SSCss Survival of SSCss in long-term culture	Kanatsu-Shinohara et al., [18] Feng et al., [19] Dann et al., 2008	Survival of mouse spermatogonial stem cells Differentiation into haploid spermatids from spermatogenic SSCss can be differentiated into early meiotic germ cells
Testicular culture with reproductive hormones such as rFSH and testosterone for differentiation of male germ cells	Dong et al., 2006, Sousa et al., [30], Tesarik et al., [31] Lee et al., [28]	Differentiation of the spermatogonia cells until spermatid stage Differentiation into haploid male germ cells	Minaee Zanganeh et al., 2013	SSCss differentiation into spermatid
Testicular cells co-culture with Sertoli cells or Vero cells as feeder cells	Sousa et al., [30], Cremades et al., [36] Tanaka et al., [35]	Meiosis in non-obstructive azoospermic patients. Meiotic differentiation primary spermatocytes into round spermatids in azoospermic men.	Virgier et al., 2004 Iwanami et al., [23] Sakai, [24] Xie et al., [25]	Positive effect on meiotic divisions of pachytene spermatocytes into round spermatids of rats. Differentiation of type A spermatogenic of rats into round spermatid-like cells with abnormal gene expression pattern. Complete process of spermatogenesis in the zebra fish (Danio rerio) Spermatogonia differentiate into morphological normal spermatids in buffaloes (Bubalus bubalis)
Three-dimential culture using collagen or soft agar	Lee et.al., [37] Lee et.al., 2006a	Differentiation of spermatocytes into spermatids in non-obstructive azoospermic patients. Expression of genes specific for post meiotic haploid state in non-obstructive azoospermic patients.	Lee et al., [28] Stukenborg et al., [27] Elhija et al., [26]	Meiosis and differentiation of rat germ cells into post-meiotic stages Differentation and meiosis of spermatogonia into morphologically mature spermatozoa in mice. Differentation and meiosis of spermatogonia into morphologically mature spermatozoa in mice.

Table 1: Overview *in vitro* spermatogenesis between human and animals.

Phases of spermatogenesis	Human markers	Rodent markers
Pre-meiotic	OCT4, GFRA1, C-Kit, CD9, ITGA6, ITGB1, GPR125, THY-1, C-KIT, CD133, MAGE-A4, CHEK2, NSE, alkaline phosphatase and TSPY	OCT4, GFRA1, C-Kit, CD9, ITGA6, ITGB1, GPR125, THY-1, NGN3, RET, CDH1 (CD324) and Stra8
Meiotic	TH2B, SCP3	LDH, Crem1
Post-meiotic	TP1	Protamine, Acrosin and SP-10

Table 2: A comparison of markers for human and rodent in pre-meiotic, meiotic and post-meiotic spermatogenesis.

Ethical Issue

In vitro spermatogenesis would be useful for chemical screens, identify the causes of infertility, the critical pathways of spermatogenesis and the imprinting disorder that would be inherited to the offspring. Ethical issue should be concern in utilizing *in vitro* spermatogenesis before proceed to clinical trial such as the source of from animal cells or other human cells into recipient human cells or the use of adult stem cells or embryonic stem cells. There could be a risk of malignancy cells transplantation or abnormalities in genetic modification from these studies

[4,56-58]. The potential development of spermatozoa *in vitro* culture will results in passing own genetic material to his offspring. However, the status of the haploid produce from the *in vitro* spermatogenesis should explore further to maintain the normality of the sperm.

Conclusion

There is a potential to develop spermatozoa *in vitro* spermatogenesis in treating male infertility. However, this research needs to explore further and improve the methodology needs to generate the normal haploid sperm before use in clinical trial.

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