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POSSIBLE USE OF SPERMATOGONIAL STEM CELLS IN THE TREATMENT OF MALE INFERTILITY

N.N. Skaletsky, G.N. Skaletskaya, V.I. Sevastianov

Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russian Federation

Spermatogonial stem cells, which are already present at birth in the testicles, are the progenitors of male gametes. These cells cannot produce mature sperm before puberty due to their dependence on hormonal stimuli. This feature of the reproductive system limits preservation of fertility only to males who can produce an ejaculate. Therefore, the use of cancer treatment which can lead to fertility loss has made sperm cryopreservation a standard practice. Prepubertal cancer boys – who are prescribed chemotherapy that is toxic to their reproductive system – are deprived of this fertility management procedure. This review focuses on the problem of obtaining and preserving spermatogonial stem cells for future transplantation to restore spermatogenesis. Development of these methods is becoming increasingly urgent due to higher survival rates in childhood cancer over the past decades thanks to improvements in diagnosis and effective treatment. Restoring and preserving fertility using spermatogonial stem cells may be the only option for such patients.

Keywords: spermatogonial stem cells, fertility, cell culture.

INTRODUCTION

Male infertility is a pressing issue, whose solution is of medical and psychosocial importance. It can affect the future of a nation to a certain extent. Properly understanding the pathological processes underlying fertility disorders is extremely important since various etiological factors and pathogenetic mechanisms disrupt the quantitative and qualitative parameters of sperm. According to estimates, over 8% of men of reproductive age seek medical help for infertility problems. In about half of subfertile (with reduced fecundity) couples, the male factor is the main cause. In about 12% of subfertile men, severe oligozoospermia or azoospermia is detected. In oligozoospermia, the semen ejaculated during an orgasm contains fewer sperm than normal for fertility. Genetic disorders, inflammatory, endocrine and infectious diseases, alcohol and drug abuse, radiation and use of certain gonadotoxic drugs, heavy metal and carbon dioxide poisoning can all lead to low sperm count. Azoospermia is a medical condition where the semen contains no sperm. There are two types of azoospermia – obstructive and non-obstructive. With obstructive azoospermia, sperm cannot get into the semen due to impaired patency or absence of vas deferens. The ducts located in the epididymis can also be affected. Various infectious and inflammatory diseases, injuries, varicocele, or congenital anomalies of the genitourinary tract sometimes lead to obstruction of ejaculatory ducts. Non-obstructive azoospermia results from endocrine and genetic diseases, after radiation exposure, with some metabolic disorders (diabetes mellitus), and oncological diseases. In the treatment of most cases of azoospermia, especially the non-obstructive forms of genetic origin, assisted reproductive technologies takes priority, with the aim of restoring the qualitative and quantitative parameters of sperm or ensuring sperm maturation *ex vivo*. Therefore, the possibility of obtaining the required amount of mature sperm cells via *in vitro* manipulation gives hope to men with severe spermatogenesis of becoming biological fathers.

Spermatogonial stem cells (SSCs), which are already present at birth, are stem cells of the testicle [1, 2]. These cells cannot produce mature sperm before puberty due to their dependence on hormonal stimuli. This biological characteristic of the reproductive system limits fertility preservation only to males capable of producing an ejaculate, because the standard procedure for fertility preservation is semen cryopreservation, which usually guarantees future achievement of paternity [3–5]. This possibility becomes very important in men whose reproductive system has been exposed to the toxic effects of infertility-causing chemotherapy and radiation for cancer [2, 6].

Over the past decades, childhood cancer survival rates have increased due to improved diagnosis and more effective therapy [4]. However, anti-cancer treatment often has a devastating effect on the reproductive system of prepubertal boys. Preliminary sperm preservation in the boys is impossible since there is no sperm to preserve. Cryopreservation of testicular tissue for future extraction and transplantation of SSCs is an option currently being developed for restoration of spermatogenesis after chemotherapy in prepubertal boys. Successes

Corresponding author: Skaletskiy Nikolay Nikolaevich. Address: 1, Shchukinskaya str., Moscow, 123182, Russian Federation. Tel.: (499) 190-42-66, (903) 790-95-39. E-mail: NSkaletsky@mail.ru

recorded in restoring spermatogenesis in animals after SSC transplantation hold out hope that this direction will succeed [7–11]. Development of autologous transplantation technology for SSCs capable of differentiating into mature spermatozoa will be of great importance for practical medicine [12]. With successful implementation of necessary research on SSC production, storage and transplantation, prepubertal boys with cancer will be able to father children in the future [3]. The following describes the basics of obtaining (isolating), identifying and cultivating SSCs *in vitro* for proliferation. Prospects for using SSCs to preserve fertility in prepubertal boys are assessed.

ISOLATION OF SPERMATOGONIAL STEM CELLS

Cell isolation studies are typically based on their ability to self-renew [13, 14] and on optimization of procedures increasing the purity of SSCs obtained, primarily by preventing contamination with other cell types. Generally, SSCs are isolated by enzymatic digestion, usually involving a combination of enzymes such as collagenase, trypsin, and DNase I [15]. Various methods have been developed to obtain highly pure population of SSCs, such as morphology-based selection associated with differential precipitation, extracellular matrix selection, fluorescence-activated cell sorting (FACS), and magnetic-activated cell sorting (MACS).

Morphology-based selection of SSCs is the simplest and cheapest method, but it has the lowest efficiency [16]. Since this method is based on enzymatic isolation of cells and subsequent precipitation at different times, the resulting samples are contaminated with various types of testicular cells, such as Sertoli cells, Leydig cells, myoid cells, and fibroblasts [16, 17]. These cells may release growth factors, hormones and extracellular matrix elements to some extent and thus interfere with *in vitro* self-renewal and proliferation of SSCs [17]. Extracellular matrix selection uses various extracellular proteins, such as laminin and fibronectin, to stimulate SSCs adhesion. These substrates are capable of binding other extracellular matrix components, and they are widely used in *in vitro* cell culture to facilitate cell attachment and stimulate cell proliferation [18]. Since SSCs have weak adhesion potential, it is necessary to facilitate their attachment by coating with substrates to maintain viability *in vitro* [18]. A high-pure SSC population can be obtained using sorting assays such as FACS and MACS. Although the described methods can basically provide SSC cultures of high purity, they have drawbacks due to the resource-intensive procedures and technical difficulties, which often lead to low cell production and insufficient cell viability [15, 18].

To obtain highly pure cell suspensions, it is advisable to use a combination of different SSC isolation methods and look for new methodological approaches. Analysis of the protocols used has shown that most of them involve differential seeding of the initial cell suspension to eliminate other types of testicular cells. This method separates the cells according to their distinctive attachment features. Cells are seeded directly on tissue culture plates or matrix-coated plates using gelatin, laminin, fibronectin or collagen [16, 17]. A recent study has demonstrated effective removal of contaminating cells and high-precision selection of SSCs using two-step purification. First, the collected SSCs were cultured on somatic and Sertoli cells, and after isolation, the cells were purified by centrifugation in bovine serum albumin density gradient. With this method, most unnecessary cells were removed, and the purity of the SSC culture reached above 91.5% [18].

Given that the main purpose of isolating, culturing, and transplanting SSCs is to preserve and restore fertility, and that most patients who benefit from SSCs transplantation have cancer at the time of testicular biopsy, the risk of re-introducing malignant cells must be eliminated. It is necessary to obtain high-pure SSC cultures by a certain combination of identified positive and negative selection markers in order to exclude malignant contamination (1). Despite this, it has not yet been possible to identify – with enough certainty – a combination of positive and negative markers that would obtain a pure SSC suspension [19]. However, a recent study showed that with a combination of CD90 (positive marker) and CD45 (negative marker), the germ cell suspension appeared to be free of cancer cells [12].

CULTIVATION OF SPERMATOGONIAL STEM CELLS *IN VITRO*

As with stem cells in many tissues, the proportion of SSCs is substantially lower than that of surrounding somatic cells. In mice, SSCs located in the epithelium of the seminiferous tubules account for only about [12, 20]. To get enough SSCs for future autotransplantation, their mass needs to be artificially increased, given that the volume of an adult testis is approximately 60 times larger than the biopsy taken from a prepubertal boy. The table shows the steps for obtaining and transplanting SSCs.

Successful long-term *in vitro* proliferation of SSCs was first demonstrated in mice and rats [21–27]. It was shown that during cultivation, the quantity of rodent SSCs can increase exponentially, and they can maintain their biological potential for productive spermatogenesis and restoration of fertility after transplantation into the testes of infertile recipient mice [9, 10, 24–28]. Preservation of non-human primate SSCs by short-term cultivation was then reported [29], and several groups reported both short-term and long-term cultivation of human SSCs, both in adult males and in prepubertal boys [30–36]. In one study [30], cultivation of human testis cells for 64 days increased SSC numbers by more than 18,000 times.

Table

Key achievements in *in vitro* production of SSCs for use in restoring fertility

Year	Authors	Most significant research	Type
1971	Huckins	Spermatogonia renewal and differentiation model and spermatogonial stem cell detection	Rat
1994	Brinster and Avarbock	First successful transplantation of testicular cells from one mouse to another, emergence of offspring from a donor	Mouse
1998	Nagano et al.	Preserving *in vitro* SSCs for 4 months using a somatic cell feeder layer	Mouse
1999	Schlatt et al.	Xenotransplantation of a testicular cell suspension obtained from one primate into the testes of another	Monkey
2002	Nagano et al.	First report on successful cell colonization in mouse testes after xenotransplantation of human SSCs	Human
2003	Kanatsu-Shinohara et al.	Prolonged *in vitro* SSC propagation using GDNF without cell immortalization in culture	Mouse
2005	Keros et al.	Evidence of successful cryopreservation of testis biopsies without compromising their structural integrity	Human
2005	Kanatsu-Shinohara et al.	Long-term propagation of SSCs in serum-free feeder medium	Mouse
2009	Sadri-Ardekani et al.	Long-term *in vitro* propagation of SSCs obtained from adult testis, while maintaining functionality	Human
2011	Sadri-Ardekani et al.	Long-term propagation of SSCs obtained from prepubertal testes, while maintaining their function	Human
2012	Hermann et al.	Production of functionally active sperm capable of fertilizing oocytes by immature macaques subjected to SSC autotransplantation	Monkey
2014	Langenstroth et al.	Separation of somatic and germ cells to create SSC cultures	Monkey
2018	Sharma et al.	Differentiation of xenograft SSCs of monkeys depending on gender and fertility of recipient mice	Monkey

The culture system developed for SSCs is usually based on the use of a feeder medium supplemented with hormones and growth factors, as well as feeder layer of somatic cells [35]. A key factor for most cell cultures is animal fetal serum, but it has proven to be unfavorable for SSC proliferation [25]. Various serum concentrations in the growth medium were used in SSC cultivation, but none of them was able to enhance SSC proliferation compared to the serum-free medium [25]. Purified proteins and additives are required to compensate for the absence of hormones or growth factors supplied by serum [37]. Some of the identified growth factors required for SSC proliferation are the glial cell-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 [14, 26, 38].

An increase in the number of SSCs in the *in vitro* culture system should ideally resemble the *in vivo* situation [39]. *In vivo*, there is a complex niche environment where SSCs and somatic support cells interact to establish the necessary intracellular signaling. Various factors required for stem cell preservation have been identified. Artificially simulating a niche environment is a very difficult task because there are many factors behind interaction between SSCs and somatic cells, and most of them are not well characterized. The use of a feeder layer consisting of somatic cells (often inactivated mouse embryonic fibroblasts, MEFs) is considered important for successful SSC propagation [8, 40]. Growth of spermatogonia on the feeder layer can lead to formation of three-dimensional aggregates, called clusters, which contain many cell types, including SSCs [41]. In order to increase their number, it is necessary to provide *in vitro* microenvironment that is as close as possible to the SSC niche in the testes [13, 39]. SSC niche consists of various supporting cells, such as Sertoli, peritubular, myoid and Leydig cells [18]. However, enzymatic treatment to dissociate tissue and isolate cells destroys the integrity of such an important microenvironment. Feeder layer has a positive effect on SSC preservation, since the cells contained in it produce growth factors and cytokines, which contribute to the conditioning of the medium [8, 42–44]. Similarly, a feeder layer (MEF) provides a convenient surface for SSC attachment. Like any other type of cell, they directly depend on the topography, roughness, and stiffness of substrates [45, 46]. The use of several techniques that improve SSC cultivation conditions has facilitated long-term increase in the pool of cells obtained from different strains of mice of different age groups. SSCs remained undifferentiated for 6 months without loss of function and ability to restore normal spermatogenesis after transplantation [26]. However, clinical use of SSCs requires development of culture systems without xenogenic and feeder culture systems in order to avoid pathogenic contamination [47].

POTENTIAL USE OF SPERMATOGONIAL STEM CELLS IN THE FUTURE

Since the late 1990s, scientists have been able to restore spermatogenesis in animal models. Since then, great

progress has been made in studying the characteristic features of spermatogonial stem cells and the possibility of preserving them *in vitro* by developing culture systems [42, 47, 48].

For future clinical use in humans, the culture medium should preferably not contain animal serum due to possible zoonotic or xenotoxic effects. The use of somatic cells existing in testicular biopsies helps preserve SSCs and can replace the use of exogenous cells located in the feeder layer. On the other hand, it can be imagined that cultivation in a medium in which serum is absent [49] or some growth factors [40] can affect the function of SSCs and decrease their potential [44]. Intervention in cultural conditions is a "double-edged sword": on one hand, there is increased quantity of SSCs due to addition of certain factors, while on the other hand, their functionality may be impaired by the same additives. Ability to modulate *in vitro* conditions involved in self-renewal control, as opposed to SSC differentiation, can lead to production of functionally active gametes *in vitro* [42]. These products, associated with transplantation methods of animal and human models, will facilitate the study of molecular and cellular biology of differentiation of male germ cells and make it possible to develop new infertility treatment strategies [16, 42].

Prospects for restoring male fertility have great potential in basic and applied science [15, 50]. Development of culturing techniques may offer future hope for preserving fertility in cases with no other way out, for example, in prepubertal boys with cancer. Many clinics already cryopreserve testicular tissue from men with cancer. However, methods should be developed to eliminate the risk of reintroducing malignant cells during SSC transplantation [2].

In addition, various studies have shown that SSCs can differentiate into various types of *in vitro* cells, such as cardiomyocytes and nerve cells. It is important to note that these cells have several advantages over embryonic stem cells, such has absence of ethical concerns about their use and origin. In addition, they have lower incidence of tumorigenesis and graft rejection. Based on the-

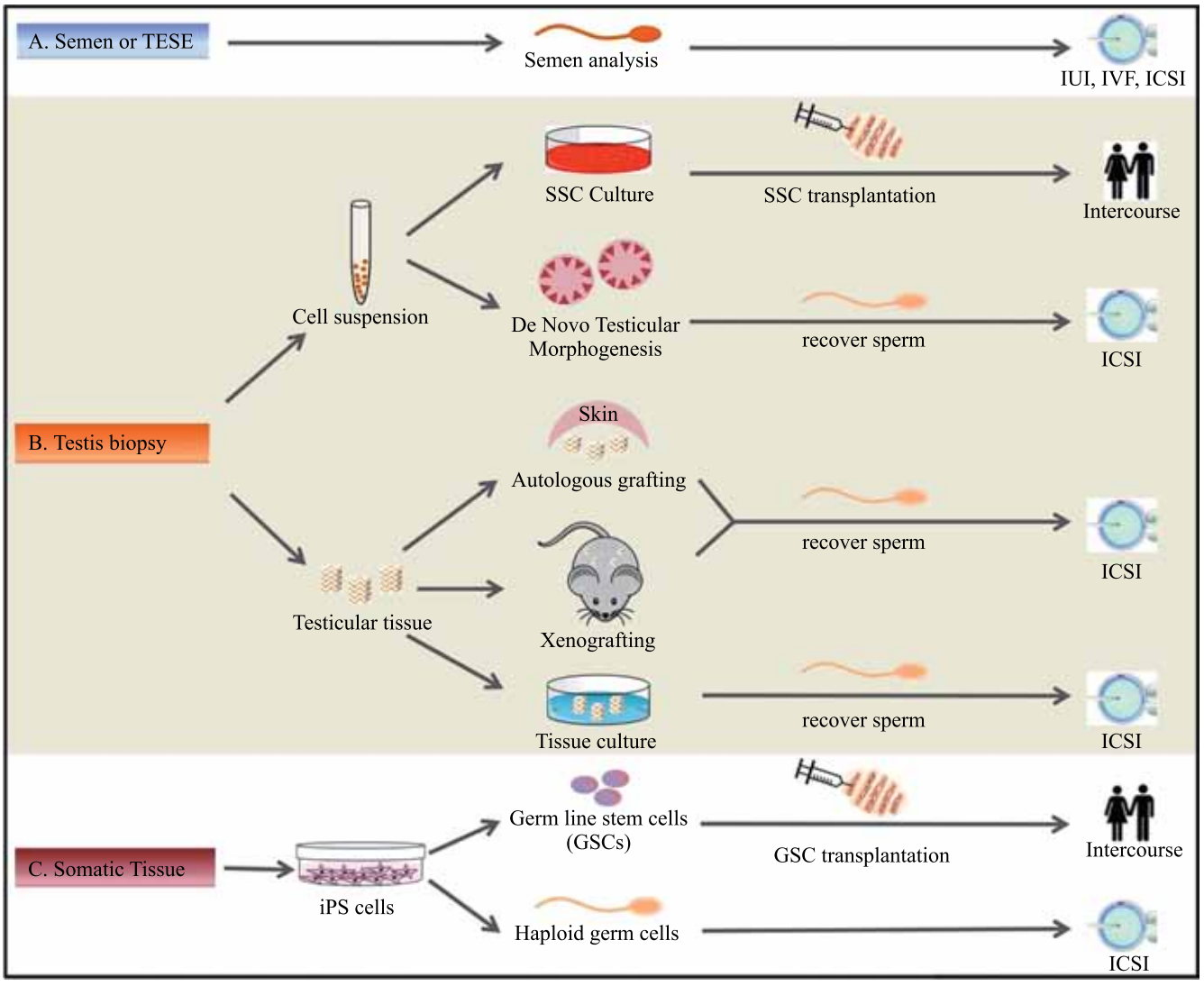

Fig. Standard and experimental treatment options for male infertility (by K. Gassei, K.E. Orwig, 2015)

se assumptions, SSCs may be one of the most promising candidates for clinical applications in cell therapy [23].

As mentioned above, the size of biopsy that can be obtained from prepubertal male testis is relatively small and may contain small amount of SSCs. The amount of SSCs that will be required to regenerate spermatogenesis and achieve fertility in humans is not exactly yet known. However, it is reasonable to assume that the number should be substantially increased in culture prior to transplantation to ensure reliable engraftment and effective spermatogenesis. Each group of researchers reporting on human SSC culture used different cell isolation and cultivation techniques, different feeder media and matrix substrates [51, 52], different sets of growth factors and different methods for evaluating results. Until recently, no method for obtaining human SSC cultures has been independently replicated by another research group. Replication is needed in order to confirm true success and achieve real progress [19]. Besides, while SSC transplantation to regenerate spermatogenesis using functionally active spermatozoa and production of offspring is the gold standard for assessing the quality of rodent SSCs obtained, there is no equivalent analysis of human SSCs. Molecular markers and xenotransplantation between humans and mice may be reasonable surrogate analyzes, but so far there is no agreement within the scientific community that would allow experiments with human SSCs. Perhaps, implementation of *de novo* morphogenesis of testis and/or use of decellularized testes will help create a complete model of human spermatogenesis and conduct the necessary experiments.

Generalizing the numerous male infertility studies described in this review to a certain extent, they can be shown in the form of a small diagram (Fig.) and comments, which are presented below [25].

A. Spermatozoa obtained from ejaculated sperm or by testicular sperm extraction (TESE) from the testes of infertile men can be used to facilitate pregnancy via intrauterine insemination (IUI), *in vitro* fertilization (IVF) or IVF with intracytoplasmic sperm injection (ICSI).

B. Where it is not possible to obtain spermatozoa by biopsy, testicular tissue containing SSCs can be obtained. Testicular tissue can be digested with enzymes to produce a cell suspension from which significant amount of SSCs can be obtained by culturing, which, in turn, can be transplanted into the patient's testes. This method can regenerate spermatogenesis, and possibly natural fertility. Heterogeneous suspensions of testicular cells also have the potential for *de novo* morphogenesis of testis with seminiferous tubules and polarized epithelium surrounded by basal membrane with germ cells inside and interstitial cells outside the tubules. Sperm generated in "rebuilt" testes can be used to fertilize eggs by ICSI. Intact testis tissues from prepubertal animals can be auto- or xenotransplanted under the skin or in the scrotum and produce mature sperm that can be used to fertilize eggs by ICSI. Sperm can also be generated when immature testicular tissue is preserved in culture and used to fertilize eggs via ICSI.

C. Patient-specific induced pluripotent stem cells (iPSCs) can be derived from the patient's somatic tissues (e.g., skin or blood) and differentiated into germline stem cells (GSCs) for further introduction into the patient's testes. This technique may have the potential for regeneration of spermatogenesis and natural fertility. It is also possible to differentiate iPSCs cells into spermatozoa, which can be used to fertilize eggs by ICSI.

Apparently, help from social efforts can play a significant role in initiating and supporting scientific research on prevention and treatment of male infertility. The society is beginning to realize the benefits of fertility preservation in pediatric and adolescent patients suffering from cancer and undergoing gonadotoxic chemotherapy, as well as those with severe sexual development problems [53]. Certain efforts are being made to create multidisciplinary community groups that would assist in developing studies on fertility preservation for pediatric patients and assessing relevant ethical issues and necessary material costs. In particular, the Oncofertility Consortium has been established to provide guidance for health care providers aiming to develop programs at institutions lacking pediatric fertility preservation services [54].

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