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Optimizing Immature Testicular Tissue and Cell Transplantation Results: Comparing Transplantation Sites and Scaffolds

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Abstract

For patients who had testicular tissue cryopreserved before receiving gonadotoxic therapies, transplantation of testicular tissues and cells has been recommended as a potential therapeutic option. There are no studies that indicate the generation of sperm after human immature testicular tissue (ITT) or spermatogonial stem cells (SSCs) transplantation. The use of releasing scaffolds and localized drug delivery systems as well as the optimizing transplantation site can play an effective role in increasing the efficiency and improving the quality of testicular tissue and cell transplantation in animal models. Current research is focused on optimizing ITT and cell transplantation, the use of releasing scaffolds, and the selection of the right transplantation site that might restore sperm production or male infertility treatment. By searching the PubMed and Google Scholar databases, original and review papers were collected. Search terms were relevant for SSCs and tissue transplantation. In this review, we'll focus on the potential advantages of using scaffolds and choosing the right transplantation site to improve transplantation outcomes.

Keywords: Injection, Scaffold, Spermatogonial Stem Cells, Transplantation

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Introduction

Chemotherapeutic drugs and radiation are proven to damage the gonads of prepubertal boys, despite their growing effectiveness in treating childhood cancers. As long-term survival rates for children with cancer keep going up, methods to maintain fertility in prepubertal boys who do not so far produce spermatozoa are urgently needed (1). According to a survey among adolescent cancer patients between the ages of 14 and 40 at the time of diagnosis, 51% wished to have their own children in the future (2). Prepubertal boys can't freeze a sample of their semen before beginning fertility-threatening therapies, while men can freeze a sample of their sperm (3). Numerous strategies utilizing the patient's own frozen-thawed immature testicular tissue (ITT) have been suggested to restore fertility after treatment, including autotransplantation of testicular tissue fragments or spermatogonial stem cells (SSC), *in vitro* maturation, and production of testicular organoids for use *in vivo* or *in vitro* (4). When a patient undergoes therapy, the transplantation of tissue or cells into the patient provides a promising method for the restoration of fertility. Cryopreservation of ITT for future transplantation has been recommended for a variety of patient populations; nevertheless, considerable obstacles must be overcome before this becomes a reality in clinical practice for fertility restoration. Today, there are no studies demonstrating the production of sperm following the transplantation of human ITT or SSC (5). ITT transplantation is one of the most effective methods for maintaining SSC in their niche and ensuring their interaction with germ cells and other supporting cells, resulting in a suitable environment for cell maturation, growth, and differentiation (6).

This review summarizes the progress made in SSC and tissue transplantation, particularly in animal models. The prospects for developing a transplantable scaffold to assist the development and differentiation of isolated testicular cells are also discussed in this study.

The PRISMA 2020 guideline forms the basis for the present narrative review. We conducted a literature search on testicular tissue and SSC transplantation as a treatment for male infertility. From 1938 until 2021, PubMed and Google Scholar were utilized as search engines. The search terms included: terms 'Immature testicular tissue', 'Injection', '*In vivo* spermatogenesis', 'Scaffold', 'Spermatogonial stem cells', 'Transplantation'' and a combination of words with the AND, OR functions, along with their equivalents in Mesh. Original and review studies

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were both included in this study. In addition to manually searching the reference lists of relevant papers to find additional studies that the electronic search missed, all of the aforementioned databases were searched for articles using the appropriate keywords. The following was the research question: Which scaffolds and transplanting sites are best?

The selection of the papers was presented using the PRISMA 2020 flowchart for narrative reviews (Fig.1).

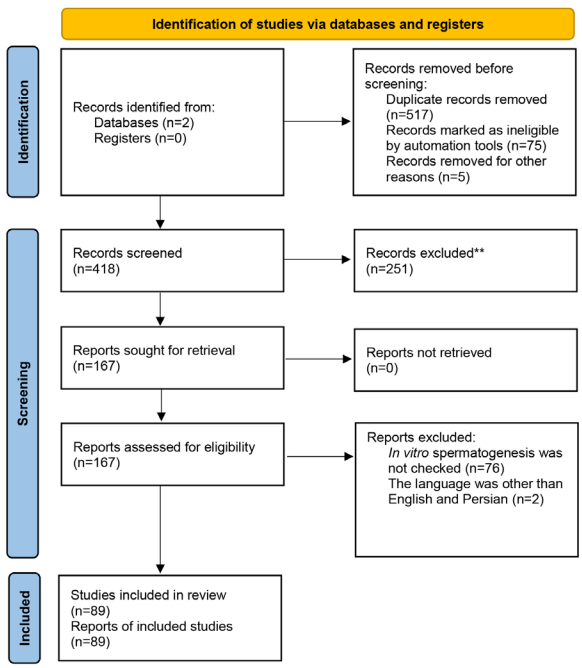

Fig.1: PRISMA 2020 Flow diagram of the study selection for narrative review.

SSC transplantation has recently been proposed as a possible therapeutic option for preserving fertility. Implanting SSC from a fertile donor into an infertile recipient is one therapeutic strategy. The method of transplanting SSC into seminiferous tubules has been extensively studied, and it is helpful and suitable for a wide range of species (Fig.2A) (7). In another approach, after collecting samples of testicular tissue, SSC are isolated and cultured in vitro before being injected into the patient's testes (8). SSC collected from cell suspensions could be cultured in vitro to generate a sufficient number of spermatogonial cells for a clinical application of SSC transplantation (9). Another hybrid procedure is *in vitro* transplantation, in which donor SSC are cultivated *in vitro*, then injected into the recipient testis, and ultimately re-cultured *in vitro* as a donor-host mix of tissue fragments. This technique allows relatively easier cell observation than pure *in vivo* xenografting (10).

In 1994, Brinster and Avarbock (11) and Brinster and Zimmermann (12) were the first to perform SSC transplantation in mice. They discovered that SSCs from donors could resumption spermatogenesis in host mice that have been treated with chemotherapy, resulting in the birth of offspring. Anjamrooz et al. (13) transplanted SSC from newborn mice into the seminiferous tubules of host mice two weeks after culture. According to their findings, enrichment of type A spermatogonial cells through an *in vitro* co-culture method can enhance recipient mice's epididymal sperm count. Koruji et al. (14) isolated Sertoli and spermatogonial cells, which were subsequently transferred, through rete testis, into the other irradiated testis of the same mouse. The results demonstrated that autotransplantation of SSC could result in sperm generation in the recipient's testes.

Experiments on animals, especially non-human primates, have shown progress in the field of SSC graft. In numerous rodent species, SSC graft has proven to be effective, including the hamster (15), rabbit (16), mouse (8, 11, 17, 18), and rat (19-22). Other animals utilized for donor cell injection into rete testis that colonized and differentiated successfully were goats (23, 24), sheep (25-27), and dogs (Table 1) (16, 28, 29).

Table 1: Spermatogonial stem cell transplantation

Doner	Recipient	Grafting site	Outcome: germ cell maturation/ offspring/ others	References
Mice	Mice	Seminiferous tubules	Offspring generation	Brinster (11, 12)
Mice	Mice	Rete testis	Sperm generation	Koruji et al. (14)
Hamster	Mice	Seminiferous tubules	Sperm generation	Ogawa et al. (15)
Rat	Mice	Seminiferous tubules	Sperm generation	Clouthier et al. (19)
Goats	Goats	Rete testis	Offspring generation	Honaramooz et al. (23)
Sheep	Sheep	Rete testis	Sperm generation	Herrid et al. (25)
Dog	Dog	Rete testis	Sperm generation	Kim et al. (28)
Rhesus	Rhesus	Rete testis	Sperm generation	Hermann et al. (30)
Rhesus	Rhesus	Rete testis	Offspring generation	Goossens and Tournaye (31)
Human	Mice	Rete testis	SSC survived in the mouse testis for as long as six months without showing any signs of differentiation	Nagano et al. (32)
Human	Mice	Rete testis	Human SSC was effectively transplanted into azoospermic mouse testis *in vitro*, and tissue culture conditions supported the homing of human SSC in the testis	Mohaqiq et al. (33)

SSC; Spermatogonial stem cell.

Studies on non-human primates provide an extremely useful insight into clinical transplantation (34). Using SSC injected into the recipient's seminiferous tubules, sperm was successfully produced in monkeys (30). In 1999, the first SSC transplants were performed on monkeys. The injection procedure has been used on bigger animals with great effectiveness. Injecting SSC into the efferent duct in the rete testis in small animals, the testes must be exposed. However, the technique is less invasive for larger animals (35). In the testes of macaques, spermatogenesis was resumed after both autologous and allogeneic SSC transplantations. The generated spermatozoa were capable of fertilizing oocytes. This study indicates the ability of SSC grafting in such a primate species, increasing the feasibility that this technique may be used in clinical practice in the future (31).

The first case of human SSC xenografting was reported in 2002. Nagano et al. (32) isolated human SSC from six infertile men and injected them into nude mice treated with busulfan. It was demonstrated that human SSC migrated into the basal membrane of mouse testicular seminiferous tubules, although their numbers significantly decreased one month after transplantation. However, some SSC survived in the mouse testis for as long as six months without showing any signs of differentiation. In a study, after xenotransplantation to mouse testes, SSCs were obtained from prepubescent boys with cancer and showed stem cell activity similar to that of human adult SSC (36). Mohaqiq et al. (33) transplanted human SSC into the testes of azoospermic adult mice. According to their findings, human SSC were effectively transplanted into azoospermic mouse testis in vitro, and tissue culture conditions supported the homing of human SSC in the testis (Table 1).

There are several examples of xenotransplantation that utilize *in vitro* procedures. SSC samples are typically cultured *in vitro* before being injected into the recipient. This method has also been tested on humans. In one experiment, human SSCs were cultured *in vitro* and subsequently injected into the rete testis of immune-deficient mice. The results revealed that SSC proliferated throughout the basement membrane of the host, but no sperm were identified. This suggests that human signaling factors may be essential for full differentiation (37).

In vitro proliferation and differentiation of SSC utilizing exogenous factors offers a platform for researching germ cell biology and enables germ-line transplantation for the treatment of infertility. In vitro, the differentiation of embryonic stem cells (ESC) into SSC and the ability to use transplantation techniques can help find ways to treat infertility (38). According to the findings of Rahmani et al. (39), it is possible to induce SSC-like cell differentiation from induced pluripotent stem cells (iPSC) after *in vitro* transplantation, 3D testicular organ cultivation, and *in vivo* transplantation. Dashtaki et al. (40) demonstrated that the adipose tissue-derived mesenchymal stem cells (AT-MSCs) that were transplanted within seminiferous tubules were localized in the basement membrane, and the testicles of mice treated with AT-MSCs expressed spermatogenesis-specific markers. Overexpression of germ cell-specific markers was seen in mice receiving cells cultured in the presence of growth factors.

Because of the unestablished domain of human SSC proliferation *in vitro* and the poor co-transplanted microenvironment for maintaining SSC, there are various limitations for SSC transplantation in humans (41). However, considering the effectiveness of the SSC transplantation method in several large animal species and the fact that many patients have previously cryopreserved testicular tissue or cells, a clinical translation of the approach is imminent.

The best site for SSC injection in the host is one of several unanswered questions. The findings demonstrate that microinjection of cell suspensions into the efferent ducts seminiferous tubules, efferent ducts, or rete testis is equally successful in producing donor-cell-derived spermatogenesis in the host. Each method is advantageous in a variety of species for different experimental reasons (42). Since ultrasonography can detect the rete testis while avoiding open surgery, it has become the most successful injection location. SSC transplantation in various big animal species, including nonhuman primates, has now been performed via ultrasound-guided rete testis injection (25, 35, 43, 44). There is no need for surgery when doing an ultrasound-guided rete testis injection. To summarize, the rete testis is seen via ultrasonography, and the injection needle is directed into the space of the rete testis through the scrotal skin (30). In the only human study, it was shown that the injection of contrast material into the rete testis, near the epididymis's caput, was the most effective site (45). Therefore, it seems that one of the suitable sites for SSC transplantation is rete testis.

SSC suspensions can be replaced with testicular tissue fragments transplanted into the recipient. This strategy preserves the reaction between germ cells and somatic cells by maintaining the SSC within their native environment (46). It appears that the most promising option for providing these patients with the opportunity to become the biological parents of their own kids is to transplant the cryopreserved patient's testicular tissue back into the patient after they have been cured (4). In several studies, orthotopic or heterotopic grafting of ITT from various animal species was performed on immunodeficient mice, which resulted in tissue maturation and full spermatogenesis (47-52). For the first time, Liu et al. (53) demonstrated that sperm from adult monkey testis xenotransplants can be used to produce non-human primate offspring. In 2019, Fayomi et al. (54) allotransplanted Rhesus macaque ITT into the scrotal and dorsal skin. According to the findings, grafts grew and released testosterone. This procedure requires appropriate vascularization of transplanted tissue to be successful (55). During transplantation, the supply of nutrition, oxygen, and factors that promote cell survival, proliferation, and differentiation is essential (56). It has yet to be demonstrated that xenotransplantation of

human ITT obtained from human fetuses or prepubertal boys has resulted in sperm production (57-65). After xenografting human ITT into mice, differentiation up to the pachytene spermatocyte stage was seen (Table 2) (58-64). Transplantation of ITT generally leads to the resumption of spermatogenesis, which may be significant when considering the possible future applications of transplanting testicular tissue from pubertal patients.

It will be critical to develop successful testicular transplantation protocols that will most probably include biomaterial scaffolds for providing a suitable microenvironment for testicular cells and *in vivo* tissue development inside microfluidic devices. To date, in comparison to ovarian transplantation, the application of modified biomaterials to enhance transplant results has been comparatively underrepresented in testicular transplantation (66).

Before a pilot clinical trial and clinical implementation of ITT transplantation, the viability of the procedure and optimum environment for producing spermatogenesis utilizing ITT transplants, including the transplantation site, must be investigated in a human-relevant preclinical model (6). In ITT transplantation, temperature and the grafting site are two crucial parameters. Xenografts could be transplanted into the testis or scrotum, known as orthotopic or homotopic grafting, or into other parts of the body, such as the intra-abdominal cavity or subcutaneous, known as ectopic or heterotopic grafting (69).

Full spermatogenesis has resulted from the transplantation of testicular tissue from various animal species into mice or rats in a variety of locations, including the back skin (15, 51, 70-72), the ear tip (67, 72), the intratesticular testis (6, 47, 73), scrotum (54, 74), and the anterior chamber of the eye (75). Recent research by Eyni et al. (68) demonstrated that when mouse ITT is transplanted into the epididymal fat of castrated adult mice, it survives for 8 weeks and spermatogenesis can be identified up to the level of elongated spermatids (Table 2).

For the first time, Luetjens and colleagues (76) demonstrated that autologous transplantation of nonhuman monkey testicular tissues arranged similarly to human testes results in complete spermatogenesis in the scrotum of immature animals. In addition, the transplantation of cryopreserved ITT from a rhesus monkey into the scrotum led to full spermatogenesis (74). The first non-human monkey born from sperm produced after ITT transplantation was a significant milestone in the clinical translation of ITT transplantation. In this research, fresh and frozen ITT of rhesus monkeys were grafted into the scrotum and the dorsal skin of castrated males. The recovery rate of testicular transplants was 100 percent after an 8 to 12-month transplantation interval, and full spermatogenesis was proven in all transplants (54).

Human ITT xenografted into the scrotum (54-56), intratesticular (58), and dorsal skin (61) of castrated nude mice demonstrated spermatogonia's capability to survive, proliferate, and even differentiate up to the stage of pachytene spermatocyte. The neovascularization and formation of a vascular system to optimize blood flow between the transplant and the recipient are critical for the transplant's survival. Reperfusion damage and ischemia, the preservation of early spermatogonial cells, and insufficient testicular transplant neovascularization are the main challenges of human ITT transplantation (60). When comparing the temperatures of ectopic and orthotopic transplantation locations, orthotopic transplantation has a significantly lower temperature. Thus, the increased temperature at the site of ectopic transplantation may be a contributing factor to spermatogenic arrest (76, 77). Orthotopic transplantation into the intratesticular site may provide intensive blood flow and hormonal support for testicular transplants (72). Testicular tissue might be lost in heterotopic transplantation because of probable degenerative processes and ischemia as well as immune reactions. This should lead to more research on biocompatible devices that can protect the testicular tissue's structure (77).

These findings support the development of orthotopic tissue transplantation as a prospective clinical option for restoring fertility in individuals who have had gonadotoxic therapies, and they urge the conduct of additional research on human ITT transplantation.

Table 2: Immature testicular tissue transplantation

Doner	Recipient	Grafting site	Outcome: germ cell matura-tion/offspring	References
Rat	Rat	Ear tip	Full spermatogenesis	Johnson et al. (67)
Piglets/goats/mice	Mice	Back skin	Sperm generation	Honaramooz et al. (48)
Mice/rabbit	Mice	Testis	Offspring generation	Shinohara et al. (47)
Cat	Mice	Back skin	Full spermatogenesis	Snedaker et al. (51)
Macaques	Mice	Back skin	Offspring generation	Liu et al. (53)
Human	Mice	Scrotal bursa	Pachytene spermatocyte generation	Wyns et al. (58)
Human	Mice	Back skin	Pachytene spermatocyte generation	Sato et al. (10)
Human	Mice	Intratesticular	Pachytene spermatocyte generation	Van Saen et al. (55)
Macaques	Macaques	Back skin, scrotal skin	Offspring generation	Fayomi et al. (54)
Mice	Mice	Epididymal fat	Elongated spermatids generation	Eyni et al. (68)

Treatment of both male and female infertility using scaffolds has shown significant potential. Because biodegradable scaffolds can mimic extracellular matrix (ECM) conditions and provide a suitable microenvironment for cell proliferation and differentiation, they are appropriate for cell delivery systems. Additionally, these scaffolds degrade over time, reducing the possibility of immunological response in the body (76). Controlled drug delivery system (DDS) is a new strategy for regulating the bioavailability of therapeutic substances. Using controlled DDS, drugs are loaded into a polymeric network structure and released in a definite way from the polymer (78). The most basic of recent advances is the embedding of testicular cells or tissues within a scaffold for in vivo transplantation (Fig.2B).

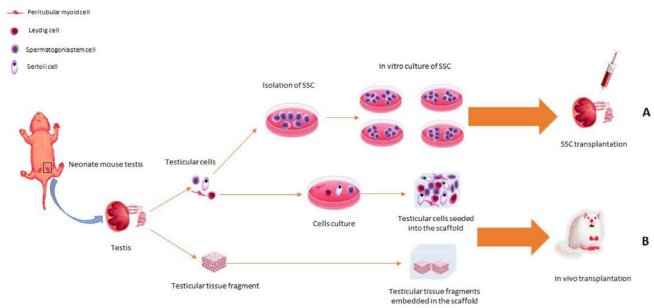

Fig.2: Schematic representation of testicular tissue and cell transplantation. **A.** In the past, spermatogonial stem cells (SSCs) were isolated and cultured before being injected into testicular azoospermia. **B.** With the development of tissue engineering and the establishment of releasing and non-releasing scaffolds, testicular tissue fragments or cells with the scaffold were transplanted into the recipient.

Hydrogels made of synthetic and natural polymers have attracted continuing interest in cell seeding, and recent advances in the field of tissue engineering have made such hydrogels particularly appealing as matrices for regenerating and repairing a broad range of organs and tissues (79). Wang et al. (80) seeded Leydig cells onto polyglycolic acid fiber scaffolds, cultivated them in vitro for 7 days and grafted the cell-scaffold structures into adult castrated rats' tunica vaginalis cavity or gastrocolic omentum. Using cells and a scaffold, a vascularized testosterone-secreting tissue was formed in both of the transplantation sites two months after transplantation. When a variety of testicular cell types, such as Leydig cells, were seeded, the serum testosterone level was raised much more. However, spermatogenesis was not achieved when a mixed community of testicular cells was used. Consequently, this strategy provided a strategy for the synthesis of androgen-secreting tissue for transplantation applications. Matrigel has also been used to encapsulate testicular cells in of several experiments (Table 3). Studies showed that seminiferous tubules were formed from testicular cells encapsulated in Matrigel, but only a small number of germ cells were found (54, 81, 82). Dores and Dobrinski (83) investigated graft development using Matrigel as a support structure to keep cells in close proximity. The improved method was then put to the test as a functional experiment to see how vascular endothelial growth factor-165 (VEGF165) affected blood supply and testicular tissue reorganization. Their findings revealed that adding Matrigel to the cell suspension improved tubule development and increased the efficiency of newly generated testis tissue, probably by promoting cell-to-cell interaction while reducing cell loss. However, adding VEGF165 to the cell suspension did not enhance blood vessel or tubule development, but it did increase the number of tubules containing spermatogonia. They found that adding Matrigel enhanced spermatogenic efficiency and that VEGF165 could preserve germ cells by promoting their survival in their niche.

Development of bioengineered scaffolds able to support testicular cells and improve ITT grafting in different species, it seems that may be a promising way to preserve fertility in humans.

To develop intelligent DDS, researchers have worked to engineer the chemical and physical features of DDS to optimize their biodegradability, surface functionality, environmental reaction, permeability, biorecognition, and biorecognition places (78). Hydrogels are an example of a DDS class that has achieved significant success in the field of intelligent drug delivery (51). It is possible to support the proliferation and differentiation of cells in newly generated tissues by using hydrogels containing growth factors. The use of hydrogels is frequently advantageous because of their high water content, quick nutrition transport, and ability to aid cell migration and angiogenesis (84). Numerous studies in regenerative medicine have investigated the advantages of local drug delivery through hydrogels (Table 3). Poels et al. (85) after transplantating testicular tissue of mice embedded in alginate hydrogel containing VEGF-nanoparticles into the scrotum, reported that VEGF-NP significantly enhanced angiogenesis but had no effect on spermatogenic cell survival, while alginate alone improved cell survival of spermatogonia. Furthermore, during long-term xenotransplantation, culture of human ITT with human VEGF-165 enhances spermatogonial viability, seminiferous tubule structure, and vascularization (56). When compared to just alginate encapsulation, testicular tissue embedded in alginate hydrogel loaded with necrosis inhibitor nanoparticles (NECINH-NP) led to a considerable enhancement in spermatogonial viability and tissue integrity after orthotopic autotransplantation (4). Alginate hydrogels supplemented with platelet-derived growth factor (PDGF)-delivery nanoparticles enhanced vascularization and vascular development in testicular tissue grafts in comparison to VEGF-only supplementation, although possible interactions with NECINH should be investigated further (86).

The successful development of bioengineered scaffolds with DDS would certainly be a significant advance toward restoring male fertility.

Table 3: Cell/tissue transplantation with scaffold

Tissue/Cell	Doner	Recipient	Grafting site	Scaffold	Factors	Outcome	References
Leydig cells	Rats	Rats	gastrocolic omentum/cavity of tunica vaginalis	Polyglycolic acid	-----	Vascularized testosterone-secreting tissue was formed and the serum testosterone level was raised much more	Wang et al. (80)
Testicular cell	Rat	Mice	back skin	Matrigel	-----	The grafts were vascularized and contained elongated seminiferous tubules, there were a few integra-tions of putative spermatogonia into the seminiferous epithelium, the development of tubule lumen, and putative Leydig cells	Gassei et al. (81)
Testicular cell	Canine	Mice	Seminiferous tubules	Matrigel	-----	De novo seminiferous tubules were formed and some germ cells were localized in the basement membrane of seminiferous tubules	Lee et al. (82)
Testicular cell	Porcine	Mice	Back skin	Matrigel	-----	Adding matrigel to the cell suspen-sion improved tubule development and increased the efficiency of newly generated testis tissue	Dores and Dobrinski. (83)
Immature testicular tissue	Mice	Mice	Scrotum	Alginate hydrogel	VEGF-NPs	VEGF-NP significantly enhanced angiogenesis but did not affect spermatogenic cell survival, while alginate alone improved cell sur-vival of spermatogonia	Poels et al. (85)
Immature testicular tissue	Mice	Mice	Scrotum	Alginate hydrogel	NECINH-NP	Testicular tissue embedded in alginate hydrogel loaded with NECINH-NP led to a considerable enhancement in spermatogonial viability and tissue integrity a	Del Vento et al. (4)
Testicular tissue frag-ments	Mice	Mice	Scrotum	Alginate hydrogel	NECINH, VEGF and PDGF-NP	PDGF-delivery nanoparticles enhanced vascularization and vascular development in testicular tissue grafts in comparison to VEGF-only supplementation although possible interactions with NECINH should be investigated further	Del Vento et al. (86)

Conclusion

Under experimental conditions, there has always been an obstacle to differentiation at the diploid cell stage so far. With the assistance of testicular tissue and cell transplantation, significant progress has been made toward the creation of therapeutic solutions for human fertility preservation. Animal studies demonstrate the feasibility of autologous transplanting of SSC or testicular tissue to produce gametes for the development of healthy offspring. The purpose of the current study is to apply this data in the clinic, especially for patients who will be receiving gonadotoxic treatments. However, for the method to be considered effective, differentiation must be achieved up to the haploid cell stage. Thus, further research is required to investigate the biology of spermatogenesis and to determine whether procedures that have proven successful with animal models may also be applied to human tissue. Tissue engineering and bioengineering could be viable options. Reproductive tissues can be designed to act as *in vitro* experimental research as well as substitute or regenerate damaged tissues to maintain reproductive ability. However, the risk of deleterious impacts of novel biomaterials on reproductive health, such as the quality and development of gametes, is typically disregarded. Hopefully, the discovery of new information from the engineered procedure mentioned here will make it possible to meet the reproductive demands of present patients and future generations.

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Authors' Contributions

A.A.; Contributed to data collection, Classification, Paper Summarization, Explicit literature search, and Manuscript writing. M.M.; Supervised the research and Contributed to writing the manuscript and Revision. M.H.; Contributed to manuscript writing and Paper summarization. All authors read and approved the final manuscript.

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