European Society of Human Reproduction and
Embryology

Course 2

Special Interest Group "Andrology

"Testicular stem cells"

27 June 2004

Berlin - Germany

Evaluation Form
Course 2 - Andrology
27 June 2004   Berlin/Germany

DEMOGRAPHICS

Are you a physician? ___ Yes   ___ No

Years since completion of training: ___ less than 5   ___ 5-9   ___ 10-19 ___ 20/over ___ currently in training

GENERAL INFORMATION

	Strongly Agree	Moderately Agree	Moderately Disagree	Strongly Disagree
1. The course objectives were clearly stated.	_____	_____	_____	_____
2. The course objectives were clearly met.	_____	_____	_____	_____
3. Faculty/participant interaction was satisfactory.	_____	_____	_____	_____
4. The course was well organized.	_____	_____	_____	_____
5. Site accessibility was not a problem.	_____	_____	_____	_____
6. Meeting facilities were adequate.	_____	_____	_____	_____
7. Course director conducted program well.	_____	_____	_____	_____
8. Overall course grade (circle one)	A	B	C	D
9. Overall syllabus grade (circle one)	A	B	C	D

EDUCATIONAL VALUE

1. I learned something new that was important	_____	_____	_____	_____
2. I verified some important information	_____	_____	_____	_____
3. I plan to discuss some of this information with my colleagues.	_____	_____	_____	_____
4. I plan to seek more information on this topic	_____	_____	_____	_____
5. My attitude about this topic changed in some way	_____	_____	_____	_____
6. This course is likely to have an impact on my teaching	_____	_____	_____	_____

FACULTY EVALUATIONS

Strongly Agree = 4   Moderately Agree = 3
Moderately Disagree = 2   Strongly Disagree = 1

	ALISON	DE ROOIJ	SMITH	SOUSA	WOELDERS	SCHLATT	TOURNAYE	BAHADUR
1. Sufficient depth								
2. Concepts clearly explained								
3. Enhanced understanding of key matters								
4. Without commercial bias								
5. Slides readable								
6. Learning objectives clear								
7. Helpful for future reference								

COMMENTS

1.   What change(s), if any, do you plan to make in your teaching as a result of this course?_____
_____

2.   Future suggested meeting topics and speakers? _____
_____

3.   Additional comments _____
_____

OPTIONAL Would you be willing to be contacted in the future regarding how this course influenced your practice?

          YES   **NO**

Name _____ Phone _____

E-mail _____

Content

Program

Sunday, 27 June – Hall 5
Course 2: SIG Andrology
"Testicular Stem Cells"

Course co-ordinator: H. Tournaye (BE)

Course description:
This course aims at giving an overview of the current insights and concepts on testicular stem cell biology and its clinical applications.

09.00 - 09.30:	Stem and progenitor cell biology: an introduction – M. Alison (UK)
09.30 - 10.00:	Testicular stem cells – D. De Rooij (NL)
10.00 - 10.30:	Discussion
10.30 - 11.00:	Coffee break
11.00 - 11.30:	Identification, selection and enrichment of testicular stem cells - G. Smith (US)
11.30 - 12.00:	In-vitro culture of testicular (stem) cells – M. Sousa (P)
12.00 - 12.30:	Discussion
12.30 - 13.30:	Lunch
13.30 - 14.00:	Cryopreservation of testicular stem cells – H. Woelders (NL)
14.00 - 14.30:	Transplantation of testicular stem cell suspensions and testicular grafting – S. Schlatt (D)
14.30 - 15.00:	Discussion
15.00 - 15.30:	Coffee break
15.30 - 16.00:	Translating testicular stem cell biology to the clinic – H. Tournaye (B)
16.00 - 16.30:	Ethics of testicular stem cell medicine – G. Bahadur (UK)
16.30 - 17.00:	Discussion and Conclusions
17:00 - 18:30:	Business meeting Special Interest Group in Andrology

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Stem and progenitor cell biology: an introduction

Malcolm R. Alison
Department of Histopathology
Imperial College
London

Learning objectives

• To list the major types of stem cells
• To describe the common attributes of adult stem cells
• To critically evaluate the claims for adult stem cell plasticity
• To summarize the role of adult stem cells in pathology, in particular in fibrosis and cancer.

Stem cell research comes of age

Morbidity and mortality as a result of malfunctions in vital organs plague even the most technologically advanced societies. Because of a dearth in transplantable organs there is a growing hope that stem cells may be the answer to mankind's prayer to be able to replace tissues worn out by old age and ravaged by disease. Indeed, it is impossible to open a newspaper today without seeing yet another apparent 'breakthrough' in stem cell research; the more optimistic hoping for an elixir of life – the promise of immortality. Embryonic stem (ES) cells can come from the ICM of the early blastocyst or foetal gonadal tissue. Blastocysts are usually 'spares' from IVF programmes, though some have been deliberately created. Tissues generated from such ES cells have still to overcome the problem of histocompatibility, but somatic cell nuclear transfer techniques (SCNT – also called therapeutic cloning) offer the possibility of using the patient's own genome to generate ES cells and so overcome this obstacle (1)

Legislation regarding the use of ES cells also varies around the globe adding to the problems. In countries like the UK and Australia new cell lines can be created from spare embryos, but in the US federal funds (taxpayers' money) can only be used on ES cell lines created before the 9th August 2001 (~ 60 existing cell lines); the rationale being that such cells while exhibiting pluripotency have not the ability to develop into a whole human being, thus the sanctity of human life is not compromised by their use

While no one really doubts that ES cells are likely to be the most flexible of all stem cells, the ethical issues surrounding their use have prompted the search for alternative adult sources.

Adult stem cell properties

A hierarchy of potential: Stem cells have varying potential. At the top of the tree (the most primitive) is the fertilized oocyte (the zygote) and the descendants of the first 2 divisions. These cells are ***totipotent***, able to form the embryo and the trophoblasts of the placenta. After about four days these totipotent cells begin to specialize forming a hollow ball of cells, the blastocyst, and a cluster of cells called the inner cell mass (ICM) from which the embryo develops. The ICM cells are considered to be ***pluripotent***, able to differentiate into almost all cells that arise from the three germ layers, but not the embryo because they are unable to give rise to the placenta and supporting tissues: ES cells are considered to be pluripotential. Most adult tissues have ***multipotential*** stem cells, cells capable of producing a limited range of differentiated cell lineages appropriate to their location, e.g. small intestinal stem cells can produce all four indigenous lineages (Paneth, goblet, absorptive columnar and enteroendocrine), CNS stem cells have trilineage potential generating neurons, oligodendrocytes and astrocytes (2), while the recently discovered stem cells of the heart can give rise to cardiomyocytes, endothelial cells and smooth muscle (3). However, describing tissue-based stem cells as 'multipotential' may be incorrect if, as it appears, that some adult stem cells, when removed from their usual location can transdifferentiate into cells that arise from any of the three germ layers (so-called plasticity). At the bottom of the tree are ***unipotential*** stem cells, cells capable of generating one specific cell type. Into this category we could place epidermal stem cells in the basal layer that produce only keratinized squames and certain adult hepatocytes that have long-term repopulating ability (4). Some would argue that there is no such thing as a unipotential stem cell, and really these cells should be called ***committed progenitors***. While there is no doubt that in some tissues, e.g. the gastrointestinal tract and haematopoietic renewal systems, there are committed stem cells (progenitors) with more

limited division potential than their multipotential stem cells, in the epidermis and liver these unipotent cells do have a large clonogenic capacity.

Self-maintenance: Stem cells are usually relatively undifferentiated, not having the functional specializations of the progeny that they give rise to. They are normally located in a protective environment (niche; *Fr.* recess), and in a tissue such as the small intestine where the cell flux is in one direction, they are found at the origin of the flux. In the heart, they are located in areas of least haemodynamic stress. Though only a small percentage of a tissue's total cellularity, stem cells maintain their numbers if, on average, each stem cell division gives rise to one replacement stem cell and one transit amplifying cell (an *asymmetric* cell division). The interactions with the stem cell niche are crucial to this process (Figure 1) and the controlling factors are rapidly becoming elucidated. In the *Drosophila* ovariole, a stem cell niche known as the 'germarium' has been defined, and here germline stem cell (GSC) number is maintained by the close apposition of GSCs with cap cells; Armadillo (fly β-catenin) and decapentaplegic (DPP) (a homologue of mammalian bone morphogenetic proteins [BMPs]) signalling are involved (5). Likewise, in the *Drosophila* testis, GSC number is strictly controlled by the interaction with so-called hub cells (6) - in both the ovariole and testis, disruption of DPP signalling and/or Armadillo/APC interactions can result in supernumerary GSCs due to alterations in the orientation of the mitotic axes (see Figure 1). In mammals too, Cadherin/catenins and BMP signalling are also involved in the maintenance of haematopoietic stem cell number through interactions with osteoblasts (7).

Proliferation, clonogenicity and genomic integrity: Stem cells are slowly cycling but highly clonogenic. Teleologically it would seem prudent to restrict stem cell division because DNA synthesis can be error-prone. Thus, in many tissues stem cells divide less frequently than transit amplifying cells. In the intestine, stem cells cycle less frequently than the more luminally located transit amplifying cells, and in the human epidermis the integrin-bright cells have a lower level of proliferation than the other basal cells. In hair follicles, the hair shaft and its surrounding sheaths are produced by the hair matrix that is itself replenished by the bulge stem cells. As befits true stem cells, the bulge cells divide less frequently but are more clonogenic than the transit amplifying cells of the hair matrix. Combined to an infrequently dividing nature, stem cells would also appeared to have devised a strategy for maintaining genome integrity. Termed the immortal strand hypothesis or Cairns hypothesis, stem cells can apparently designate one of the two strands of DNA in each chromosome as a template strand, such that in each round of DNA synthesis while both strands of DNA are copied, only the template strand and its copy is allocated to the daughter cell that remains a stem cell (8). Thus, any errors in replication are readily transferred (within one generation) to transit amplifying cells that are soon lost from the population. Such a mechanism probably accounts for the ability of stem cells to be 'label retaining cells' after injection of DNA labels when stem cells are being formed (9).

Adult stem cell identity: In many tissues and organs the identity of the stem cells has remained either elusive or at least equivocal. However, in the bone marrow the recognition of cells with the properties of self-renewal and multilineage differentiation potential is well advanced. In fact such cells were recognised operationally back in 1961 by Till and McCulloch as cells that gave rise to multilineage haematopoietic colonies in the spleen (colony forming units – spleen [CFU-S]). In the human bone marrow the sialomucin CD34 is a haematopoietic cell surface antigen that has been extensively exploited for the selection of long-term repopulating cells with multilineage potential, though not all HSCs express this marker. In the mouse HSCs are known as KLS cells (c-kit$^+$lin$^-$Sca-1$^+$). An alternative method of enriching for HSCs exploits the fact that some cells have evolved a cellular protection mechanism against toxic metabolites and xenobiotics. This mechanism involves the expression of efflux pumps that belong to the ATP-binding cassette (ABC) superfamily of membrane transporters, and such cells are able to efflux a combination of Hoechst 33342 and Rhodamine123, thus appearing at the bottom left corner of a dual parameter FACS analysis – hence called the side population (SP). There are SP cells in many other tissues that might well correspond to their multipotential stem cells (10).

In the basal layer of the epidermis, patches of epithelial cells are highly expressive of the β-1 integrin, the receptor for type IV collagen, a component of the underlying basement membrane. It may well be that the epidermal stem cells are within these populations of so-called integrin-bright cells. In the central nervous system, neural stem cells and probably their transit amplifying descendants express both the intermediate filament nestin and a RNA-binding protein known as musashi 1. Musashi was first identified in *Drosophila* and thought responsible for the asymmetric divisions of sensory organ precursor cells; it may also be a marker for intestinal crypt stem cells.

Molecular control of stem cell behaviour: It appears likely that the local microenvironment, through a combination of cells and extracellular matrix components will govern all aspects of stem cell behaviour. This has led to the concept of the stem cell niche (see Figure 1) that supports and controls stem cell activity. In the intestinal mucosa the

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pericryptal myofibroblasts that ensheath the crypts serve as niche cells secreting Wnt proteins. One of the most striking observations was made through targeted disruption of the *Tcf*-4 gene. Tcf-4 is a partner protein for β catenin, and the heterodimer transactivates a number of genes involved in cell cycle progression: the absence of Tcf-4 results in the small intestinal crypts failing to maintain a proliferative zone. Paradoxically, activation of this same pathway is one of the earliest recognizable abnormalities in colonic carcinogenesis. Apart from Wnts, other proteins such as Nanog and Bmi-1 may be involved in the long-term self renewal of many stem cells.

Adult stem cell plasticity

A large body of evidence now supports the idea that certain adult stem cells, particularly those of bone marrow origin, can engraft alternative locations (e.g. non-haematopoietic organs), particularly when the recipient organ is damaged and transdifferentiate in to phenotypes appropriate to their new location (11). However the field is not without its detractors. The reason for this is twofold, 1) certain instances of so-called plasticity have now been attributed to cell fusion between bone marrow cells and cells of the recipient organ, and 2) several remarkable claims have not been able to be confirmed, most recently the inability to show that HSCs can contribute to the healing of a myocardial infarction (12).

Stem cell diseases – metaplasia, fibrosis and cancer

Under normal circumstances tissue-specific stem cells generate the range of cell types appropriate to their location. However, often accompanying chronic inflammation and damage is metaplastic change - a major switch in tissue differentiation, and it is reasonable to suppose that this switch occurs at the level of stem cells rather than between terminally differentiated cells. Examples include squamous metaplasia in the conducting airways of smokers, various types of intestinal metaplasia in the stomach often associated with gastritis, and gastric (pseudopyloric) metaplasia in the intestine affected by inflammatory bowel disease.

There may be a 'dark side' to adult stem cells. HSCs in particular appear to contribute to fibrogenesis in both pulmonary and hepatic scarring (13) and may also be responsible for allograft failure by contributing to transplant arteriosclerosis.

It is also likely that many cancers, particularly those of continually renewing tissues, are in fact a disease of stem cells since these are the only cells that persist in the tissues for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development. Moreover, tumours are heterogeneous populations in which many cells are terminally differentiated (reproductively sterile) or transit amplifying cells with limited division potential, and only tumour stem cells are capable of 'transferring the disease'. For example, in human acute myeloid leukaemia, only the CD34+CD38- cells are capable of transferring the disease to NOD/SCID mice, while in human breast cancer, the CD44+ESA+CD24$^{-/low}$ fraction has a similar potential (14).

Summary

In this lecture I have tried to illustrate that adult stem cells are involved in almost all aspects of tissue homeostasis. They are responsible for normal cell renewal and recent observations suggest that some stem cells, notably HSCs, may have a role in regenerative medicine by virtue of their ability to transdifferentiate. On the other hand, HSCs may have a role in organ scarring and be targets for neoplastic transformation. Stem cells also exist in tumours and successful cancer therapy will depend on eradication of this crucial subpopulation.

References

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(First demonstration of human ES cells derived from nuclear transfer)
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(Critical review of the role of HSCs in liver regeneration)

5. Gonzalez-Reyes A. (2003) Stem cells, niches and cadherins: a view from Drosophila.
J Cell Sci., 116, 949-954. *(Beautifully illustrated review of the stem cell niche in the fly ovarioles)*

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(Superb account of the control of stem cell number in the fly testis)

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(Shows that label retaining cells do proliferate but still retain their immortal DNA strands)

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(Beautiful illustration of label retaining cells in the mouse tail)

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J. Pathol., 200, 547 *(Review of ABC proteins and their role in stem cell biology)*

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J Clin Pathol., 57, 113-120. *(Critical review of claims for adult stem cell plasticity)*

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Nature. 2004 Mar 21 [Epub ahead of print] *(Contradicts the papers suggesting purified HSCs can contribute to the healing of a myocardial infarct)*

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Gastroenterology., 126, 955-963. *(First demonstration that bone marrow can contribute to human organ scarring)*

14. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A.,100, 3983-3988.
(Illustrates that only human tumour cells with a stem cell phenotype can propagate the tumour in immunodeficient mice)

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Figure 1. The stem cell niche. The niche (microenvironment) is likely to control many facets of stem cell behaviour including the rate of division, the orientation of mitotic axes and the type of division
([a] asymmetric vs. [b] symmetric) that results in new stem cells (S) or transit amplifying (TA) cells. The effectors are likely to be secreted soluble factors (growth factors such as BMPs), integral membrane proteins that require cell-cell contact such as the receptor c-kit and its ligand SCF, and cell adhesion molecules such as cadherins that maintain contact with the supportive cells of the niche
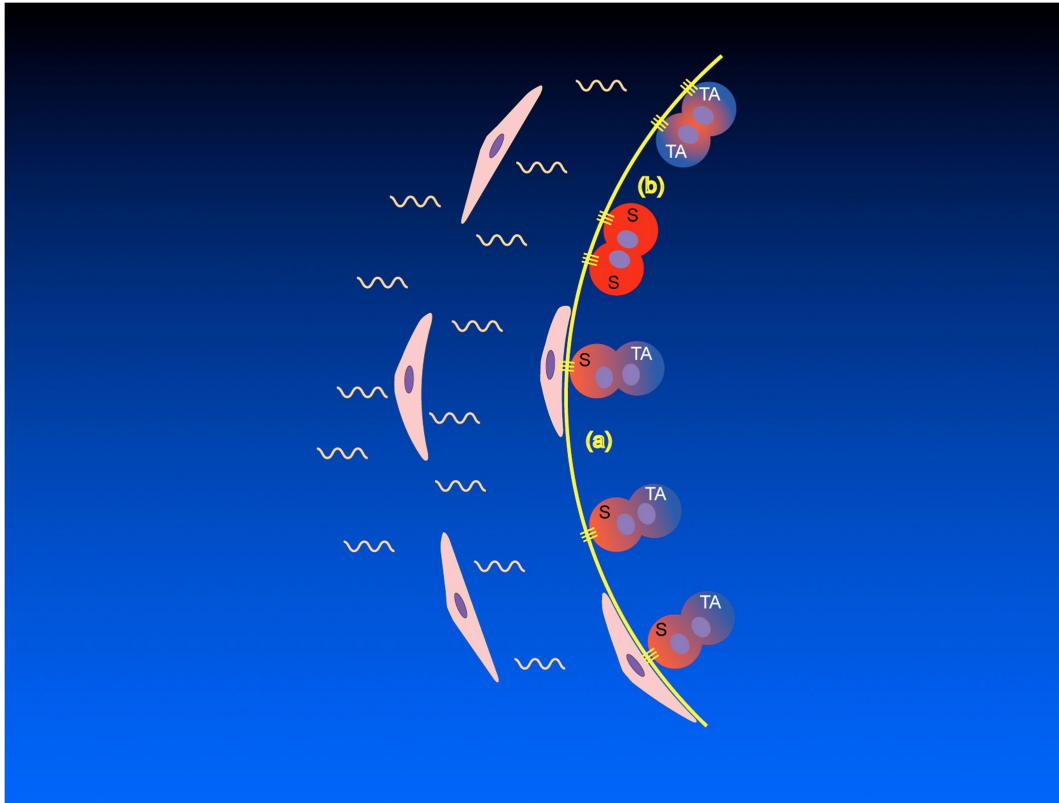
Testicular stem cells

Dirk G. de Rooij

Departments of Endocrinology, Faculty of Biology
Utrecht University and of Cell Biology
Utrecht
The Netherlands

Learning objectives

1. Knowledge on the development of the spermatogenic stem cell line
2. Principals of the nature of spermatogonial stem cells in primates and non-primates
3. Understand the principles of the regulation of differentiation and self-renewal
4. Possible important new development - Side population of spermatogonial stem cells

1. Development of the spermatogenic stem cell line

Spermatogonial stem cells originate from primordial germ cells (PGCs) which in turn derive from epiblast cells (embryonal ectoderm). PGCs migrate from the base of the allantois, along the hindgut to finally reach the genital ridges. During migration the PGCs proliferate. In the human, at about 6 weeks of gestation testis differentiation takes place and at that time there are about 3.000 PGCs in the testis (Bendsen *et al.* 2003).

Primordial germ cells are single cells that under certain culture conditions can form colonies of cells which morphologically resemble undifferentiated embryonic stem cells (ES cells. These cells can be maintained on feeder layers for extended periods of time and can give rise to embryoid bodies and to multiple differentiated cell pheno-types in monolayer culture and in tumors in nude mice. Primordial-germ-cell-derived ES cells can also contribute to chimeras when injected into host blastocysts. Hence, all PGCs are true stem cells, still having the capacity to renew themselves and to differentiate in various directions (review(de Rooij 1998)).

Once arrived in the genital ridges, the PGCs become enclosed by the differentiating Sertoli cells, and seminiferous cords are formed. The germ cells present within the seminiferous cords differ morphologically from PGCs and are called gonocytes or prespermatogonia. The gonocytes first go through a period of proliferation and at 9 weeks p.c. there are about 30.000 germ cells in the human testis (Bendsen *et al.* 2003). Although the proliferative activity diminishes after that, it does not stop entirely as in third trimester fetuses about 4×10^6 germ cells are found per testis. In vitro studies have demonstrated differences between gonocytes and PGCs. For example, rodent gonocytes can only survive in the presence of Sertoli cells, while PGCs can be co-cultured with other types of somatic cells. This suggests that the gonocytes are restricted in their differentiation potential in comparison to the PGCs. Furthermore, at the mitoses of the gonocytes, cytokinesis is often not complete and many gonocytes remain interconnected by intercellular bridges. As discussed below, in the adult testis the first visible sign of differentiation of stem cells is the formation of a pair of cells interconnected by an intercellular bridge. These paired spermatogonia are destined to ultimately become spermatozoa. Likely, the gonocytes are a heterogenous population of cells, only the single cells of which still have stem cell properties, while the rest are destined to become differentiating cells after the start of spermatogenesis.

Upon transplantation of gonocytes to testes of adult mice of which the endogenous spermatogenesis was removed, it appeared that these cells are not quite able to restore spermatogenesis in host animals (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). As spermatogonial stem cells of postnatal mice readily establish donor spermatogenesis in recipient testes it can be inferred that single gonocytes still have to undergo a specific differentiation step to become competent spermatogonial stem cells.

In rodents, gonocytes are quiescent until after birth and then all of these cells become active within a period of a couple of days and then start spermatogenesis. It has been concluded that during this period part of the gonocytes become differentiating spermatogonia (probably the ones in interconnected clones) while others become spermatogo-nial stem cells (the single cells) (review(de Rooij 1998)). In mammals in which spermatogenesis does not start shortly after birth, the transition into active spermatogenesis seems more gradual. In primates, A_{pale} and A_{dark} spermatogonia are found way before puberty. In monkeys, already at birth the seminiferous epithelium was found to consist of A_{pale}

and A_{dark} spermatogonia (Kluin *et al.* 1983), indicating that the transition of gonocytes into adult type spermatogonia takes place much earlier than the actual start of sperm production. Interestingly, in these young monkeys there were many more A_{dark}, which are quiescent cells, than A_{pale} spermatogonia illustrating a lack of stimulation of spermatogonial proliferation or an inhibition of this process. However, through the years before puberty spermatogonial numbers were found to increase, indicating that new spermatogonia kept being produced (Kluin *et al.* 1983).

Identity of spermatogonial stem cells in mammals

In general, spermatogonia in the adult testis are distinghuished in type A, when no or very little heterochromatin is present in the nuclei, and type B spermatogonia when the nuclei contain a great deal of heterochromatin. In some, but not all animals, an intermediate type is present called Intermediate (In) spermatogonia, accordingly. Spermatogonial stem cells are A spermatogonia and using whole mounts of seminiferous tubules have been identified to be single cells in various rodents, boar and ram. Accordingly, in these animals these cells were called A-single spermatogonia. Upon division, the daughter cells of the A-single spermatogonia can migrate away from each other and become two new stem cells, in which case self-renewal takes place. Alternatively, the daughter cells can stay together connected by an intercellular bridge, in which case they are called A-paired spermatogonia. The formation of the pair of spermatogonia comprises the first differentiation step in the spermatogenic lineage, and these cells are destined to ultimately become spermatozoa. It has been established that the spermatogonial stem cells in rodents divide on the average 2 to 3 times each cycle of the seminiferous epithelium (review (de Rooij and Russell 2000)).

In primates too, A and B spermatogonia are distinguished. However, the situation seems more complicated in primates than in other mammals. First, the density of the A spermatogonia is too high to allow a proper study of their topographical arrangement in the normal testis. Therefore, it is impossible to discern single spermatogonia or clones of 2, 4, etc. Second, similar to non-primate mammals the nuclei of the A spermatogonia do not show heterochromatin but some are very dense, stain heavily with hematoxylin and have a non-staining nuclear vacuole and and are called A_{dark} spermatogonia, while others stain less heavily and are termed A_{pale} (Figure 1). Generally, in the adult there are equal numbers of pale and dark staining A spermatogonia (review (de Rooij and Russell 2000)).
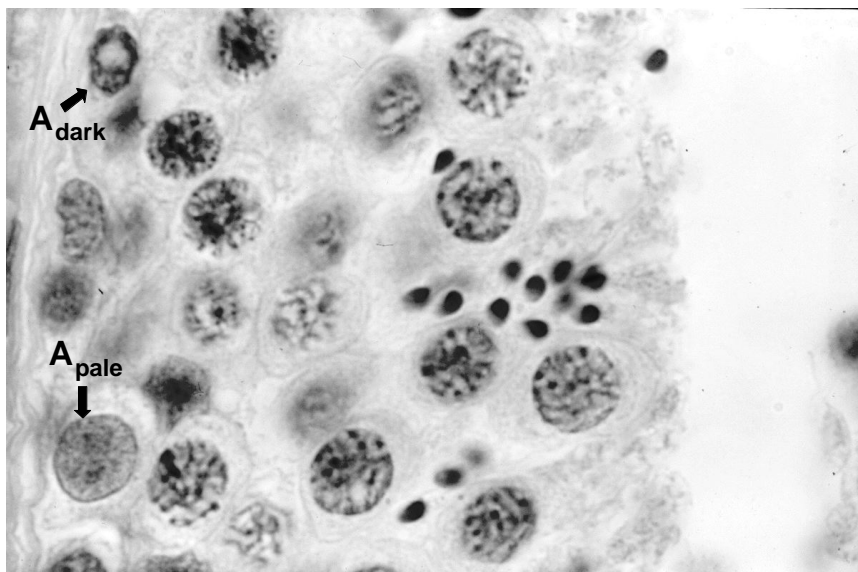

Figure 1. Area of a human seminiferous tubule showing A_{pale} and A_{dark} spermatogonia.

The A_{pale} spermatogonia divide once every epithelial cycle, which in the human means, once every 16 days. Hence, spermatogonia in primates divide much less frequently than in other mammals. The A_{dark} spermatogonia normally do not divide and are apparently quiescent for very long periods of time. The A_{dark} spermatogonia do not comprise a separate class of spermatogonia that renew themselves and give rise to differentiating type spermatogonia. In the monkey, after irradiation the A_{dark} spermatogonia transform into A_{pale} spermatogonia, probably in order to replenish the pool of A_{pale} spermatogonia that became depleted by the cell killing effects of the irradiation. Subsequently, after transition into A_{pale} the cells start to proliferate again. During repopulation after irradiation, new A_{dark} spermatogonia are formed by A_{pale} spermatogonia. A_{dark} spermatogonia seem to be cells set aside by the A_{pale} spermatogonia that can be recruited to become A_{pale} again when needed. Hence, the A_{dark} spermatogonia are supposed to be reserve sper-

matogonia that are activated upon cell loss in the population of active spermatogonia. This phenomenon does not occur in the non-primate testis.

While in the normal epithelium the density of the A_{dark} and A_{pale} spermatogonia is such that in whole mounts it is not possible to distinghuish the clones of these cells separately, after irradiation when density is lower, it can be seen that both these types of A spermatogonia consist of single cells, pairs and chains. This suggests that spermatogonial multiplication and stem cell renewal in primates is principally similar to that in other mammals, the single cells being the stem cells. So, likely only the single A_{dark} and A_{pale} spermatogonia have stem cell properties.

Regulation of differentiation and self-renewal

Like in all other renewing tissues, the seminiferous epithelium is able to react to (stem) cell loss by enhanced stem cell renewal in order to replace lost stem cells. After a high dose of irradiation surviving spermatogonial stem cells almost only self-renew during at least their first 6 divisions, leading to a rapid recovery of stem cell numbers. This indicates that there are mechanisms that can inhibit stem cell differentiation and/or enhance self-renewal in situations of cell loss. The question then arises which molecular pathways are involved. Recent data indicate a role for glial cell line derived neurotrophic factor (GDNF). Normally, GDNF is secreted by Sertoli cells while a subset of spermatogonia express both receptors for this growth factor, Ret and GFR-alpha1. Ectopic expression of GDNF in spermatogonia or overexpression in Sertoli cells induces the formation of large clusters of single type A spermatogonia, while normal spermatogenesis is suppressed (Meng *et al*. 2000; Yomogida *et al*. 2003). Moreover, in mice overexpressing GDNF in spermatogonia, germ cell tumors that resemble human seminoma, are formed at about one year of age. In heterozygotic GDNF deficient mice, spermatogenesis deteriorates with age, as germ cells become depleted. It was concluded that GDNF has a role in the regulation of self-renewal and differentiation of spermatogonial stem cells. Too high levels of GDNF inhibit stem cell differentiation and cause an accumulation of stem cells and low levels stimulate differentiation and cause stem cell depletion.

Another interesting recent finding in this field is that in the classical spontaneous mouse mutant *luxoid*, adult males exhibit a progressive loss of spermatogonial stem cells. Apparently, the as yet unknown gene(s) involved in this mutation also has a role in the regulation of spermatogonial stem cell renewal and differentiation.

In several renewing tissues, stem cells occupy specific areas. For example in the intestine, stem cells reside near the bottom of the crypts and stem cells in the bone marrow also occupy specific niches. Until recently, in the seminiferous epithelium no such niches were found for spermatogonial stem cells. Now it has become clear that most spermatogonial stem cells are preferably present in those areas of seminiferous tubules that border on interstitial tissue (Chiarini-Garcia *et al*. 2001). Apparently, the interstitial tissue affects stem cell behavior in such a way that differentiation is less likely to occur when stem cells lie close to it. Interestingly, high testosterone levels have been found to prevent spermatogonial differentiation.

Side population (SP) of spermatogonial stem cells

In recent years it has become clear that in several tissues, like hemopoiesis, liver, mammary gland and brain, there is a special kind of stem cells that are called side population stem cells. These cells can be distinguished by their ability to expell the fluorescent DNA-binding dye Hoechst 33342. In hemopoiesis it was found that SP cells possess a longterm reconstituting ability. A first report also indicated the presence of SP cells in the testis (Kubota *et al*. 2003). However, surprisingly these cells were found unable to colonize a recipient testis after transplantation. In two subsequent reports by other groups testicular SP cells were also found and in both these cases the cells were found to colonize recipient mouse testes after transplantation (Falciatori *et al*. 2004; Lassalle *et al*. 2004). In hemopoiesis, the SP cells were found to be primitive hemopoietic stem cells and exquisitely capable of restoring hemopoiesis in recipient animals. Hence, the finding of SP cells in the testis might well be important, but further work will have to be done to sort out the controversy about their ability to give rise to repopulation of a spermatogenesis-less testis.

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Identification, selection, and enrichment of testicular stem cells

Gary D. Smith[1,2,3,4] and Herkanwal Khaira[3]
Departments of Obstetrics and Gynecology[1],
Molecular and Integrated Physiology[2], and Urology[3];
Reproductive Sciences Program[4].
University of Michigan
Ann Arbor
USA

Learning Objectives

At the conclusion of this presentation, the participant should be able to:

1. Cite the different methods that have been attempted to identify spermatogonial stem cells and understand the limitations of each technique.
2. Describe the main approaches to improving isolation, selection, enrichment, and colonization of spermatogonial stem cells.
3. Describe the different approaches used to increase the quantity of As spermatogonia in a transplanted solution.
4. Understand the potential therapeutic uses and limitation of human spermatogonia transplantation.

Introduction

The past 30 years have been marked by unparalleled accomplishments in the medical treatment of malignancy. Prior to advances in chemotherapeutic and radiation treatment, many oncologic conditions had dismal survival rates. Today, medical interventions have success rates that approach complete remission for many malignancies. An inadvertent complication of these therapies, however, has been the high rates of infertility following treatment. Male germinal tissue, like many malignancies, is mitotically active and therefore is particularly susceptible to the toxic effects of chemo- and radiotherapies (Meistrich *et al*., 1982; Meistrich, 1993). Consequently, post-treatment patients often develop severe oligospermia or azoospermia (Wallace *et al*., 1991). Potential infertility complications are anticipated and adult male patients interested in future procreation are counseled to cryopreserve semen prior to instituting treatment. With present-day capabilities of *in vitro* fertilization, particularly intracytoplasmic injection, male patients can maintain post-treatment fertility. Pre-treatment sperm banking, however, is not a viable option for pre-pubescent males. These individuals having not yet begun spermatogenesis and thus lack viable spermatozoa. It is estimated that by the end of the decade, 1 in 250 young men will be childhood cancer survivors (Blatt, 1999). For these patients, infertility has often been an accepted consequence of their life-saving treatment. Testicular autologous transplantation is a newly-explored intervention that may provide a future therapeutic fertility option for these individuals. Having been successfully demonstrated in rodent models, investigators have now begun to explore the possibility of using testicular auto-transplantation to restore fertility in humans.

History of spermatogonial transplantation

In 1994, Brinster and colleagues published their landmark findings in the field of testicular tissue transplantation. Using a mixed cellular solution, obtained from dissociated testicular parenchyma, they infiltrated recipient mouse seminiferous tubules with the donor cells (Brinster and Zimmermann, 1994). Among the hallmark findings of this experiment, was the discovery that donor spermatogonial cells could interact with the host environment, migrate from the adluminal compartment, and negotiate past Sertoli-Sertoli tight junctions to enter the basal compartment. These researchers demonstrated successful donor spermatogenesis from testicular tissue transplanted between mouse subjects. They utilized donor testicular tissue from post-natal mice between days 4 and 12 of life. The assumption was that immature mice would have the highest concentration of undifferentiated spermatozoal progenitor cells or gonocytes, thereby providing the largest quantity of viable cells for transplantation. Testicular tissue was mechanically and enzymatically dissociated into a cellular suspension. The suspension was microinjected into mice pre-treated with busulfan to eliminate native spermatogenesis. Donor cells came from transgenic mice expressing the

LacZ (E. Coli B-galactosidase) gene; these cells when differentiated to the round spermatid phase stained blue, distinguishing them from the recipient's native sperm cells. The authors identified restored spermatogenesis in the recipient mouse with colonization and differentiation of the donor tissue (Brinster and Zimmermann, 1994). Brinster and Avarbock in 1994 reported successful spermatogenesis in a mouse allogenic spermatogonial cell transplantation. They found that the donor-derived spermatogonia were responsible for generating offspring; transmission was confirmed by presence of donor haplotype in progeny (Brinster and Avarbock, 1994).

Further investigations found that the intra-luminal transplanted germ cells degenerated and disappeared by 1 month's time. The successfully transplanted spermatogonia localized at the basement membrane and began to show evidence of division by the first week after transplant. Donor spermatogonia migrated to the basal compartment through the first month and donor spermatozoa were noted by that time (Parreira *et al.*, 1998; Nagano *et al.*, 1999).

The limits of spermatogonia transplantation were noted during more distant, xenogeneic transplantations. Although some limited colonization did occur with rabbit, monkey, bull and human transplantation, no spermatozoa or post-meiotic germ cells were found after these transplantations. Schlatt and colleagues in 1999 transplanted germinal tissue in primates to find evidence of spermatogonial survival at 4 weeks. Broodeoxyuridine (BrdU) was introduced into donor tissue prior to transplant. Using immunostaining at 4 weeks, cells located in the interstitium and seminiferous tubules were identified with the BrdU label in their nuclei. Morphologic criteria indicated these were type B or differentiated spermatogonia (Schlatt *et al.*, 1999). Nagano and colleagues reported in 2001 transplantation of baboon testicular tissue into nude mice. In this investigation, the authors identified survival and propagation of the transplanted cells for up to 6 months. They used a rabbit-produced anti-baboon antibody in conjunction with an anti-human antibody to identify the baboon cells. They noted that baboon cells had migrated to the basement membrane of the seminiferous tubules—indicating that mouse Sertoli cells had somehow interacted with the baboon spermatogonia, and allowed passage through the blood testis barrier. Despite this evidence of favorable interactions between the two tissue types, the baboon spermatogonia showed no signs of spermatogenesis (Nagano *et al.*, 2001).

To date, successful donor-derived spermatogenesis has been primarily limited to pylogenetically similar species. In addition to mouse to mouse transplants, spermatogenesis has been noted in rat to immunodeficient mouse (Clouthier *et al.*, 1996), hamster to immunodeficient mouse, and mouse to rat transfers (Ogawa *et al.*, 1999). It has been theorized that evolutionary distance is primarily responsible for failure of more distant xenogolous transplantations. Transplants with animals separated by greater evolutionary distances have been less successful. This is likely due to failed spermatogonia and sertoli cell structural association and functional interactions.

Identification, selection, and enrichment of spermatogonial stem cells

Approximately 2 in 10^4 testicular cells are spermatogonial stem cells. Currently most testicular transplantation cellular solutions contain an estimated 100-200 stem cells and yield a 7-20% success rate in generating colonies (Nagano *et al.*, 1999). By increasing the fraction of spermatogonia in the transplanted solution, investigators have hypothesized that a greater quantity of undifferentiated, stem cell spermatogonia can be introduced into the host. Increasing the percent quantity of spermatogonial stem cells in a transplanted solution increases the likelihood of colonization with the donor germinal line. Therefore, isolation of spermatogonia, particularly spermatogonia As or stem cells has been an active pursuit. Among the methods available to isolate more spermatogonial stem cells are using or creating animals with spermatogonial maturation failure (without intrinsic germ cell defect), *in vitro* culturing of spermatogonia, and cellular isolation of stem cells based on cell markers or other factors (e.g. density).

Increased undifferentiated spermatogonia of donor

Cryptorchid animals typically have seminiferous tubules populated with only undifferentiated spermatogonia. Furthermore, it is estimated that 1 in 2000 cells in a cryptorchid teste is a spermatogonial stem cell (Shinohara and Brinster, 2000). An effective method to increase the undifferentiated spermatogonial content for donor transplantation has been utilizing tissue from iatrogenically-derived cryptorchid mice. C57/B1 mice made cryptorchid were found, after 2 months, to have testes containing only type A spermatogonia (Aizawa and Nishimune, 1979; Haneji and Nishimune, 1982).

Steel (Sl) mutant mice are another source of testis thought to contain higher fractions of undifferentiated spermatogonia. The Sl mutation involves a defect in the production of c-kit ligand. Phenotypical Sl mutant mice demonstrate spermatogonial maturation arrest. As a consequence, their testes are likely populated by a higher percentage of undifferentiated spermatogonia than wild type mice (Ogawa et al., 2000). However, recent investigations have called

into question the exact quantity of stem cells in these subjects (Shinohara and Brinster, 2000). Using spermatogonial transplantation as a functional assay for the presence of spermatogonial cells, transplantations from cryptorchid donors resulted in a 25-fold increase in the number of spermatogonial colonies. Colonization was increased, but less dramatically, with donor cells from Sl mutant mice (Shinohara *et al.,* 2000).

Additionally, the percentage of undifferentiated spermatogonia can be affected by medical intervention. Vitamin A deficient mice and rats have only undifferentiated spermatogonia and may serve as sources for these cells. It is believed that Vitamin A influences the differentiation of spermatogonia via a highly specific interaction with cells in stage VIII of the epithelial cycle. Vitamin A deficient diets have been demonstrated to influence spermatogonial stem cell transplantation efficiency and colonization (McLean *et al.*, 2002).

Isolation based on markers or density

Because of the small numbers of spermatogonial stem cells in the normal testis, many believe that specific antibody probes or advances in cell culturing will be necessary to make spermatogonial transplantation a therapeutic option. The c-kit receptor is a marker that has been investigated in attempts to isolate undifferentiated spermatogonia. C-kit antibody studies demonstrate undifferentiated spermatogonia are c-kit independent, while differentiated spermatogonia are dependent (Yoshinaga *et al.,* 1991; Dym *et al.,* 1995).

Attempts to isolate spermatogonial stem cells have found some success based on the light scatter qualities of these cells. Fluorescent activated cell sorting is a cell separation technique based on light scattering of cells and cell surface molecules. In a cryptorchid mouse, fluorescent sorting resulted in a 166-fold enrichment of spermatogonia stem cells determined by subsequent transplantation assay. In the previously described experiment, spermatogonia were selected based on low side scatter. This was because cells with low intracellular complexity, a suspected quality of undifferentiated spermatogonia, would be isolated. These cells also lacked or have low levels of alpha v integrin and they were positive for alpha 6 integrin. Interestingly, presence of c-kit did not increase stem cell isolation (Shinohara *et al.*, 1999; Shinohara *et al.,* 2000).

Magnetic cell sorting is another technique that may be used to better isolate spermatogonia. Von Schonfeldt and colleagues reported isolation of c-kit positive spermatogonia via a magnetic cell sorter. A solution of testicular cells was exposed to anti-c-kit IgG antibodies. Magnetic labeling was then performed using anti-IgG antibodies conjugated with ferro-magnetic microbeads. Twenty five-55% of the isolated magnetically labeled cells were c-kit positive (Von Schonfeldt *et al.,* 1999).

Sedimentation velocity (separating based on size, shape) and differential adhesion were used by Dirami and colleagues to create an isolate of cells containing 95-98% porcine type A spermatogonia (Dirami *et al.,* 1999). Utilizing adhesion to laminin, isolation of spermatogonial stem cells has been noted to increase 3 to 4 fold (Shinohara *et al.,* 1999). Morena and colleagues utilized sedimentation velocity in conjunction with differential adhesion to attain an 85% isolate of Type A, c-kit positive spermatogonia (Morena *et al.*, 1996).

Future clinical application and caveats

The most likely clinical application of testicular transplantation will involve pre-pubertal males facing systemic chemotherapy with sterilizing side effects. These individuals would undergo pre-treatment testicular biopsy. The extracted tissue would be isolated for spermatogonial stem cells, which would then be cultured *ex vivo*. Upon completion of treatment with no evidence of recurrence, these patients would undergo auto-transplantation with their own cultured spermatogonia. Subsequent repopulation of their testes with germinal tissue would result in spermatogenesis and fertility.

Testicular transplantation in patients with malignant diseases that are blood related like leukemia, sarcomas and lymphomas should be considered a great risk for reintroduction of malignancy. On the other hand, non-blood related malignancies such as Hodgkin's lymphoma may not pose a serious risk for patients undergoing autologous transplantations (Aslam *et al.*, 2000). Jahnukainen and colleagues reported on the transmission of rat T-cell leukemia via testicular transplantation from diseased donors. These findings demonstrate the profound importance of developing accurate *ex vivo* spermatogonial isolation and quantification assays. The investigators noted that as few as 20 lymphoblastic cells introduced into the recipient testis were capable of transmitting acute leukemia into healthy hosts (Jannukainen *et al.*, 2001).

Additional caution must be exhibited with the application of human xenogeneic transplantation; as a clinical adjunct it is an unlikely prospect. In the past, porcine retroviruses have been found to infect human kidney cells (Patience *et al.*, 1997). Thus, the possibility of introducing a xenologous viral genotype into human germ line makes the risk of such clinical investigations imposing.

Among topics requiring further research are refinement of spermatogonial stem cell identification, isolation, enrichment, and standardized and efficient pre-transplant cryopreservation. This list emphasizes the need for continued basic research in the arena of spermatogonial stem cell biology.

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In vitro culture of testicular (stem) cells

Mario Sousa, MD, PhD
Lab Cell Biology, ICBAS
University of Porto
Portugal

Abstract

Background: In-vitro long-term cocultures of the normal human seminiferous epithelium remain to be established and characterized. *Methods*: Sertoli cells (SC) and premeiotic diploid germ cells (DGC) were isolated by micromanipulation from testicular biopsies of patients with conserved spermatogenesis, normal karyotypes and absence of Yq11.2-AZF microdeletions, and cocultured for 2-weeks in Vero-cell conditioned-medium (CM) only or supplemented with recombinant follicle-stimulating hormone (rFSH) and Testosterone (T). *Results*: rFSH+T elicited 6.7% of meiotic index and differentiation into elongating (48.5%) and late (16.7%) spermatids. Transmission electron microscopy demonstrated partial reestablishment of cell-junctions only for DGC. Fluorescent in situ hybridization and mRNA expression confirmed cell-stage diagnosis and demonstrated normal meiotic chromosomal synapsis and segregation in vitro. DNA replication (4%) of DGC occurred only in the first week of cultures. Although inhibited by rFSH+T, cultures appeared limited by apoptosis, which caused degeneration of DGC (90% at day 12) and developmental arrest of round (46.2%) and elongating (31.8%) spermatids. *Conclusions*: In vitro long-term cocultures of the normal human seminiferous epithelium sustain full germ-cell differentiation but limited cell proliferation and meiosis, with apoptosis being the major limiting factor.

Learning objectives

-Cytogenetical and molecular selection of patients.
-How to dissociate germ cells from the seminiferous tubules.
-How to recognize the different germ cell populations.
-How to culture germ cells in vitro under highly controlled conditions.
-How to characterize stage-specific germ cells using FISH and RTPCR.
-How to characterize meiosis using FISH.
-How to study germ cell proliferation.
-How to study germ cell apoptosis by live-cell assays, immunocytochemistry and RTPCR.

introduction

Restoration of the spermatogenic cycle in vitro remains to be successfully achieved in mammals. This has been explained by the existence of complex mixtures of germ cells at different stages and of complex relationships between SC, basal lamina and germ cells, whith germ cell development appearing also dependent on numerous hormones, growth factors and interleukins secreted by intraepithelial cells and by cells located in the surrounding connective tissue. In rodents, culture of seminiferous tubule fragments, which preserve SC and germ cell contacts, can be maintained viable for several months, but after 2-3 weeks only SC remain alive and no round spermatids were ever formed in vitro. More recent in vitro experiments then demonstrated that 2 week in vitro cultures of dissociated germ cells and SC enable a few late pachytene spermatocytes to complete the two meiotic divisions, but the newly formed round spermatids appeared unable to differentiate into normal elongating spermatids. In vitro studies also revealed that most of the germ cells rapidly degenerate in the first 2 days, especially if contacts with SC were lost, with successful preparation of Sertoli-spermatogenic cell cocultures depending on minimal cell-junction disruption during enzymatic dissociation, cell-plating at maximum density, supplementation with hormones, growth factors and vitamins, frequent replenishment of culture medium and simultaneous removal of metabolic waste products (1-6). We have recently shown that in vitro cocultures of SC and DGC from azoospermic patients elicit in vitro differentiation of late spermatids in the presence of Vero cell CM supplemented with rFSH and T (5,6). In the present study, long-term cocultures were established to characterize the limiting steps of cell proliferation, meiosis and differentiation of the normal human seminiferous epithelium in vitro.

Materials and Methods

Male patients

Selection criteria: 15 patients with anejaculation and obstructive azoospermia, with conserved spermatogenesis in diagnostic histopathology, normal karyotypes and absence of Yq11.2-AZF microdeletions. Cells used for experiments were those that remained in the tissue plates after microinjection treatments under patient informed consent.

Germ cell isolation and culture

Open testicular biopsy was performed on the day before oocyte pick-up. Each biopsy was collected in sperm preparation medium (SPM-Hepes), squeezed with surgical blades, and the resultant fluid washed in SPM, 2x5min at 1000rpm (500-600g). After 5min incubation in 2ml of erythrocyte-lysing buffer, cells were dissociated by incubation, for 1h at 37ºC, in SPM containing 25µg/ml of DNase and 1000U/ml of collagenase-IV. After washing, the pellet was resuspended in 50-100µl of IVF medium and incubated at 32ºC, 5% CO_2 (6). Cells of specific stages were selected by micromanipulation and plated in (5 cases per medium) Vero cell-CM (5), CM+25 UI/L rFSH, and CM+rFSH+1 mmol/L T (4). Cells were cocultured for 2 weeks (32ºC, 5% CO_2) and counted every 24 h (under double-blind conditions).

Cell morphology

SC easily ruptured during manipulation, releasing their nuclei and lipid droplets. Germ cells had a clear smooth rim of cytoplasm and a smooth nucleus without chromatin clumps: elongated A-spermatogonia (SGA) displayed an elipsoidal shape, primary spermatocytes (ST1:19-24mm diam), secondary spermatocytes (ST2:14±1mm), and early (without flagellum) round spermatids (Sa1:8-10mm). Sa1 were at the Golgi (round acrosomal vesicle) or cap (elongated AV) phase and were distinguined from other similar sized structures based on: nuclei of SC exhibited an elevated border, a large nucleolus, and shrunk when transferred to polyvinylpyrrolidone (10% PVP-SPM); cytoplasmic droplets, blebbed out from damaged SC, displayed no visible organelles and shrunk in PVP; small lymphocytes had irregular chromatin patches, sticked to the micropipette tip when aspirated, and spread and attached to the tissue culture dish in about 24h.

Confirmation of germ cell stages

mRNA expression

Total RNA was extracted from whole testicular tissue cell suspensions and from stage-specific cells isolated by micromanipulation (600-1000 cells per stage) using RNeasy Mini Kit and RNeasy Micro Kit (Qiagen), respectively. Total RNA obtained was primed with 100 ng of random hexamer primers and reverse transcribed with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Two RT reactions were performed and pooled to obtain enough amount of cDNA. Synthesized cDNAs were used for PCR in a final volume of 25 l. PCR reactions were optimized using 2 l of cDNA, 1.5 mM $MgCl_2$, 10mM of each dNTP, 1mM primers and 1U Taq DNA Polymerase (MBI Fermentas). PCR cycling: one cycle, 95ºC, 5 min; 40 cycles, 95ºC, 30 s; various annealing temperature upon different genes, 30s (*Oct4* , *c-kit* and *MAGE* for SGA, *SCF* for SC, *HSP* for DGC, *MLH1* for ST1, *SPAN-X* for Sa1); and 72ºC, 1 min. PCR products were analyzed by electrophoresis on agarose gels.

Fluorescent in situ hybridization

Fluorescence in situ hybridisation (FISH) was performed using -satellite probes for the centromeric regions of chromosomes X (DXZ1, Xp11.1-q11.1), Y (DYZ3, Yp11.1-q11.1) and 18 (D18Z1, 18p11.1-q11.1). Slides were mounted with Vectashield antifade medium containing DAPI to counterstain DNA and observed by epifluorescence microscopy. Normal male lymphocytes were used as controls (6).

DNA replication

The pyrimidine analogue of thymidine, 5-bromo-2-deoxyuridine (stocks prepared in d_6-DMSO; Sigma), which is selectively incorporated into DNA at the S-phase of the cell cycle, was incubated (18h, 100mM) in cell cultures. DGC were then isolated, washed, alocated to PBS in poly-L-lysine coated glass slides and air-dried. After methanol treatment (20min, -20ºC), they were permeabilized (3x10min) in PBS-0.1%Triton-X100, fixed (20min, 4ºC) with 1% paraformaldehyde, washed, denatured (45min, 37ºC) in 4N-HCl, washed, blocked (37ºC, 30min, dark moist-chamber) with 10% goat serum, incubated (1:20 PBS, 1h, rt, dark) with monoclonal anti-BrdU antibody, washed, incubated (1:600 PBS, 30min, rt, dark) with goat anti-mouse antibody conjugated with CY3, washed, air-dried, mounted with Vectashield-DAPI and observed by epifluorescence microscopy.

Apoptosis detection in stage-specific germ cells
Caspase 3-like activity detection in live cells
Cells were transferred to 50μl sperm preparation medium containing 5μM of the cell-permeable profluorescent caspase substrate PhiPhiLux, which becomes fluorescent after being cleaved by activated caspase-3-like proteases. After incubation (30min), cells were washed, transferred to PBS in slides and air-dried. After mounting with Vectashield-DAPI, they were observed by epifluorescence microscopy.

Apoptosis gene mRNA expression
RNA extraction and RT were performed as above. PCR reactions were performed for *caspase 8*, *casp9* and *casp3*, and for *Fas-Receptor*, *Bcl2* and *Bax*. Peripheral blood lymphocytes from a leukemic patient at remission were used as a positive control.

Transmission electron microscopy
Cells were aspirated with fine Pasteur pipettes, pelleted (1500rpm, 10min), fixed (4ºC, 2h) with 6% glutaraldehyde in 0.2M Na-cacodylate buffer, pH 7.2, washed, postfixed in 2% OsO_4 in buffer, dehydrated in an ethanol series followed by propylene oxide and embedded in Epon. Ultrathin sections were cut with a diamond knife, collected on copper grids, stained with alchoolic concentrated uranyl acetate (20min) and Reynolds lead citrate (10min), and studied at 60 kV in a JEOL 100CXII transmission electron microscope (TEM).

Statistics
Proportions and 95%CI were calculated and compared using the Difference Between two Proportions Test (Statistica, vs. 5á1).

Results

In 1-2 days, a few cell clusters formed in cultures supplemented with hormones, of which some tended to dettach from the tissue culture plate. Cell junctions were shown by TEM to be partially reacquired only between SC and DGC. Completion of meiosis and spermatid differentiation were mainly restricted to cell aggregates or side-connected germ cells. After 1-3 days, a few SGA, some STI and several STII were observed at telophase. In general, newly formed Sa1 appeared in the first 1-2 days, transition to late (with flagellum) round spermatids (Sa2) took another 1-2 days, maturation of the latter into normal elongating spermatids (Sb) required 2-3 days, and from these to elongated spermatids (Sd) needed 5-7 days. Thus, in total, meiosis completion and spermiogenesis took about 9-14 days.

In CM, meiosis did not proceed and no new Sa1 formed. However, plated Sa1 differentiated in vitro to Sa2 (12.9%), Sb (10.8%) and Sd (3.2%). In CM+rFSH, new Sa1 formed in only two cases. Of the Sa1 that extruded a flagellum, about half were derived from the newly formed Sa1; similarly, about one third of the normal Sb and 4 of the normal Sd (in one of the cases) originated from the newly formed Sa1. In CM+rFSH+T, newly formed Sa1 were observed in all five cases, with the majority of the in-vitro differentiated Sb and Sd coming (in all cases) from the newly in-vitro formed Sa1. Comparisons between groups suggest that if meiosis (MI-meiotic index = new Sa1/DGC) is induced by rFSH (3%), it is highly potentiated by rFSH+T (6.7%), and that if rFSH seems to slightly induce maturation to Sa2 (22.7%), no effects were noticed on Sb (18%) and Sd (4%) differentiation. On the contrary, rFSH+T induced significant increases of maturation rates in early (53.8% Sa2) and late (48.5% Sb, 16.7% Sd) spermiogenesis, either in relation to controls or to rFSH. Independently of these effects, in all groups spermatid differentiation appeared especially halted during maturation of Sa1to Sa2 and of Sb to Sd.

FISH analysis revealed: a normal 1818,XY signal in 179/200 (89.5%) SC, a normal paired 18,XY signal in 195/200 (97.5%) ST1, a 18,X/18,Y normal signal in 193/200 (96.5%) ST2, a normal 18,X/18,Y signal, with a sex ratio near 1:1, in 178/200 (89%) Sa1, a normal haploid signal in 237/255 (92.9%) arrested-Sa1/Sa2, in 59/70 (84.3%) arrested/abnormal-Sb and in 28/31 (90.3%) normal-Sd.

DNA replication (SGA proliferation) was stimulated only with rFSH+T (4%), but decreased to 1% at day 6 and became undetectable by day 12. SC degeneration appeared well limited and was only inhibited by rFSH+T (live cells: 87.5%-day2, 81.3%-day6, 80%-day12). Cell degeneration was particularly intense marked on DGC in the first 2 days of cocultures (CM-live cells: 10%-d2, 5%-d6, 2%-d12). This could only be partially inhibitted by rFSH (live cells: 30%-d2, 17%-d6, 5%-d12), and especially with rFSH+T (live cells: 50%-d2, 30%-d6, 10%-d12). Spermatids did not

enter cell degeneration but most became arrested or exhibited abnormal development. This could be only partially inhibited by rFSH+T for Sa1, with no further effect on other spermiogenic stages (arrested cells: 46.2%-Sa1, 9.9%-Sa2, 65.6%-Sb). Cases where less cell degeneration occurred in the first 2 days also exhibited the highest potential for in-vitro HGC differentiation. If SC or DGC were left isolated, cell aggregation was inhibitted and cell death was complete after 2-3 days. Formation of cell aggregates, HGC differentiation (at a very low rate) and decreased cell degeneration was nevertheless obtained with isolated ST and Sa in case cells were plated at higher densities. Isolated SC cultures plated at high density also formed aggregates and survived for more than 2 weeks, although in some cases giant cell masses formed. TEM analysis of degenerated cells showed SC with signs of cytoplasmic degeneration, and DGCs with apoptotic condensed chromosomes and degenerating cytoplasm. Detection of executioner caspase-3 (casp3) activity was used to confirm nuclear and cytoplasmic apoptotic events in living cell cultures. All SC and DGC with clear signs of degeneration evidenced casp3 activity mainly in nuclei. Arrested and abnormal spermatids showed fluorescence in both the nucleus and the cytoplasm, with about 40% of the abnormal Sb also showing casp3 activity in the acrosomal vesicle. Of the in-vitro differentiated normal Sd, half exhibited casp3 activity in midpiece. Expression mRNA studies showed active caspases 9, 8 and 3 in SC, ST1 and Sa1 but absence of casp9 activation in SGA.

Discussion

Spermatogenesis is a hormonal-sensitive process, with FSH and T being needed to support germ cell differentiation through direct or indirect actions on SC. Besides preventing degeneration of germ cells, FSH was shown to stimulate SGA proliferation, and suggested to modulate meiotic divisions and differentiation of Sa1 to Sd. Similarly, T was shown to be implicated in germ cell survival, induction of SGA proliferation and meiosis, although it mainly induces and controls Sa1 maturation to Sd (1-3). Experiments in humans showed that about 22% of isolated Sa1 cultured in non-supplemented IVF medium can grow flagella in 1-2 days, but then become arrested. On the contrary, by using Vero cell monolayers, isolated Sa1 were shown to mature into Sd and spermatozoa in about 7-12 days (5). In other studies, culture of testicle tissue suspensions suggested that FSH stimulates spermatogenesis and T inhibits SC apoptosis (4). However, differentiation of Sd occurred within 1-2 days, highly contrasting with the normal cycle that needs 16 days to evolve from the late pachytene ST1 or ST2 stages to Sd. These hormonal effects were later confirmed in cases of non-obstructive azoospermia, but Sd differentiation was demonstrated to need about 2 weeks of culturing (6).

In the present study, long-term 2-week cocultures were established to test and characterize the limiting steps of cell proliferation, meiosis and differentiation of the normal human seminiferous epithelium in vitro. Because spermatogenesis needs a complex set of growth factors and interleukins (1-3), we have used Vero cell-CM, once it contains several of those factors and detoxicating substances (5). To avoid any possibility of contamination with a hiden focus of spermatids, SC and DGC were isolated and then cocultured. Results show that both FSH and T are needed to support cell survival and junctions, meiosis completion and spermatid differentiation in vitro; that the association between rFSH and T further inhibits culture-induced apoptosis, increases the meiotic index, and improves maturation of round to elongated spermatids; and first suggest that FSH acts over conversion of ST1 to Sa1 and of Sa1 to Sa2 (flagellum extrusion). These results confirm previous evidence in humans that T inhibits SC and germ cell apoptosis (1-4), and results in rodents which demonstrated that FSH plays a determinant role in the survival of the seminiferous epithelium and that T induces premeiotic DGC proliferation and conversion of Sa1 to Sd (1-3).

The present experiments also reproduced the physiological in vivo time delays of spermatogenesis, with appearance of Sa1 and their subsequent differentiation into Sd taking about 9-14 days (6-12 days in vivo). In this process, some ST1 (probably at late pachytene) finished meiosis I in 2-3 days, and ST2 took also 2-3 days to finish meiosis II and give rise to Sa1, which also fits into in vivo findings (4-6 days). However, cocultures did not allow extended SGA proliferation nor meiosis reinitiation. In comparison with non-obstructive azoospermic patients (6), if spermiogenesis was clearly improved using cells from normal seminiferous epithelia, completion of meiosis was not increased. This points to the fact that most of the newly formed Sa1 must have been originated from late pachytene ST1 and especially from ST2, which is in accordance with studies in rodents that showed the information needed for meiosis completion and to begin spermiogenesis is only translated at the late pachytene stage (1-3). Nevertheless, the present data show that meiosis can be reinitiated in vitro at a rate of 6.7%, at least from samples containing late pachytene ST1 and ST2, and that Sd can differentiate at a rate of 16.7% from Sa1.

Germ cell apoptosis was not sufficiently inhibitted, and only 46.7% (7/15) of the cases were able to progress through complete spermiogenesis, which suggests that at present, even with control samples, in vitro cultures and the maturation

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process is not reprodutible. The main causes for these failure might be attributed to rupture of cell junctions during cell dissociation, the use of low cell densities, absence of a supporting basal lamina and of specific paracrine factors, and deficient renewal of the culture medium (1-3). This might be overappreciated in the present study because cultures of cell suspensions were not performed. Instead, the present investigation was conducted with isolated cells in microdrops to assure the absence of hiden spermatids at plating, with cell densities being limited by patient tissue availability. We also first show that connections between SC and DGC were partially reacquired, but the same did not apply to spermatids. This suggests that FSH, as potentiated by T, was unable to render SC competent to bind Sa1 (1-3), which might explain why so many Sa1 exhibited an abnormal developmental pattern as also why Sd showed a high rate of head abnormalities. Abnormal synapsis and chromosomal segregation could also impair spermatogenesis and induce apoptosis in vitro (1-3). However, we here first show that most of SC, ST1, ST2 and in vitro differentiated spermatids have a normal chromosomal constitution, which further supports that in vitro cultures progressed normally through meiosis without aneuploidy induction. Classical morphological signs of apoptosis were described in premeiotic DGC but not in SC, whereas both cell types exhibited evident degeneration during in vitro cultures. In azoospermic patients, apoptosis is further increased, with DNA fragmentation having been described in nuclei of spermatids and sperm, and annexin-V labeling being negative in Sa1 but present in sperm (1-4). Classical morphological signs of apoptosis during cultures were here demonstrated by light and electron microscopy. Although the exact mechanism by which apoptotic cells are cleared remains unknown, studies suggested either a SC phagocytating process or sloughing of cells to excretory ducts (1-3). Both observations are here supported, as SC were observed to phagocytate premeiotic DGC but not spermatids. Caspase activity was here also first applied to the study of human germ cells cultured in vitro. Whereas in SC and DGC caspase activity was present mainly in nuclei, arrested and abnormal spermatids displayed caspase activity in both the cytoplasm, nucleus and acrosome, and about half of the normal Sd exhibited caspase activity in midpiece. This suggests that apoptosis in SC and DGC is mainly triggered by deficiency in exogenous substances, that both pathways are implicated during early spermatid differentiation, and that in Sd it seems related to an endogenous metabolic insult.

In conclusion, the present experiments ensured 2-week in-vitro cocultures of the normal human seminiferous epithelium, enabling, at a physiological pace, some degree of SGA proliferation, meiosis completion from late ST1 and ST2, and differentiation of Sa1 to Sd. Further developments will be aimed to improve media supplementation to more effectively control the apoptotic limiting steps.

Acknowledgements

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Cryopreservation of testicular stem cells

Henri Woelders
Animal Sciences Group
Wageningen UR
Division of Animal Resources Development
Lelystad
The Netherlands

Learning objectives

General cryobiology, Physical and chemical events during freezing
Theoretical cryobiology; Phase diagrams
Theoretical cryobiology; Modelling of osmotic events
Methods for stem cells
Methods for spermatogonial stem cells

General cryobiology: Physical and chemical events during freezing
For references pertaining to this chapter and further reading see: [Mazur, 1966; Woelders et al 1997].

During the process of cooling, freezing, and thawing, cells are subjected to a series of drastic changes in their physical and chemical environment. The first change that the cells have to cope with is cooling below body temperature, causing phase transitions of the lipids in the membranes of the sperm cell. As different lipids have different phase transition temperatures some types of lipid will aggregate in domains of gel-like ("frozen") lipid, thus excluding other lipid species that still remain in the liquid-crystalline (melted) state. Membrane proteins are also excluded from these gel domains and consequently find themselves in an unphysiological lipid environment (lateral phase separation, Figure 1). This is believed to impair the function of membrane proteins that are involved in structural integrity (cytoskeleton) or in ion metabolism (ion pumps). When cells are unstable at low unphysiological temperatures, this is referred to as chilling injury. Cells may also be harmed by a sudden change of temperature. This is referred to as Cold Shock.

A second change in the environment of the sperm takes place when liquid water is converted into ice. Spontaneous ice nucleation will usually occur after the solution is supercooled to a temperature between "5 and "15 C. This is due to the fact that small ice crystals have a large surface tension, which makes them thermodynamically unstable. Therefore, spontaneous ice nucleation is a random process, occurring only when, by chance, enough molecules with a lower-than-average kinetic energy get together and form a stable ice nucleus. Once this has happened, the ice crystals will grow rapidly in all directions. The release of the latent heat of fusion then causes the sample to warm up abruptly, until the freezing/melting temperature of the solution (of the remaining unfrozen fraction) is reached. At this point, the ice formation stops, or will proceed at a rate then governed by the rate at which heat is extracted from the sample. The formation of ice means that part of the (liquid) water is removed from the solution. What remains is the so-called unfrozen fraction, in which all cells and all solutes are confined. The concentrations of the solutes and the osmotic pressure of the unfrozen fraction increase rapidly. At the same time, the volume of the unfrozen fraction decreases rapidly. The increase of the osmotic strength causes an efflux of water from the cells, resulting in shrinking of the cells and increase of the intracellular concentration of solutes. As cooling continues, these processes will continue, until the viscosity of the unfrozen fraction becomes so high that further growth of the ice mass is halted. This means the remaining 'unfrozen fraction' solidifies in a glassy state. This is referred to as vitrification. The "colligative" action of cryoprotectants can be understood as follows. The total solute concentration of the unfrozen fraction is a function of subzero temperature. When the freezing medium has a high (initial) concentration of solutes, there is less water to form ice and the volume of unfrozen fraction remains higher. When a high proportion of these solutes are non-electrolytes, this will in addition lead to a lower final concentration of (damaging) electrolytes in the unfrozen fraction (Figure 3). When, on top of that, these non-electrolytes are membrane-permeant (e.g.. glycerol), this will in addition lead to less shrinking of the cells, and a lower intracellular salt concentration. So the use of a permeating cryoprotectant, e.g. glycerol, leads to a larger unfrozen fraction, less shrunken cells, and a lower intracellular and intracellular salt concentration
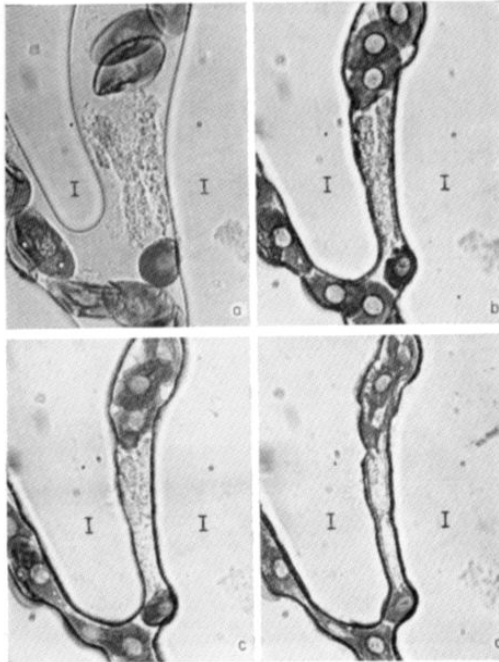
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Figure 2. Frog erythrocytes at "1.5, "5, "7, "10 ºC [Rapatz *et al.*, 1966]
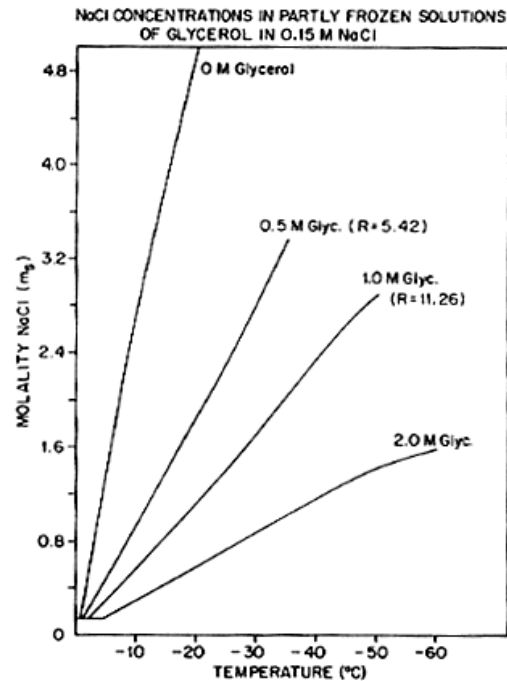

Figure 3. Cryoprotectants like glycerol result in a larger volume and a lower electrolyte concentration of the unfrozen fraction. [Mazur and Rigopoulos, 1983]

Cryopreservation in a broader perspective; Phase diagrams

As said above, at a certain temperature (and osmolality) the remaining unfrozen fraction will solidify in a stable glassy state. A glass state confers stability because (by definition) molecules have lost the ability of translation movement. However, glass transition is not necessarily a function of temperature, but mainly of the activity of water in the sample. In very dry conditions, glass transition is possible at temperatures above zero. This can be seen in the phase diagram for trehalose (a sugar) shown in Figure 4. It can be seen that the glass transition temperature is a function of the water concentration (or the solute concentration) of the sample. The different preservation techniques (drying, freeze drying, slow freezing, vitrification) all aim at reaching glass transition. For storage at subzero temperatures, glass transition must be reached while preventing intracellular ice formation.

Cells like spermatogonia have a very high water content. Glass transition without reducing the water content is only a theoretical possibility, because cooling rates must be extremely high (> 50.000 ¡C/min) to prevent, or outrun the initiation and growth of ice crystals. Other techniques that could apply to spermatogonial stem cells, like vitrification and slow freezing, rely on withdrawal of water from the cells in order to prevent IIF. In vitrification techniques, withdrawal of water is done by adding cryoprotectants to the cell suspension to a very high final concentration, which can then be safely plunged into liquid nitrogen. In slow freezing techniques withdrawal of water is done by controlled extracellular ice formation. To date, vitrification methods have not been tried for freezing stem cells, except for embryonic stem cells [Reubinoff *et al.* 2001]. Conventional slow freezing is still the most common cryopreservation principle.
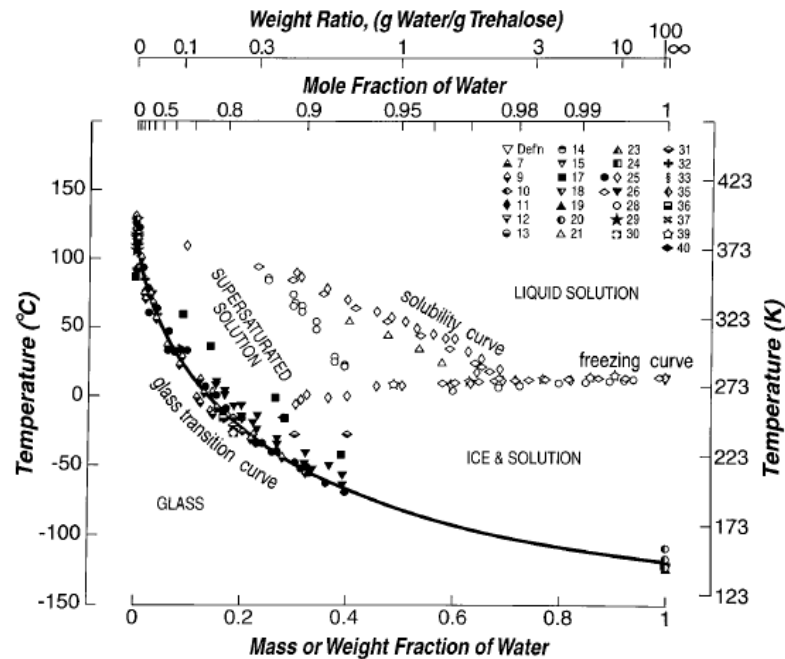
Figure 4. Phase diagram of a solute (trehalose) and water [Chen et al 2000]. Normally we start with 'wet' cells" at a temperature above zero (in the area 'liquid solution'). To reach the stable glass we must either plunge through the region of 'ice-plus-solution' (risk of IIF) or slow-freeze along the freezing curve (dehydration because of ice formation), or dehydrate by drying or by addition of very high concentration of solutes.

Modelling of osmotic events during slow-freezing

In 'slow cooling methods' the rate of cooling can be an important factor. It is a general observation in cryopreservation that cells and other biological systems may have a specific range of cooling rates at which post-thaw survival is optimal. As explained by Mazur in 1966 This can be explained by two sets of mechanisms of cryoinjury at too low and at too high cooling rates, respectively [Mazur, 1966]. In between the start of the freezing programme and stabilization of the cells in the glassy state, cells are exposed to unfavourable unphysiological conditions, most notably the rise of the solute concentration in the unfrozen fraction, the very small volume of unfrozen fraction, and the shrinking of the cells. Slow freezing can exacerbate this by prolonged exposure of the cells to these unfavourable conditions and because of increased shrinking and dehydration of the cells. The latter is due to the fact that at slow cooling rates, plenty time is available for the efflux of water, and cells will shrink close to osmotic equilibrium. Too fast cooling, however, does not provide enough time for the cells to dehydrate. Water is then 'supercooled' which can be seen as 'supersaturated' and the chance of intracellular ice formation (IIF) becomes appreciable. Also, it has been proposed that fast cooling damage could result from a too high rate of water efflux or a too high osmotic pressure difference across the cell membrane, or from mechanical stress caused by a too rapid shrinking of the cells. Anyway, this means that 'fast cooling damage' remains limited when the level of supercooling remains limited, because the level of supercooling (or osmotic disequilibrium) governs both the chance of IIF and the rate of membrane water flux.

The osmotic events during freezing and thawing can be modelled mathematically, provided that the necessary parameters and coefficients are available, like the osmotically active cell water volume, the cell membrane surface, the permeability coefficients for water (hydraulic conductivity; L_p), and for CPA (P_s), and their respective Arrhenius activation energy (E_a), and the initial concentrations of permeable (CPA) and impermeable solutes. L_p, P_s, and E_a of a specific cell type can be determined by measuring the rate of volume changes of cells under anisosmotic conditions. Volume changes can be monitored by measuring the fluorescence of an entrapped, concentration dependent fluorophore (carboxyfluorescein).

Such theoretical models have been applied in a number of studies. In these models it was necessary to assume a fixed (constant) cooling rate. However, a constant cooling rate inside the sample may not at all be optimal, as both the amount of water that solidifies per degree of cooling and the membrane permeability coefficients decrease with decreasing subzero temperature.

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We have recently derived a model that allows to predict the optimal cooling rate as a function of subzero temperature [Woelders and Chaveiro 2003; Woelders and Chaveiro 2004]. The model is based on the assumption that the cooling rate should be as high as possible (to prevent slow cooling damage) but not so fast that there is a chance of fast cooling damage. This translates into the boundary condition that the level of intracellular supercooling is kept constant at a chosen value of p ¡C, e.g. 2 ¡C. It is generally accepted that an intracellular supercooling by 2 ¡C is sufficiently low to preclude IIF.
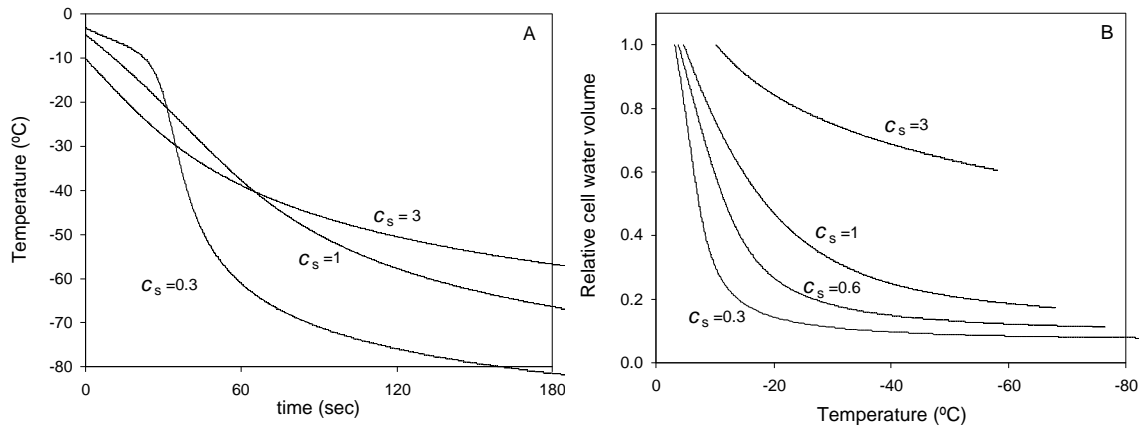

Figure 5. (A) Theoretical prediction of the optimal freezing protocol for bovine spermatozoa according to Woelders and Chaveiro, 2003; 2004. (B) the corresponding simulation of the dehydration of the cells during freezing. The curves show the influence of concentrations of glycerol of 0.3, 0.6, 1, and 3 mol.litre[-1] respectively.

The model predicts that a non-linear cooling curve is optimal for cell survival. The model is able to calculate the optimal cooling rate as a function of subzero temperature, and the corresponding time course of temperature. Thus, one can design the optimal cooling programme for a given cell type in a medium with given solute and CPA concentrations (Figure 5).

Methods for stem cells

Spermatozoa were the first mammalian cells to be cryopreserved successfully. Cryopreservation of somatic cells soon proved to be possible for a number of cell types. In early studies, the methods came down to adding 5 to 10% of a suitable cryoprotectant, like glycerol or dimethylsulfoxide (DMSO), to the suspension of cells in culture medium, and place tubes with a few ml of that suspension at "80 °C in a mechanical freezer. In fact this simple procedure is still effectively used today. Obviously, with this simple procedure the rate of cooling cannot be controlled; in fact in many publications the cooling rate is unknown. There are only a few studies in which controlled rate freezers were used, e.g. with skin fibroblast.

Stem cells

Methods that have later been adopted for stem cells were not very much different from those used for other somatic cells. Blood stem cells are usually frozen in a simple physiological saline or a culture medium, with the addition of 5 or 10% DMSO. Additionally, freezing media may contain non-permeant cryoprotectants. For instance, hydroxy ethyl starch and serum or human albumin have been shown to have a cryoprotective effect, and may be used in addition to the permeant cryoprotectant DMSO, or can replace part of the DMSO. Again, some publications report the use of controlled rate freezers. e.g. in the case of human myeloid stem cells, which had optimal survival at cooling rates of 1-3 °C/min.

As explained above, mathematic modelling of the osmotic events during freezing can be a helpful instrument for optimising freezing protocols. Such studies predict that there may be a strong interaction between cryoprotectant concentration and cooling rate. Woods *et al.* [2003] used a theoretical model (assuming a constant cooling rate), to predict the combination of cooling rate and DMSO concentration that would minimise the duration between ice nucleation and glass transition while preventing conditions of intracellular ice formation. This optimal combination was 0.7 mol.liter[-1] DMSO (5% v/v) and a cooling rate of 4 °C/min. With this combination, cells could be plunged in LN_2 at a temperature of "44 °C. This method gave improved engraftment of the stem cells compared to the standard method. The theoretical study demonstrates that it is important to address cooling rate and cryoprotectant concentration

together, because it may depend on the cooling rate whether or not it is possible to reduce the (toxic) DMSO concentration.

Spermatogenic stem cells

There have been few reports of efforts to cryopreserve male germ line stem cells. In one study, as early as 1954, transplantation of frozen-thawed immature rat testes was reported that resulted in spermatogenesis from the transplanted cells. Recently, birth of a healthy human child after transplantation of frozen minced testis tissue was reported [Res *et al.*, 2000]. Cryopreservation of non-purified spermatogonial cell populations was demonstrated in 1996 by Avarbock *et al.*, using cells from mouse testes [reference 11 in Nagano et al 2002]. Likewise, nonpurified spermatogonial cell populations from a number of large domestic animals (boars, bulls, and stallions) were cryopreserved successfully [Dobrinski *et al.* 2000, reference 21 in nagano et al 2002]. With the same procedure human spermatogonial cells were frozen successfully [Nagano et al, 2002]. Avarbock *et al.* used routine freezing procedures as have been used widely for somatic cells. In these studies, non-controlled rate freezing was used, and little effort seemed to have been done to optimise the procedures as to freezing medium composition, cryoprotectant concentration, and cooling rate.

Using a controlled rate freezer, Izadyar *et al.* [2002] investigated the role of cooling rate and medium composition in cryopreservation of purified bovine type A spermatogonia. These spermatogonia had a significantly higher survival at a relatively low cooling rate of 1 °C/min compared to 5 °C/min. Using a non-controlled rate freezing method, in which the average cooling rate between +5 and "40 °C was similar to that of the controlled rate freezer at 1 ¡C/min, the results were even slightly better (not significantly). Looking at the range of temperatures between +5 and "40 °C, the non-controlled protocol had cooling rates varying between 0.5 (shortly after ice nucleation), and 4 °C/min (between "30 and "40 °C). As to the medium composition, it was found that 1.4 mol.l"1 DMSO (= 10% (v/v)) gave survival far superior to that obtained using 1.4 M glycerol. An important finding was that the post-thaw percentage live spermatogonia could be increased by adding sucrose or trehalose (0.07 mol.l"1) to the freezing medium. The survival increased from 49% to 68%. More importantly, freezing in medium containing sucrose resulted in a significant increase of the percentage of tubule cross sections containing type A spermatogonia, with more than twice as many type A spermatogonia per tubule cross section after transplantation into recipient mice. Thus, this study indicated that the composition of the freezing medium was very important for post-thaw survival and successful transplantation of cryopreserved type A spermatogonia. Autologous transplantation of bovine spermatogonia frozen by the procedure developed by Izadyar et al [2002] (as described above) resulted in a complete regeneration of spermatogenesis [Izadyar et al 2003].

The controlled rate experiments showed that the cooling rate has a significant and important influence on cell survival. The non-controlled rate freezing protocol was very successful. 'Non-controlled rate' does no mean that the cooling rate doesn't matter and needs not be controlled. In fact, the conditions of non-controlled rate freezing were chosen carefully to obtain the 'just right' non-linear cooling curve. In the non-controlled rate curve (see Figure 6) the heat flux from the cell suspension depends on the level of insulation of the cryovial and the Styrofoam box, and decreases with decreasing temperature. This, and the release of heat of fusion together result in the sigmoidal shape of the cooling curve between onset of ice formation and "80 °C. The theoretical model presented recently by Woelders and Chaveiro [2003; 2004] clearly indicates that the cooling rate should not be constant. In fact, depending on a number of cell characteristics the model predicts that a non-linear cooling curve, like that obtained in the 'non-controlled rate method' could give better cell survival than a linear cooling curve as is used in most controlled rate freezers. The non-controlled rate curve is characterised by a slow cooling rate directly after ice nucleation, thus allowing enough time for the efflux of water from the cells, followed by a much higher cooling rate at lower subzero temperatures, at which water efflux is less important. Thus the time needed to reach the stable glassy state is minimised while conditions of intracellular ice formation are prevented. Thus we can conclude that the theoretical model provides a rationale to explain the very good survival obtained with the non-linear cooling curve obtained with the non-controlled rate freezing method. In the controlled rate freezing programs (Figure 6) it can be seen that the cooling curve is linear, except for a brief spike of temperature directly after ice nucleation.

This is due to the supercooling that precedes ice nucleation, and the subsequent release of heat of fusion. Extrapolating from the notion of an optimal cooling rate for a specific cell type, some researchers have erroneously concluded that they should rigidly enforce that cooling rate at all phases of the freezing process. Consequently, protocols have been used in which extra liquid nitrogen is pumped into the freezing chamber to absorb the heat of fusion as rapid as possible and to bring back the cooling curve to the programmed straight line. This is most likely not beneficial for the cells. This could cause the rate of cooling directly after ice nucleation to be extremely high, and possibly to high for optimal cell survival.
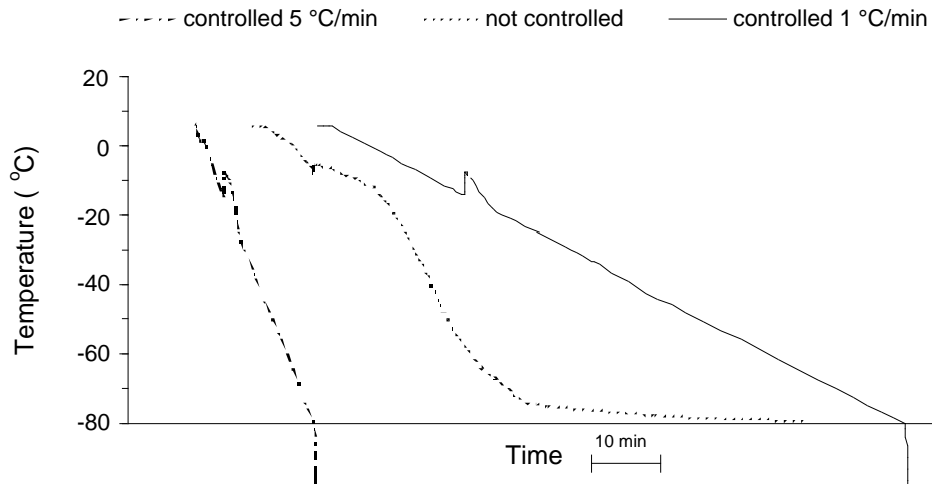
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Figure 6. Cooling curves used for freezing bovine A-type spermatogonia [Izadyar et al, 2002] (temperature measured inside the cryotubes as function of time) obtained using a non-controlled rate method (middle curve, or using a controlled rate freezer with constant cooling at 5 °C/min, or 1 °C/min.

Conclusion

Spermatogonial stem cells of mice, boars, bulls, stallions and humans have been frozen successfully. The cells can be frozen using the slow freezing procedure in culture medium with 10% (v/v) DMSO + 10% FCS. Post-thaw ability of purified type A spermatogonia (from bulls) to colonize recipient testes was strongly improved by using 0.07 sucrose in the freezing medium. A non-controlled rate freezing method is convenient and can give good results. However, packaging and other conditions must be carefully selected to obtain the 'just-right' cooling curve. For optimal recovery of fertility of patients by transplantation of frozen-thawed spermatogonia, the best possible freezing protocol should be selected and should be standardized. A theoretical approach as proposed by Woelders and Chaveiro [2003;2004] is needed to predict the optimal shape and steepness of the non-linear freezing curve, which then can be validated and implemented as a non-linear freezing programme in a controlled rate freezer.

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Transplantation of testicular stem cells and grafting

Stefan Schlatt, Ph.D.
Assistant Professor, Department of Cell Biology and Physiology
University of Pittsburgh School of Medicine
Pittsburgh, PA
USA

Learning objectives

1) The attendants will be introduced to the biology and physiology of male germ line stem cells
2) The participant distinguishes the adverse effects of oncological treatment on male fertility and debates the options for fertility preservation in male patients.
3) The participant evaluates new approaches on spermatogonia in basic research and clinical application.
4) The attendants will learn about the complexity and the future perspective of germ cell transplantation and testicular grafting as a tool for fertility preservation in patients.

Introduction

Spermatogonia are the diploid germ cells in the testis located at the basement membrane of the seminiferous tubules. Spermatogonia are important cells (Meachem *et al.*, 2001) since they are needed for the initiation of spermatogenesis and hence sperm output. The integrity of the male genome relies on their continuous ãerror free“ proliferation and differentiation. They are ancestors of the primordial germ cells colonising the embryonic gonad early during development. Spermatogonia represent a heterogeneous subset of cells containing undifferentiated (stem cells) and differentiated cells (Meistrich and van Beek, 1993). An excellent description of spermatogonial subtypes and their physiology as well as a suggestion for a non-confusing nomenclature was presented by de Rooij and Russell (2000). The number of true spermatogonial stem cells in the fully functional adult testis appears to be very low (Tegelenbosch and de Rooij, 1993; de Rooij and Russell, 2000).

Several environmental or pathological conditions are known to have detrimental effects on the testis (Brinkworth and Handelsman, 2001). Various toxic substances damage or destroy spermatogonial stem cells and do effectively eliminate the highly sensitive differentiating spermatogonia (Fig. 1). The process of spermatogenesis is therefore disrupted at its initiation point. Some of these gonadotoxic substances are used for tumour therapies and – depending on the dose or intensity of exposure - are responsible for the gonadotoxic site effects leading to periods of temporary or sometimes permanent infertility in oncological patients. One out of 650 children develops cancer by the age of 15 and suffer from the gonadotoxic effect of the therapy (Bahadur and Ralph, 1999). Similarly, the accidental or therapeutic exposure of the testis to X-rays (>1.2 Gy) or other forms of high energy irradiation induces a loss of stem cells with the consequence of male infertility but less effect on androgen producing Leydig cells. Since these treatments target the testicular stem cell pool, testes at any stage of development are at risk. Other environmental influences are known to affect testis function. Heating of the testis or intake of toxic chemicals destroy germ cells and lead, dependent on the extent of exposure, to damage of the seminiferous epithelium and male sub- or infertility.

Since cure of the male infertile patient is no realistic goal, establishment of a reserve of gametes was and still is the only appropriate option which could be offered to adult patients undergoing a gonadotoxic therapy or having a strong risk of facing infertility due to other reasons (Kliesch et al., 2001). Although cryopreservation of semen has been extensively used, the success rates before the use of intracytoplsmic sperm injection (ICSI) were not promising but have improved afterwards (Kelleher et al., 2001). Alternative strategies for fertility preservation in male patients are germ cell transplantation, testicular grafting or protection of the testis by hormonal pretreatment. Table 1 presents an overview of four strategies. These present und future options for the preservation of testis tissue were recently reviewed (Nordhoff and Schlatt, 2003).

Germ cell transplantation

Successful germ cell transplantation in rodent models was first performed by Brinster and Zimmermann (1994) and Brinster and Avarbock (1994). Microinjection of germ cell suspensions into the seminiferous tubules at the surface of

the testis restimulated spermatogenesis from spermatogonial stem cells (Brinster and Zimmermann, 1994). Most of the transplanted germ cells degenerate and disappear from the testis during the first few weeks (Parreira *et al.*, 1998). The first meiotic germ cells arising from transplanted stem cells appeared after 1 month and increased permanently thereafter, indicating that the restoration of donor spermatogenesis is a slow and extended process. Xenotransfer of rat germ cells into mouse testis prompted rat germ cells to associate with mouse Sertoli cells and to induce qualitatively normal rat spermatogenesis in the mouse testis (Clouthier *et al.*, 1996). The xenotransplantation of spermatogonia from several other species has revealed that full and intact spermatogenesis can not be achieved when less related species like dog, boar, bull, stallion, monkey and human are used as germ cell donors (Dobrinski *et al.*, 1999; 2000; Reis *et al.*, 2000; Nagano *et al.*, 2001). Since repopulation and proliferation of stem cells is observed in most cases, the mechanisms of stem cell colonisation appear to be less species specific than the differentiation of germ cells and spermatogonial stem cells from a variety of species, including human (Nagano et al. 2002) are able to recolonize a testis as spermatogonial stem cells. Germ cell transplantation has become an assay to estimate the potential of germ cell development and the site of action in transgenic animals with disturbed fertility. The importance of c-kit expression of spermatogonia and stem cell factor expression of Sertoli cells was shown through transplantation experiments. Mahato *et al.,* (2000) transplanted estrogen receptor a (ERa) deficient germ cells into wild type testes, showing that the mutated germ cells induced qualitatively normal spermatogenesis. In contrast, after transplantation of germ cells from mice carrying the *jsd* mutation, no donor derived spermatogenesis can be established (Boettger-Tong *et al.,* 2000). The use of GFP-positive spermatogonia allowed the real time observation of transplanted cell clones and opens new pathways for the detailed study of stem cell colonisation and germ cell development (Ohta *et al.,* 2000).

Germ cell transplantation as a clinical tool

Male germ cell transplantation is an experimental approach with a strong clinical perspective (Schlatt *et al.*, 2000, Fig. 2). Our studies aimed to develop a clinically relevant approach for autologous germ cell transplantation in primates. The easiest, least invasive and most efficient transfer of germ cells into the seminiferous tubules was achieved via the rete testis. Ultrasonography was used to localize the rete testis as well as the injection needle. Autologous germ cell transplantation into the involuted testes of two cynomolgus monkeys was used to show that the principle of germ cell transplantation is applicable to primates. The detection of transplanted germ cells four weeks after autologous germ cell transfer revealed the success (Schlatt *et al.*, 1999). Our germ cell transfer technique was also applicable to surgically removed human testes. Studies using macaque monkeys as animal models and mimicking the gonadotoxic treatment of oncological patients by testicular irradiation show that germ cell retrieval and cryopreservation of testicular tissue before and transfer of germ cells after irradiation evokes an earlier and better recovery of spermatogenesis (Schlatt *et al.*, 2002).

Safety and efficiency of germ cell transplantation

One major limitation to the application of germ cell transplantation in oncological patients is the safety aspect. In lymphoma and leukaemia patients, the testis is a likely organ for the settlement of metastasising cells. Reintroducing malignant cells into a patient who was previously cured of the disease must be excluded. The high incidence of the transmission of the oncological disease was shown by intratesticular infusion of testicular cells from leukemic rats causing leukemia in the recipients (Jahnukainen *et al.*, 2001). Therefore, strategies for safe and efficient germ cell transfers have to be developed to minimise the risk and optimise the refertilisation capacity. An easy and efficient method for the enrichment of spermatogonia from testicular cell suspensions is immunomagnetic cell sorting which allowed us to achieve a considerable enrichment of spermatogonia from rat, hamster and marmoset monkey testes (Von Sch nfeldt *et al.*, 1999).

Extracorporal storage of the cells or tissues is an important part of the treatment. Cryopreservation of testicular cell suspensions seems to be the easiest and most favourable approach for the storage of germ cell transplants. Cryopreservation of germ cell preparations prior to germ cell transfer did not interfere with repopulation of the testis and reinitiation of spermatogenesis (Avarbock *et al.*, 1996). Various strategies for testicular cell preparations from murine and human testes have been performed and did not lead to major differences in cell survival when four commonly used cryoprotective agents were compared (Brook *et al.*, 2001). However, retrieval and storage of human testicular tissue evoke several ethical and legal issues (Bahadur *et al.*, 2000). It seems noteworthy to develop guidelines which standardise and control the procedures used in a clinical setting.

Testicular grafting

Grafting of tissue can be considered as an extreme form of tissue culture. Not only is the environment well controlled but often, also the blood supply to the grafted tissue is fully restored. Grafting might therefore present another tool for preservation of testis tissue. In females cryopreservation and grafting of ovarian tissue and the culture and *in vitro* maturation of follicles have successfully been developed in animal models and these techniques are tested in clinical research trials. We have recently shown that grafting of testicular tissue allows to differentiate immature testes up to the level of complete spermatogenesis (Honaramooz et al., 2002). Using immunodeficient mouse strains, newborn testicular tissue from various species developed into fully active spermatogenic tubules. Sperm from these grafts was used in combination with ICSI to produce healthy progeny (Schlatt et al., 2003). In the future, grafting might therefore become an important tool for initiation of testis function and generation of sperm from immature donors.

Conclusion

Since the testis contains stem cells, the preservation of male fertility can be subdivided into two aspects: 1) preservation of the male germ line and 2) preservation of fertility. In addition to the approaches discussed here, additional tools and various combinations of them can be applied to allow the preservation of male fertility. The testis carries male germ line stem cells throughout life. Therefore, the neonatal and prepubertal period of childhood should be an important period when options for male fertility preservation are discussed (Nordhoff and Schlatt, 2003). Fig. 3 highlights the co-existence of germ line preservation and fertility preservation as two independent but related aspects in the context of male infertility.

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Table 1: Comparison of four approaches used for fertility preservation in the male

	Cryopreservation of sperm	Testicular Grafting	Hormonal protection	Germ Cell transplantation
Patients	Adults	Children	Children and adults	Children and adults
Aim of treatment	Maintain a limited reserve of gametes	Generate a limited reserve of gametes	Cure of the patient	Cure of the patient
Achievement of pregnancy	Artificial, ICSI and IVF	Artificial, ICSI and IVF	Intercourse	Intercourse
Risks	**None, not invasive**	**Invasive retrieval of tissue, risk of malignant cells, retroviruses**	**Permanent damage of testes, time consuming**	**Invasive retrieval of stem cells; risk of mailgnant cells**
Status	Routine clinical practice	Experimental	Experimental	Experimental

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Fig. 1: Illustration of environmental or cytotoxic insults on spermatogenesis. The temporary or permanent loss of differentiating spermatogonia and the damage of male germ line stem cells leads to disruption of germ cell production and infertility.
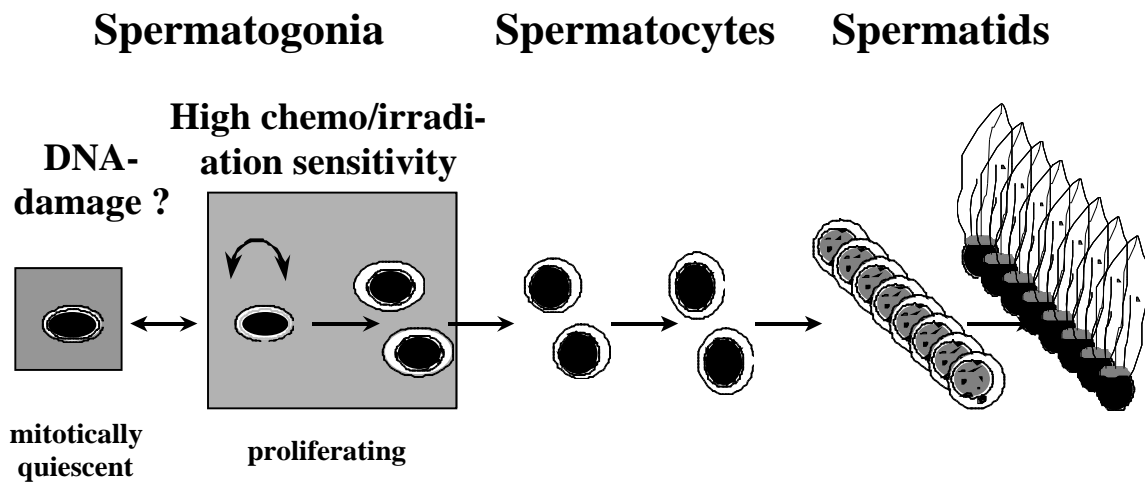
Spermatogonia   Spermatocytes   Spermatids

Fig. 2: Schematic presentation of the treatment regimen of germ cell transplantation for fertilty protection of oncological patients.
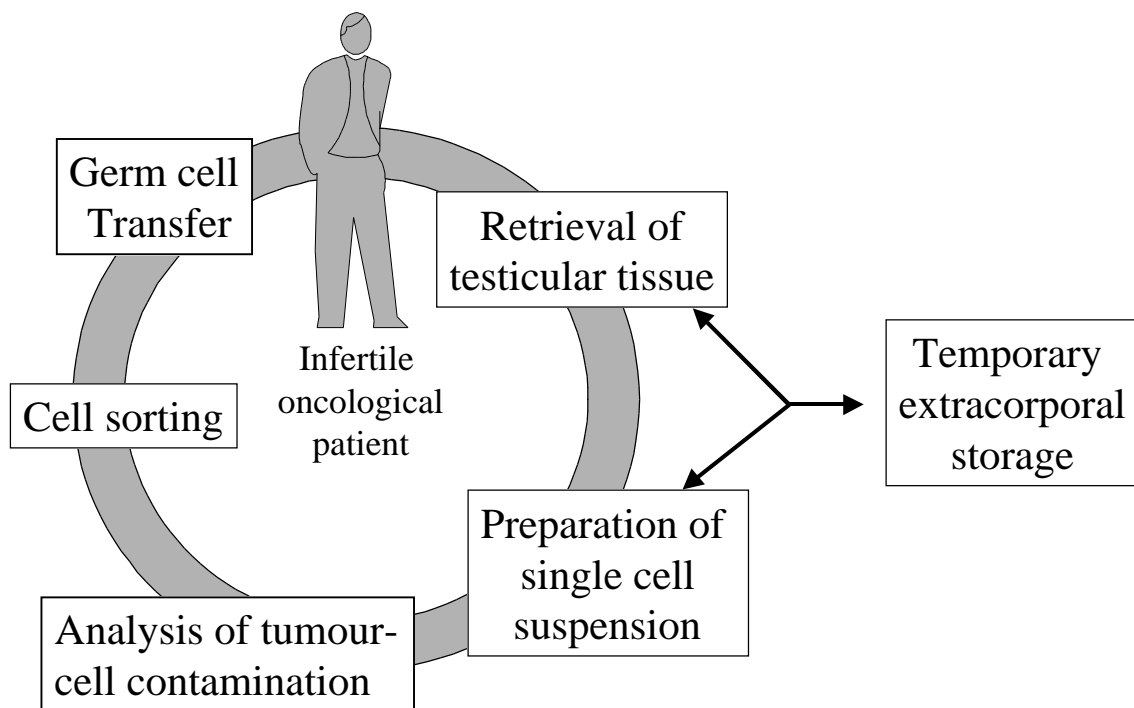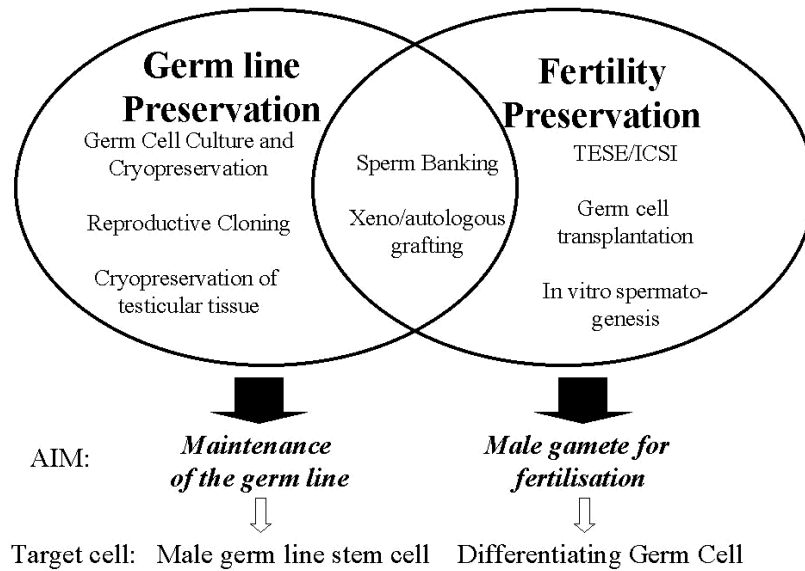
Fig. 3: Schematic illustration of various interventions allowing to preserve the male germ line and/or to produce gametes. The combination of various techniques might be applied to allow efficient fertility preservation in male patients.

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Translating testicular stem cell biology to the clinic

Herman Tournaye, MD PhD
Centre for Reproductive Medicine
University Hospital
Dutch-speaking Brussels Free University
Brussels
Belgium

Learning objectives

• Childhood cancer has become a curable disease, however, in many patients sterility is the price to be paid for their cure.
• In the next decade, prevention of sterility in childhood cancer survivors will become a major challenge in reproductive medicine.
• In theory, testicular stem cell banking is the only method by which to preserve the future fertility of boys undergoing a sterilizing chemotherapy.
• In animal models, testicular stem cell transplantation has proven efficacious, however, it remains to be proven that this technique is clinically efficient too, especially when frozen-thawed cells are to be transplanted.
• There is a need for improvements in both the cryopreservation and transplantation technique
• Prevention of recurrence of malignancy is an important prerequisite for any clinical application of testicular stem cell transplantation

Lecture summary

About 1 in every 600 children will develop cancer before the age of 15. In recent years, remarkable progress has been made in the treatment of cancer in infants and children and up to 75% of them can now be cured. Thanks to better treatment options, today, the cancer death rate in children has decreased more than that for any other age group. At present one in 1000 adults in the age group of 20-30 years old is a childhood cancer survivor (Hawkins and Stevens, 1996) and it has been estimated that by 2010 this proportion will raise to one in 250 (Bleyer 1990). From these figures it is evident that prevention of sterility in childhood cancer survivors will become a major challenge in reproductive medicine.

When an adult man undergoes a sterilising cytotoxic treatment, spermatozoa can be frozen in order to circumvent sterility after his treatment. However, no such prevention is possible before puberty since no active spermatogenesis is present. Attempts to prevent gonadal damage to the testicular germ line by GnRH agonist or antagonist administration have failed in the human (Krause and Pfluger, 1989)

A decade ago, it was demonstrated that spermatogenesis can be re-initiated after transplanting testicular stem cells (Brinster and Zimmerman, 1994): spermatogonia from pre-pubertal mice were transplanted into the seminiferous tubules of adult mice with a Sertoli-cell only syndrome induced by a cytotoxic treatment. Using a donor mouse with a transgenic marker (lacZ), Brinster and Zimmerman proved that the adult recipient mice produced spermatozoa derived from the donor mice. It was also shown that the recipient mice could reproduce in-vivo after transplantation and produce transgenic offspring (Brinster and Avarbock, 1994). Subsequently, these experiments were performed using stem cells that had been frozen and thawed (Avarbock et al., 1996).

Testicular stem cell banking and subsequent transplantation after thawing (autologous transplantation) could thus theoretically circumvent sterility induced by chemotherapy in prepubertal boys cured of cancer. To date, transplantation has only been successfully applied in animal models mainly for fundamental research purposes. Apart from the preservation of the fertility potential of prepubertal boys or even adult men, testicular stem cell transplantation has also been proposed as a means by which to treat male subfertility in adult men (Ogawa et al. 2000; Kanatsu-Shinohara et al. 2003).

However, any clinical application of this transplantation procedure requires not only a thorough ethical reflection but also a critical assessment of the feasibility of the transplantation technique and an assessment of its safety regarding both normality of meiosis after cryopreserving and transplanting stem cells and the risks of transplanting carcinogenous cells into an otherwise cured patient.

To date, restoration of fertility after transplanting testicular stem cells has only been demonstrated in mice (Brinster and Avarbock, 1994) and rats (Hamra et al. 2002; Zhang et al. 2003).

Some years ago, human autologous transplantations of frozen-thawed testicular cell suspensions have been performed in adults (Radford et al. 1999), but this application, proposed as an alternative to sperm banking, has not been reported to be successful so far. Most work has thus been performed in mice. From this work it is clear that testicular stem cell transplantation is efficacious, both after transplantation of fresh suspensions (Brinster and Avarbock, 1994) or frozen-thawed suspensions (Kanatsu-Shinohara et al. 2003). We evaluated the fertilising and developmental abilities of spermatozoa obtained after transplantation of fresh testicular stem cells in a mouse model (Goossens et al., 2003). After in-vivo conception we observed that 90% of the mice with a copulating plug became pregnant, however, after germ cell transplantation only 35% of the mice became pregnant. We therefore evaluated in-vitro fertilization (IVF) using either spermatozoa obtained after germ cell transplantation or spermatozoa obtained directly from the donor males. We found lower fertilization and blastocyst developmental rates in the transplanted group compared to controls. After intracytoplasmic sperm injection (ICSI), fertilization and blastocyst developmental rates were comparable to control spermatozoa. This observed decrease in efficiency of donor-derived fertilizing capacity certainly needs further study. Any clinical application will need an assessment of efficiency at a certain point, certainly when testicular germ cell banking will be proposed as an alternative to a long-established successful strategy such as sperm banking in men with active spermatogenesis. Blind extrapolation of findings in the mouse to the human situation is impossible, e.g. because of differences in the stem cell population, differences in testicular anatomy or the risks involved.

In mouse models, several dissected donor testes are pooled, then minced and enzymatically dissociated in order to prepare a testicular cell suspension containing stem cells. However, in a clinical setting only a small testicular biopsy can be obtained. Because further scarring and fibrosis after chemotherapy of the remaining part of the testis may be anticipated, it may be acceptable to perform a unilateral orchidectomy. But even then, at the utmost one small prepubertal testis can be removed. It has been estimated that in the mouse only 0.03% of testicular cells are stem cells (Tegelenbosch and De Rooij, 1993). However, this proportion is assumed to be higher in the human. Here only one generation of spermatogonia following the A-pale/dark spermatogonia is described and the developing clones of germ cells are limited which may imply that the density of spermatonial stem cells in the human seminiferous epithelium may well be higher than in the mouse. Enrichment of stem cells may nevertheless be needed. In mice spermatogonial enrichment can be obtained by surgically inducing cryptorchidism or by inducing vitamin-A deficiency (McLean et al. 2002), but these approaches are not practicable in a clinical setting. Enrichment by magnetic cell sorting or fluorescent-activated cell sorting (FACS) has been shown to enrich the proportion of testicular stem cells by 7 and 166 times respectively in the mouse (Shinohara et al. 2000), but it has still to be proven which surface markers are expressed on human testicular stem cells in order to obtain enriched cell populations.

An optimal cryopreservation protocol is another prerequisite for clinical application. In the mouse, testicular stem cells seem to survive simple freezing procedures much better than mature spermatozoa (Nakagata 2000; Avarbock et al. 1996). Yet few data exist on the efficiency of cryopreservation of testicular stem cells. A study by Brook et al. (2001) compared different cryopreservation protocols but failed to show any effect of the cryoprotectant used on the survival of human testicular cells after cryopreservation. However, they showed that the cooling rate did affect survival when freezing was faster than -0.4¡C/min. Unfortunately, they did not transplanted the frozen-thawed cells in order to assess survival of the stem cells. A paper by Hovatta (2001) reports on six different freezing and thawing protocols for both mouse and human testicular cell suspensions. Here, a slow-programmed protocol using propanediol and sucrose as cryoprotective agents yielded the best cell survival. Unfortunately, again no functional assessment by transplantation was performed. In the bovine, non-controlled freezing using DMSO with sucrose provided the best cell survival of isolated bovine type A spermatogonia (Izadyar et al. 2002). The frozen-thawed bovine spermatogonia retained their ability to proliferate in-vitro and to survive for up to 3 months after xenotransplantation into a mouse testis.

In a stepwise comparative study, we evaluated different cryopreservation protocols for cell survival (Frederickx et al. 2004). In addition we applied testicular cell transplantation as a functional assay for the optimised protocol, i.e. a controlled freezing protocol using ethylene glycol, and compared donor-specific spermatogenesis after transplanting

both fresh and frozen-thawed germ cell preparations. The proportion of mice showing restoration of spermatogenesis was lower compared with fresh controls which may indicate that the functional capacity of the stem cells may be compromised by cryopreservation despite good survival of the testicular cells. In contrast, another recent study in the mouse reported that frozen-thawed testicular stem cell suspensions demonstrated a higher stem cell activity after transplantation than fresh controls when a simple non-controlled DMSO-cryopreservation protocol was used (Kanatsu-Shinohara et al. 2003). It is clear that further study for optimizing cryopreservation protocols for human cell suspensions will be needed.

The transfer technique itself has been subject to further study (Schlatt et al. 1999; Brooke et al. 2001). It has been shown that because of the anatomical differences between man and mouse, infusion under gravity pressure via multiple punctures into the rete testis may provide the best results in terms of filling the seminiferous tubules with a stem cell suspension. In order to reduce the backpressure from intratubular fluid secreted by the Sertoli cells, priming recipients with GnRH-antagonists before infusion may be necessary as shown in non-human primate models (Schlatt et al. 1999).

Another important issue is the timing of the re-introduction of the testicular stem cells in case of autologeous transplantation. In mice it has been shown that transplantation of frozen-thawed stem cell suspensions to prepubertal recipients was more successful than to adult recipients especially after busulphan treatment (Kanatsu-Shinohara et al. 2003). Although it is not clear whether this difference was due to a deficient stem cell repopulation of the seminiferous tubules in the adult recipients, in a clinical setting too it may be preferable to transplant testicular stem cells to a testis of cured prepubertal patients than to a postpubertal testis. Again more research is needed in this field.

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer, representing about one third of all cancers in children under 15 year olds. Testicular leukemic infiltration can be expected and if remaining undiagnosed when treatment ends it may adversely affect treatment outcome (Heaney et al. 1983).

In a rat model, it has been shown that testicular cell suspensions from T-cell leukemic donor rats can induce testicular leukemia in recipients undergoing testicular transplantation of fresh or frozen-thawed donor samples (Jahnukainen et al., 2001). In theory, however, testicular stem cell transplantation can introduce a risk for cancer recurrence in every metastatic childhood cancer because of contamination of the cell suspensions with carcinogenous cells contained in the testicular blood vessels.

In order to circumvent the problem of malignous contamination, cell sorting may be a strategy. At present, FACS has been shown to enrich testicular stem cells, but it has still to be shown that malignant cells can be negatively sorted from the cell suspensions preventing any contamination by malignant cells.

Developing human testicular stem cells to maturity in surrogate animals could be another strategy. So far, preliminary experiments on xenogeneic transplantation have not been invariably successful. Transplantation of the stem cells from hamsters, rabbits and dogs has failed to initiate spermatogenesis in recipient mice, probably because the phylogenetic gap between these species is too wide (Dobrinski et al., 1999). This may explain why xenologeous transplantation from human to mouse did not work (Reis et al., 2000). However, the phylogenetic gap between great apes and humans is smaller than that between rats and mice, a model in which xenotransplantation worked!

Xenografting may be an alternative approach in order to circumvent malignous contamination. In a recent report non-human testicular xenografts were shown to support spermatogenesis in immunodeficient mice (Hoaramooz et al. 2002). Although this approach may raise ethical concerns, it may provide one day an efficacious means by which to prevent malignancies when attempting reproduction in certain childhood cancer survivors.

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Ethics of Testicular Stem Cell Medicine

G. Bahadur
Department of Obstetrics & Gynaecology
Fertility & Reproductive Medicine Laboratories
Royal Free & University College Medical School
London
United Kingdom

Abstract

The paper examines the ethical issues raised by advances in reproductive technology which allow for the transplantation of testicular stem cells so as to enable infertile men, and cancer patients, including the pre-pubertal, to have children. These ethical dilemmas involve considerations of respect for the patient's autonomy, the need for informed consent, and the health of any offspring resulting from such a procedure.

The paper considers the problems raised by cases when it is necessary to obtain consent for the transplantation of testicular stem cells from pre-pubertal and adolescent patients. It discusses the legal status of stem cells, which is not fully defined under existing regulations. While there are no property rights in tissue under the current legal framework, the paper argues that to treat such tissue as property might serve as a means of guaranteeing respect for patients' rights in disputed cases.

The paper also discusses whether such tissue might be covered by the regulations governing patents, and the ethics of allowing commercial traffic in such material. Finally, the paper raises questions as to the health and safety of using testicular tissue for medical purposes, as well as rejecting xenotranplantation technology in humans.

Keywords

Testicular stem cells; assisted reproduction; cancer patients; ethics; patents

Introduction

Advances in reproductive technology have created new possibilities for those patients who would not normally be able to have children (Averbock et al., 1996; Brinster et al., 1994; Brook et al., 2001). The ultimate goal of testicular stem cell technology is to create healthy offspring. Typically the kind of patients who may make use of the technology are infertile men who are normally thought to be azoospermic and men with cancers who are unable to ejaculate. Recently, the technique has been used to preserve gonadal tissue for pre-pubertal children.

Xenotransplantation and maturation could potentially cause genetic mismatches and contamination. It is important to understand the social, legal and ethical ramifications of the procedure. These centre on which uses of testicular tissue are ethical, what are the appropriate safeguards for people from whom testicular tissue is removed, and whether it is ethical to buy or sell human testicular tissue as with other tissue (Moore v Regents, 1990). Is it ethical to patent inventions involving testicular tissue, and how can the safety of testicular tissue used for medical purposes be ensured? The ethical issues relate directly to the core notion of respect for human beings; namely, that they should not be injured and nothing should be done to them without their consent.

The legal status of tissue is unclear; an embryo or foetus has no legal status. Work on sperm and stem cells can be less problematic than that on other tissues, but this should not in any way diminish the autonomy of individual patients or the need for informed consent. Children and adolescents present a new challenge to our understanding of informed consent; in the case of pre-pubertal subjects, parental or proxy consent may be inevitable.

Testicular stem cells, whilst having a lower status than embryos, still represent half of the unique coding of potential human beings. Maturation of such germ cells in inappropriate conditions may alter methylation patterns. As such, the

indiscriminate use of ICSI raises profound doubts about the long term health of offspring produced by the procedure. There is more potential for germ cells to be altered by different maturation processes, despite stem cells themselves being more resistant than mature sperm cells to cytotoxic damage. The genetic integrity of testicular stem cells has not been fully tested. The use of transplanted sperm in the creation of a human being could potentially have adverse consequences for the existing population.

In order to offer stem cell therapy options to cancer patients, we need to know if it is better to store purified germ cells and inject directly into the testes, or to keep the germ cells in their tissue matrix and perform ortotopic transplantation (Bahadur and Ralph, 1999). Would the former technique make the patient more prone to testicular cancer in future years? By whichever technique, would the removal of the surrounding tissue make the germ cells more prone to non-specific methylation? The public requires further convincing of the need for autotransplantation if the amount of information to be acquired does not clearly outweigh safety considerations.

The overall resistance to toxic treatment of testicular stem cells and their good freezing potential offer sufficient hope in specific cases such as a Sertoli cell-only syndrome, men who are naturally azoospermic, and perhaps in some cases of children and adults about to receive TBI treatment. However, the question of utilising such technology for men who have already had cancer treatment must remain contentious. The attempt to prove the safety of using immature testicular stem cells is complicated.

Ethical issues.

There are clearly unacceptable and unethical uses of human tissue or its extracts. Perhaps more difficult to evaluate is whether human tissue can be bought and sold, and whether the donor has any rights to or say over the tissue. Philosophers find these issues difficult to resolve and discuss them in the context of rights and utilitarianism, or more broadly consequentialism.

The main ethical issues involve the need for an avoidance of injury, to be balanced against considerations of benefits, safety and consent. The amount of tissue to be extracted, mode in which it is to be removed and the issue of informed consent all need to be taken into account. It would be unacceptable in the case of clinics which wish to offer services such as stem cell cryopreservation on a commercial basis. It is unclear at present whether the transplantation of stem cells could be done safely without long-term harm. It will be necessary to ensure that the abnormal repopulating mechanism will not cause long-term damage to patients.

If a boy protested at having tissue taken from him, despite parental consent, then there could be scope for the charge of having caused bodily harm. This needs to be avoided by engaging the child in the decision-making process.

Legal status of stem cells

To date courts in England and Wales have not addressed the issue of the legal status of human reproductive material outside the human body. Although we know there is no property status in corpses, a corpse may however still be accorded property status if it has undergone a process or the application of human skill. Gametes and embryos have an undefined status where questions such as accidental damage, modification or destruction arise.

That an embryo is not a legal person has been reiterated in several cases and reinforced by the Warnock Committee in the report of their enquiry into fertilisation and embryology. If not a person, the embryo risks being classified a chattel. The foetus, likewise, is not accorded the status of a person. The management and trade of sperm, especially across borders, makes the treatment of it as a chattel more of a likelihood. Public feeling is likely to be against the commercialisation of entities with the potential to become human beings. It is a matter of time, however, before the property status of sperm, egg and embryos becomes subject to court application or clarification.

Pre-pubertal patients and adlescents with cancer

The Legal Framework Obtaining consent in pre-pubertal and early adolescent patients remains a contentious topic (Bahadur et al., 2001), beset by confusing legal precepts and advice (Bahadur and Hindmarsh, 2000). Normally consent models refer to treatment, but the freezing of sperm is preventative and normally requires no surgical intervention. In the USA the concept of 'assent' has been developed. Early adolescents are considered 'to assent' (or,

its converse, 'dissent') when they have sufficient competence to have some appreciation of a procedure, but not enough to give fully informed consent. The age of assent is currently estimated as being twelve (Anonymous, 1977; Sigman and O'Connor, 1991; Committee on Bioethics, 1995).

If a patient is unable to produce semen by masturbation, the possibility of preserving testicular tissue arises, and two issues are at stake. Where the patient is pre-pubertal, and therefore the testicular tissue does not contain 'gametes' as defined by the HFEA (haploid cells only), the legal, practical and ethical considerations are covered by the Children's Act 1989 and the Tissues Act 1961. Under these circumstances parental consent is essential (Bahadur et al., 2000). Secondly, where in the opinion of the medical practitioner, 'gametes' are present and the patient has reached Tanner Grade 2 maturity, then under the provisions of the UK HFEAct, consent must come from the patient.

A 'family rule' model (Foreman, 1999) of consent for early adolescents has been developed. Children between six and twelve can understand, for example, the idea of psychiatric hospitalisation. This fits the model age at which UK practitioners think children can make decisions about surgery, and the age of assent. On the other hand, early adolescents lack the social independence needed to make a fully autonomous decision (Bahadur et al., 2001).

Therefore, whilst not concerning medical intervention per se, testicular stem-cell taking, cryopreservation and transplantation in pre-pubertal cancer patients require delicate, sensitive handling. We also have a duty of care to the patients' relatives and guardians, who should ultimately respect the confidentiality accorded by statute to the patient if he so chooses. If the patient refuses at any stage then this must be respected.

Rights

In most cases, when we say that someone has a right to do something, we imply that it would be wrong to interfere with his doing it, or at least that some special grounds are needed for justifying any interference (Bahadur, 2001b; Dworkin 1991; Scott, 1998). Rights can also be described as claim rights (positive rights) or liberty rights (negative rights). The HRAct 1998, article 12, incorporates the right to form a family, thereby acknowledging the unacceptability of obstructing someone in the exercise of that right, rather than demanding positive action.

English law is silent on the issue of whether a person can claim property rights in tissue which has been removed. The Human Fertilisation and Embryology Act 1990 adopted a scheme requiring consent so as to avoid addressing the issue of property and ownership. Some support for the property approach can be derived from the various statutes in existence. The HFEAct 1990 considers that the control and disposal of gametes and embryos rests with the donors. It further allows for the transfer of reproductive material between those having a licence to deal with it.

The statutory provision, S25, of the National Health Service Act 1977 also implicitly adopts a property approach. The section provides that '*where the Secretary of State has acquired: (a) supplies of bloodÉor (b) any parts of human bodyÉ, he may arrange to make such supplies or that part available (on such terms, including terms, as charges, as he thinks fit) to any personÉ*'. A logical extension to according rights is to reconsider the property status of tissue.

Property and commerce

The codification of the property concept would better protect individual autonomy, as well as clarifying legal rights and duties regarding the control of human tissue. For example, if a clinic were to destroy tissue without consent, common law property principles concerning the destruction or spoilage of materials rightfully in one's possession might prove helpful in defining legal rights and duties. Some courts and jurisdictions refer to the right of possession as a 'quasi-property' right. It empowers spouses or next of kin who are wronged by interference to sue for damages.

A hospital which has tissue in its possession, for example for transplant purposes, has such property rights over the tissue as to exclude another's claim to it. That the user acquires property rights over removed tissue does not, of course, mean that the user can do whatever he likes with the tissue.

Perhaps the limitation of greatest concern has to do with the commercial dealing in tissue. The HFEAct 1990 provides that '*no money or benefit may be given or received in respect of any supply of gametes or embryos unless authorised by directions*'. The Recommendation on Human Tissue Banking of the Council of Europe's Directing Committee on public health (Recommendation No R(94)1) specifically recommends that activities associated with human tissue '*should be carried out by non-profit making institutions*'.

Patents

Additional problems may be encountered with intellectual property rights. The requirements for patentability are as follows: novelty, inventiveness, industrial applicability and sufficiency of description. Excluded are mere discoveries, immoral inventions, biological, animal and plant varieties.

The European patent Office (EPO) has granted patents relating to inventions covering the use of processes from human tissue. A highly purified sample would meet the novelty requirement, as would for example a glycosylated derivative of the natural form. It is envisaged that patent applications for testicular stem cell technology will follow, especially where gene therapy may be involved.

Health and safety

The Medical Devices Agency was set up so as to ensure the safety of tissue storage and use. Gonadal tissue and germ cells are likely to be used for orthotopic transplantation. There is a need to clear patients for Hep B, Hep C and HIV, in order mixed storage of samples does not cause cross-contamination. Nitrogen vapour-only freezing does not give added reassurance as vapours circulate carrying any airborne pathogens. The use of automated alarms and fillers, too, have proven to offer false reassurances, and banks have gone dry due to the malfunction of complicated structures. It is important that banks are physically maintained and checked.

The Health and Safety of Work Act 1974 imposes important duties on employers. Records and auditing of samples along with good laboratory practice and accreditation collectively contribute towards staff and patient welfare.

Conclusion

The ethical issues are complex and some of these will be covered in the presentation. Testicular stem cell technology and transplantation represent a new dimension to reproductive medicine for male infertility and cancer patients, particularly for pre-pubertal boys. Safety concerns are foremost, and in the case of patients who have previously undergone cancer treatment, one must err on the side of caution; germ cell mutation, with its effects on future generations, cannot be ruled out. Xenotransplantation and maturation in humans is clearly unacceptable and this could potentially cause genetic mismatches and contamination.

Issues of consent, rights, commerce, property status, patents, health and safety all raise ethical questions which need to be taken into consideration in instances of testicular stem cell transplantation. These ethical dilemmas involve considerations of respect for the patient's autonomy, the need for informed consent, and the health of any offspring resulting from such a procedure. The issue of consent is particularly complicated in the case of pre-pubertal cancer patients, for whom the technology may prove useful.

While there are no property rights in tissue under the current legislation, to treat such tissue as property might serve as a means of guaranteeing respect for patients' rights. It is likely that patent applications for testicular stem cell technology will follow. The field is in the research phase and it would be unethical to make commercial gain from those seeking help from this technology.

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