Options for fertility preservation in prepubertal boys

Human Reproduction Update, Vol.16, No.3 pp. 312–328, 2010

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Introduction

- Due to remarkable advances in the treatment of childhood cancer → great improvements in life expectancy (up to 80% of children surviving their disease) → resulting in a growing population of adult long-term survivors of childhood malignancies
- by the year 2010, 1 in 250 young adults aged 20–29 years will be a cancer survivor (Bleyer, 1990; Blatt, 1999).
- a major concern is their adverse impact on fertility
- Currently available drugs to prevent testicular damage from cytotoxic therapy have not proved helpful in humans so far.

- Improved therapeutic regimens using less gonadotoxic protocols could enable spontaneous recovery of spermatogenesis.
- Since rapidly dividing cells are the target of chemo- and radiotherapy → act not only on cancer cells, but also on germ cells.
- there is evidence that cytotoxic treatment given to prepubertal boys affects fertility *(Rivkees and Crawford, 1988; Mackie et al., 1996; Kenney et al., 2001).*
- Recovery of sperm production after a cytotoxic insult depends on the survival and ability of mitotically quiescent stem spermatogonia (type A dark) to transform into actively dividing stem and differentiating spermatogonia (type A pale)

Gonadotoxicity after chemo- and/or radiotherapy

- Although little is known about the effects of gonadotoxic treatments on the immature testis (fertility cannot be assessed before puberty), cytotoxic damage has been extensively studied after puberty.
- The extent of damage is dependent on the *agent administered, the dose delivered, the combination of cytotoxic drugs and the potential synergic interaction of radiotherapy*
- Literature on semen quality in adult cancer patients before and after therapy was recently reviewed

Table I Long-term fertility prognosis following treatment with different agents

Good	Moderate	Poor
Azathioprine	Thiotepa	Cyclophosphamide (>7.5 g/m^2) (Meistrich et al., 1992)
Fludarabine	Gemcitabine	Ifosfamide (>60 g/m^2) (Williams et al., 2008)
Methotrexate	Cisplatin	Mustine, carmustine
6-mercaptopurine	Oxaliplatin	Busulfan
	Carboplatin	Chlorambucil (>1.4 g/m^2)
Vincristine	Doxorubicin	Melphalan (140 mg/m^2)
Vinblastine	Dacarbazine	Chlormethine
Bleomycin	Cytosine-arabinoside (cytarabine)	Procarbazine (>4 g/m^2) (Bokemeyer et al., 1994)
Actinomycin-D	Daunorubicin	Cisplatin (>600 mg/m^2) (Petersen et al., 1994; Pont and Albrecht, 1997)
Etoposide	Mitoxantrone	Mechlorethamine

Adapted from Meirow and Schenker, 1995; Howell and Shalet, 2001.

irradiation and cyclophosphamide administration

Loss of fertility: who can benefit from fertility

Table II Indications for immature testicular cryopreservation in case of malignant and non-malignant disease

Malignant	Non-Malignant
• Leukemia • Hodgkin's disease • Non-Hodgkin's lymphoma • Myelodysplastic syndromes • Solid tumors • Soft tissue sarcoma	(1) HSCT in case of: • hematological disorders: thalassemia major, sickle cell disease, aplastic anemia, Fanconi anemia • primary immunodeficiencies • severe autoimmune diseases unresponsive to immunosuppressive therapy: juvenile idiopathic arthritis, juvenile systemic lupus erythematosus, systemic sclerosis, immune cytopenias • osteopetrosis • enzyme deficiency disease: Hurler's syndrome (2) Risk of testicular degeneration • Klinefelter syndrome

HSCT, hematopoietic stem cell transplantation.

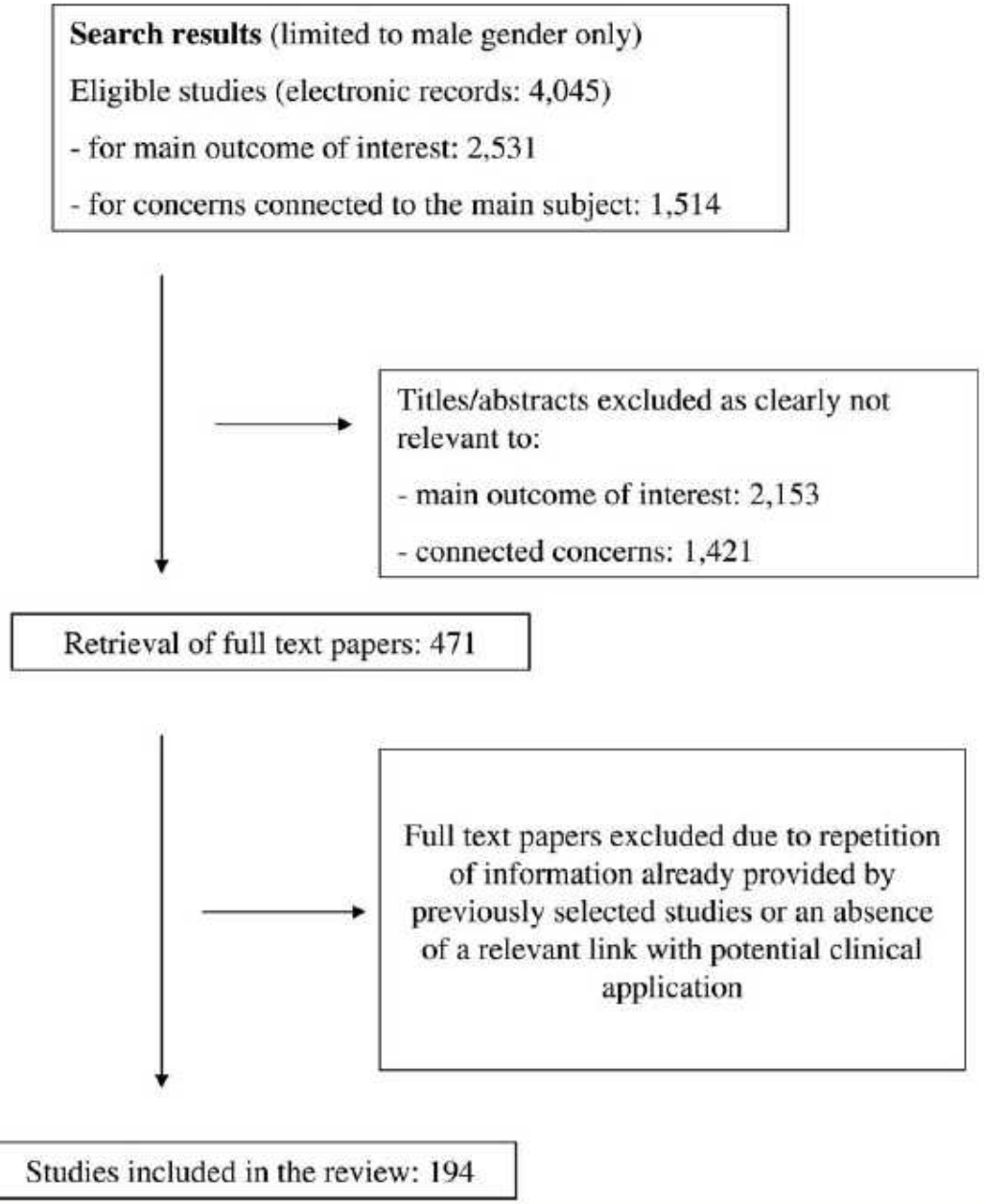

Figure 1 Flow diagram of search and inclusion criteria for studies in the review.

Results
Fertility preservation options before gonadotoxic therapies

- Two different approaches may be considered:
  - (i) minimizing the testicular damage from cancer treatment or protecting SSCs (spermatogonial stem cells) in vivo;
  - (ii) cryopreserving testicular tissue prior to gonadotoxic treatment in the form of either cell suspension, tissue fragments or whole organ.

(In vivo SSC protection)

- Limiting radiation exposure by shielding or removing the testes from the radiation field should be implemented whenever possible.
- Gonadal protection through hormonal suppression is based on the principle that disruption of gametogenesis renders the gonads less sensitive to the effects of cytotoxic drugs or irradiation.
- Promising results were obtained in rodents but not in non-human primates or humans except in one clinical trial *(Masala et al., 1997)* where only moderate stem cell death was induced by chemotherapy.

- By contrast, stimulating spermatogonial proliferation by follicle-stimulating hormone (FSH) might be an option as shown in monkeys by Van Alphen et al. (1989)
- Anti-apoptotic agents such as sphingosine-1-phosphate *(Suomalainen et al., 2003; Otala et al., 2004)* and AS101 *(Carmely et al., 2009)* and various other cytoprotective substances *(Lirdi et al., 2008; Okada et al., 2009)* have also been used with partial success in rodents.
- In conclusion, no effective gonadoprotective drugs are so far available for use in humans.

(Immature gamete cryopreservation)

- Since prepubertal boys cannot benefit from sperm banking, a potential alternative strategy for preserving their fertility involves storage of testicular tissue → future technologies will allow its safe utilization →this strategy is still experimental.
- As prepubertal testicular tissue contains SSCs →these cells can either be cryopreserved as a cell suspension or in the form of tissue

- **Cell suspensions:**
  - In various animal models, post-thaw viability of 29–82% was reported *(Geens et al., 2008).*
  - For human testicular cell suspensions, post-thaw viability of up to 60% was achieved, regardless of cryoprotective agent *(Brook et al., 2001; Hovatta et al., 2001).*

- **Tissue pieces.**
  - considered as an alternative method capable of maintaining cell-to-cell contacts between Sertoli and germinal stem cells → preserving the stem cell niche necessary for their survival and subsequent maturation
  - preservation of the Sertoli cells and Leydig cells (useful to alleviate the hormonal imbalance caused by cytotoxic therapy).

- Because of the complexity of the tissue architecture, cryopreservation protocols must strike a balance between optimal conditions for each cellular type *(water content, size and shape of cells, and the water permeability coefficient of their cytoplasmic membrane)*
- Post-thaw survival and seminiferous tubule structure are profoundly affected by both the type of cryoprotectant and freezing rates *(Milazzo et al., 2008),* so optimization of freeze-thawing protocols is mandatory.
- Furthermore, slow-programmed freezing better protects spermatogonial morphology

- Two teams have reported freezing protocols for prepubertal human testicular tissue, both yielding good structural integrity
- Using different cooling and freezing rates, Keros et al. (2007) observed a difference mainly in terms of survival of spermatogonia, with 94% of intact spermatogonia found after freeze-thawing and culture with their best protocol.

- **Whole testis.**
  - Due to the small number of SSCs contained in a testicular biopsy and the small size of a child's testis, it is possible that cryopreservation of a whole testis may be more appropriate
  - Cryopreservation methods for whole testes need to be developed

Fertility restoration after immature tissue cryopreservation

- animal studies: frozen diploid precursor cells may provide some hope of fertility restoration in prepubertal boys in the absence of haploid gametes.
- Three approaches may be considered:
  - transplantation of purified cell suspensions back to their own testes
  - autografting of testicular pieces or whole testes
  - IVM up to a stage at which they are competent for normal fertilization through intracytoplasmic sperm injection (ICSI).
- None of these approaches have proved efficient and safe in humans as yet.

- **Testicular germ cell transplantation**
  - spermatogenesis is reinitiated after transplantation
  - SSCs are recognized by Sertoli cells and relocate from the lumen onto the basement membrane of seminiferous tubules.
  - Stem cells: unlimited potential to self-renew and produce differentiating daughter cells → SSC transplantation offers the possibility of long-term restoration of natural fertility.
  - Not yet proved successful in humans (see Progress towards human clinical application).

- **Lessons learned from transplantation of fresh testicular stem cells in animals**
  - Autologous SSC transplantation has been reported in mice, rats, pigs, goats, cattle, monkeys and dogs.
  - Restoration of fertility from donor stem cells has only been achieved in mice, rats, goats and chickens
  - Heterologous transplantation does not appear to be as successful as autologous transplantation
  - Rat gonocytes produced mature spermatozoa after xenogeneic transplantation to the testes of mice but qualitative and quantitative abnormalities of sperm were observed

- Recipient age : an impact on colonization efficiency, since more and larger spermatogenic colonies were generated in preadolescent recipient mouse testes than in adult testes *(Shinohara et al., 2001).*
- Better niche accessibility and niche proliferation due to Sertoli cell multiplication, elements facilitating colony formation and an increase in seminiferous length during testicular enlargement may be involved.
- This should be taken into account to ensure optimal transplantation time in clinical practice.

- Techniques for SSC enrichment:
- Because of the small number of SSCs in a testis (2/10000 germ cells), the small size of testicular biopsies recovered for fertility preservation, and the low efficiency of recolonization after transplantation → increased the number of SSCs prior to transplantation is essential.
- Ideally, isolation of pure stem cells would be the most effective method to increase the number of SSCs in a suspension and therefore transplantation efficiency.
- Adequate purification will probably be best achieved by cell-sorting techniques, such as magnetic-activated cell-sorting (MACS) or fluorescence-activated cell sorting (FACS) based on cell characteristics and membrane antigens.

- Techniques for SSC expansion.
- Better results were achieved using culture on feeder layers with a combination of growth factors, or applying serial transplantation procedures (Kanatsu-Shinohara et al., 2003a; Ogawa et al., 2003).
- So far, strategies for in vitro expansion of SSCs have only proved successful in rodents

- **Lessons learned from transplantation of frozen testicular stem cells in animals.**
  - high survival rates do not guarantee preservation of the functionality of frozen-thawed cells → important to evaluate their capacity to self-renew and differentiate through transplantation of cell suspensions.

(Testicular tissue grafting)

- Testicular tissue grafting involves transplantation of SSCs with their intact niches and thus within their original microenvironment.
- not yet been reported in humans → available data will be reviewed on the basis of observations made in animals.

- **Lessons learned from transplantation of fresh testicular tissue in animals.**
  - Grafting of testicular tissue from several mammalian species into immunodeficient mouse hosts has resulted in varying degrees of donor-derived spermatogenesis.
  - Complete spermatogenesis following testicular grafting has been reported
  - By contrast, germ cell differentiation blockage was observed in marmosets
  - The mechanisms underlying these species-specific differences in spermatogenic differentiation remain unknown, but some hypotheses can be proposed

- 1st: differences between host and donor gonadotropic hormones → inefficient interaction between murine gonadotrophins and grafted donor testicular tissue. *(Supplementation with exogenous gonadotrophins could therefore be useful)*
- xenografts of ITT from rhesus monkeys to mice treated with exogenous gonadotrophins : showed some degree of sperm differentiation compared with blockage at the spermatogonial level observed in untreated mice (Rathi et al., 2008).

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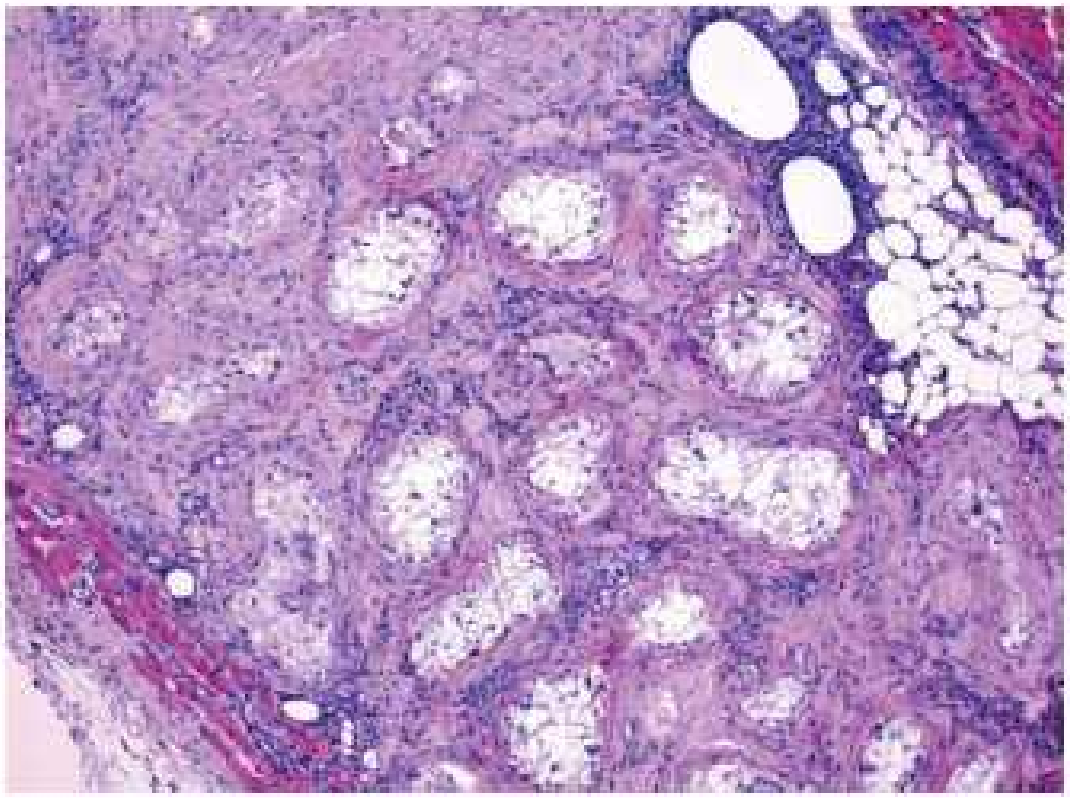

Figure 2 Histological appearance (hematoxylin–eosin sections) of donor testicular tissue from a 44-year-old man after 3 weeks' orthotopic xenografting at x200 magnification. Most tubules show degenerative changes, i.e. sclerosis, while the remaining contain mainly Sertoli cells.

- The reasons for the poor outcome of adult testicular tissue xenografts are so far unknown.
- studies in rodents : adult tissue could be more sensitive to ischemia than immature tissue
- This hypothesis was supported by studies in bovines, showing higher expression of some angiogenic factors in grafts from younger donors (Schmidt et al., 2007).
  - pretreatment of testicular tissue with vascular endothelial growth factor (a potent angiogenic factor) was found to increase the number of tubules containing elongating spermatids (Schmidt et al., 2006b).

- **Lessons learned from transplantation of frozen testicular tissue in animals.**
  - In rodents, cryopreservation of ITT led to the birth of healthy offspring *(Shinohara et al., 2002).*
  - A number of studies in animals designed to evaluate the effect of freezing on the functional capacity of germ cells have shown that freezing does not appear to affect the functional capacity of frozen germ cells on a qualitative basis
  - Loss of SSCs after cryopreservation was nevertheless suggested, since Ohta and Wakayama (2005) reported lower colonization efficiency after grafting frozen-thawed testicular pieces

- **Lessons learned from xenotransplantation of fresh human testicular tissue.**
  - Very few studies have been published on xenotransplantation of human testicular tissue *(Geens et al., 2006; Schlatt et al., 2006; Yu et al., 2006).*
  - Grafting of human ITT, either from fetuses *(Yu et al., 2006)* or prepubertal boys *(Goossens et al., 2008b),* did not result in complete spermatogenesis, although graft and germ cell survival were shown to be more favorable than in mature tissue grafts.

- **Lessons learned from xenotransplantation of frozen human testicular tissue.**
  - No studies have reported xenografting of cryopreserved adult testicular tissue in humans and only two have been published on cryopreserved ITT xenotransplantation in humans *(Wyns et al., 2007;2008).*

(IVM of germ cells)

- In vitro maturation (IVM) of germ stem cells, leading to in vitro-derived male haploid gametes available for ICSI, circumvents the risk of reintroducing the malignant cells, making this procedure potentially highly beneficial in cancer patients.
- Efforts have focused on establishing optimal in vitro culture systems to allow male germ cells to complete meiosis and spermatid elongation in experimental conditions.
- A number of studies have investigated culture systems suitable for in vitro spermatogenesis in humans *(Cremades et al., 2001; Sousa et al., 2002; Tanaka et al., 2003; Lee et al., 2007).*

- Sertoli cells : a key role in the regulation of growth/proliferation of spermatogonial cells and early stages of spermatogenesis in in vitro culture BUT not seem to be the case for later stages.
- Future research should focus on the identification of specific factors and signaling pathways that are present only in the testis, supplying an ideal microenvironment for full spermatogenic maturation.

(Safety issues)

Table III Studies on isolation of germ cells with detection of cancer cell contamination

Reference	Species	Cell-sorting technique	Markers	Evaluation after cell sorting	Outcome (% of residual contamination/number of contaminated samples or mice)
Fujita *et al.*, 2005	Mouse	FACS	H-2Kb/H2Db$^-$ (MCH cl I) CD45$^-$	Cell transplantation Histology: testis, bone marrow, peritoneal exudate of recipient mice	No contamination of recipient mice
Fujita *et al.*, 2006	Human	FACS	MCH cl I $^-$CD45$^-$	RT–PCR for germ cell markers (DAZL, HIWI, VASA, NANOG, STELLAR, OCT4)	1.45% K562 cells (CML), 0% K562 cells after IFγ (for induction of MCH cl I)
Geens *et al.*, 2007	Mouse	MACS + FACS	H2Kb$^-$ (MCH cl I) CD49f$^+$ (α6 integrin)	FACS *In vitro* culture Cell transplantation	0.39% H2Kb$^+$ cells 3.1% (1/32) contaminated cultures 1/20 contaminated mice
	Human	FACS	H2Kb$^-$ (MCH cl I)	FACS; *In vitro* culture; PCR for B cell receptor	0.58% SB$^+$ cells 1/11 contaminated samples

MCH cl I: major histocompatibility complex class I (marker of somatic cells); α6 integrin: marker of SSCs; CD45: surface marker of leukemic cells; IFγ: interferon-γ; CML: chronic myelogenous leukemia.

- One of the reasons for suboptimal cell sorting may be that the surface antigens are shared by other stem cells, namely hematopoietic stem cells involved in hematological cancers.
- Further research on surface markers should focus on the complete elimination of cancer cells from cell suspensions before sorted preparations can be safely transplantated.
- Cancer cell contamination is also a major concern in tissue autografting. (damage to testicular tissue or disturbance of vessel barriers in cryopreserved tissue may enhance hematogenous invasion of surviving malignant cells in tissue grafts)

- Infectious transmission.
  - Due to the risk of infectious transmission from animals to humans, testicular xenografting should not be considered for reproductive purposes at present.
  - The risk of animal viral transmission or contamination with animal antigens or cellular membrane-binding molecules is also present in IVM, so these systems should not be used for clinical purposes.

- Birth defect risks.
  - Goossens et al. (2003) recently reported smaller litter size, significantly lower fetal weight and reduced length in first generation mouse offspring after germ cell transplantation, suggesting imprinting disorders *(Goossens et al., 2006).*
  - Chromosomal abnormalities were found in embryos obtained after ooplasmic injection of in vitro-derived haploid germ cells issuing from diploid germ cells.

- Special attention should also be paid to the genetic and epigenetic status of in vitro-matured cells
- Although the birth of healthy offspring has been reported after IVM of immature germ cells like primary spermatocytes *(Tesarik et al., 1999),* insufficient data are currently available to allow safe clinical application.

(Ethical concerns)

- Learning that a child has cancer is devastating for all concerned, and treatment needs to begin quickly, leaving very little time for the impact of possible future sterility to sink in.
- However, the inability to father one's own genetic children might have a huge impact on the psychological well-being of patients in adulthood, so it is crucial to inform them of the potential consequences of their therapy on future fertility.
- Ethical concerns have been expressed about ITT cryopreservation, highlighting the importance of the risk/benefit balance
- Because of the small size of testes from prepubertal children, immature gonadal tissue sampling may be considered too invasive a procedure,

- study of cryptorchid boys who had undergone testicular biopsy during orchidopexy, no adverse long-term effects were reported *(Patel et al., 2005).*
- Children and their parents should be informed of the experimental nature of this approach and the fact that there is no guarantee of fertility restoration
- With continued advances in potential fertility restoration strategies, ethical guidelines will need to be established with respect to harvesting, preservation and use of prepubertal testicular tissue.

Conclusion

- Providing young people undergoing gonadotoxic treatment with adequate fertility preservation strategies is a challenging area of reproductive medicine, but every patient should be given the chance to consider fertility-sparing options because the detrimental effect of such therapy on gonadal function remains unpredictable.
- Hormonal or cytoprotective drug manipulation aimed at enhancing spontaneous recovery of spermatogenesis remains a possibility for the future.
- SSC preservation offers the prospect of several realistic applications, although none is feasible in humans at this point in time.

- Before considering the fertility restoration options, patient selection is essential, since risks vary according to disease.
- No single (or simple) algorithm can, thus far, summarize all the possible stratgies for fertility preservation and restoration in the case of gonadotoxic therapy in prepubertal boys, but the most appropriate course of action may be selected according to the scheme shown in Fig. 4.

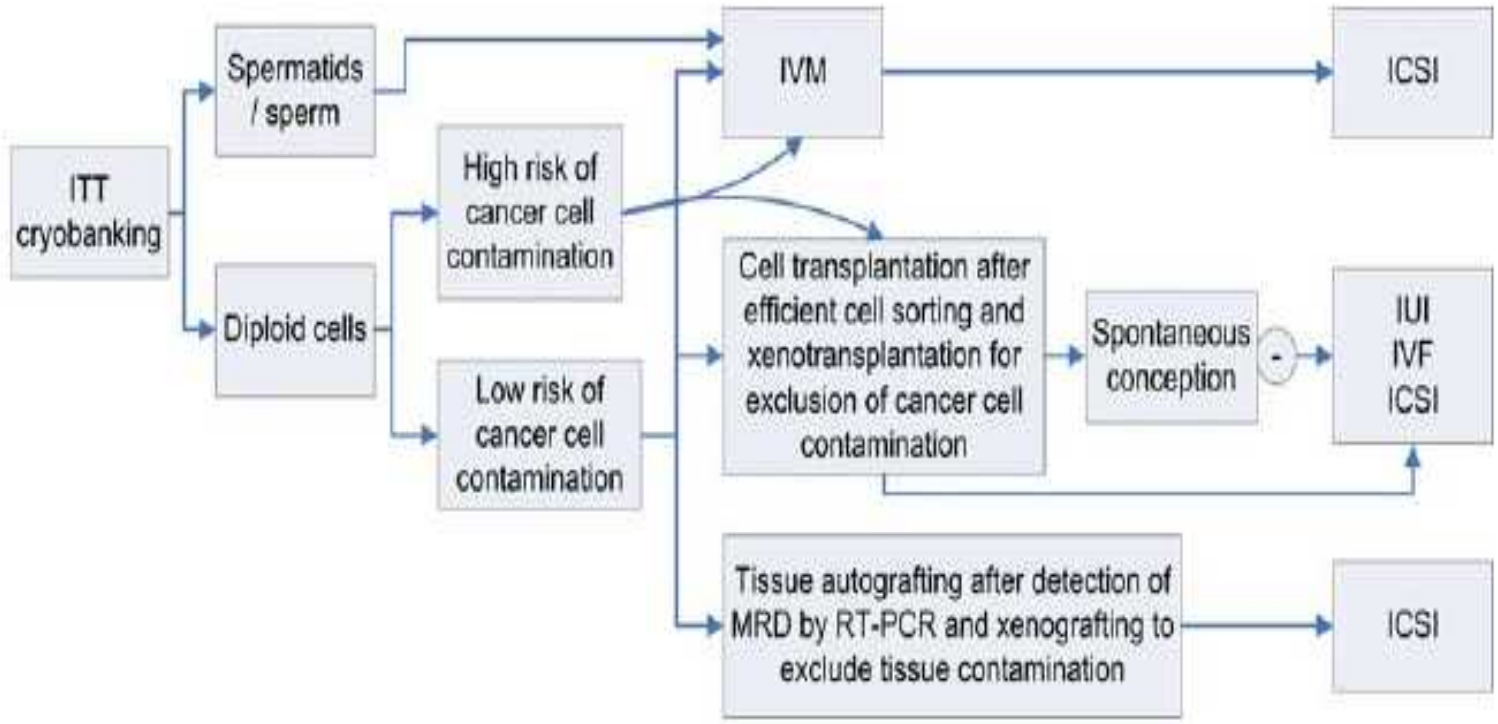

Figure 4 Fertility restoration strategy after gonadotoxic therapies in prepubertal boys. ITT, immature testicular tissue.

- Until then, samples should at least be banked after providing careful counseling and obtaining informed consent, making sure the patient understands that there is no guarantee of success (Hovatta, 2003).
- Preservation of testicular tissue from today's prepubertal patients will allow them to consider various fertility restoration options that will emerge in the next 20–30 years, giving them hope of fathering children with their own genetic heritage.

Thanks for your attention!