l'infertilità maschile oggi

male infertility today

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Giovanni M. Colpi

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HOW FAR ARE ICSI PROCEDURES USING HUMAN SPERMATOZOA GENERATED INTO ANIMAL TESTICLES? MICRO- AND MACRO-CONSEQUENCES OF GERM CELL TRANSPLANTATION TECHNIQUES

Sofikitis Nikolaos[1,2], Kaponis Apostolos[1], Giannakopoulos Xenophon[2], Miyagawa Ikuo[1]

[1]Dept. of Urology, Tottori University School of Medicine, Yonago, Japan
[2]Laboratory for Molecular Urology and Genetics of Human Reproduction,
Dept. of Urology, Ioannina University School of Medicine, Greece

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Abstract

Results from the transplantation of donor male germ cells into xenogeneic recipient seminiferous tubules indicate that donor spermatogonia are capable of differentiating to form spermatozoa morphologically characteristic of the donor species. Germ cell transplantation procedures combined with developments in freezing, culturing, or enriching germ cell populations have applications of paramount importance in medicine, basic sciences, and animal reproduction. Additionally these techniques can serve as an alternative approach for gonadal protection and fertility preservation in patients with oncological disease.

This article is a chronological critical review of the technological advances that followed the initial successful transplantation of mouse germ cells into recipient mice. Furthermore, the factors responsible for the immunological privilege properties of the testicle and the parameters influencing the potential of mammalian germ cells to undergo mitosis and meiosis within a xenogeneic testicle are described. Finally the role of human germ cell transplantation procedures in the therapeutic management of non-obstructive azoospermia is discussed.

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MECHANISMS REGULATING MALE GERM CELL MEIOSIS AND SPERMIOGENESIS

Approximately 50% of human infertility is attributable to male defects with the clinical presentation of abnormal sperm production, such as oligo-, astheno-, teratospermia, or azoospermia *(Sigman et al., 1997).* Spermatogenesis is a process that involves mitotic and meiotic division of spermatogonia to produce mature spermatids and spermatozoa. Spermatogenesis occurs within the seminiferous tubules of the testicle *(Dym, 1983)*. The spermatogonial type A stem cells in the testicle can be considered as eternal germ cells because they are present from the birth to the death and have the capacity to give rise to new subtypes of spermatogonia and form spermatozoa, as well. The latter cells represent highly motile structures that can pass genetic material from generation to generation *(McLaren, 1992)*. Among the type A spermatogonia there are distinct subpopulations, types A_1-A_4 that have been considered the renewing stem cell spermatogonia, and type A_0 that have been considered a reserve type of stem cells. The type A_4 cells divide to give rise to differentiating spermatogonia as well as to form new type cells, the A_1 cells to maintain the stem cell population *(Dym & Clermont, 1970)*. The type A_0 cells divide slowly and represent a reservoir to repopulate the testis after an injury as a fine mechanism to maintain normal numbers of types A_1-A_4 *(Clermont, 1969)*.

Numerous factors have been implicated in spermatogonial development. Several alternative hormonal mechanisms regulate the mitotic and meiotic dynamics of spermatogonia *(Meachem et al., 2001)*. Sertoli cells limit the expansion of the spermatogonial population. Each Sertoli cell supports a defined number of germ cells. Sertoli cells form niches for stem cells. These niches allow a certain number of stem cells to reside in or repopulate the seminiferous tubules. The repopulation pattern of donor spermatogonia post-transplantation supports the presence of niches of donor germ cells *(Ohta et al., 2000).* FSH has an important role in the regulation of the kinetics of

adult spermatogonia. The mechanism via which FSH mediates its effect is probably by acting both as a survival factor and as a mitogen *(Meachem et al., 1997).* Withdrawal of FSH in normal adult rats showed a time-dependent decline in the number of early germ cell types *(Meachem et al., 1997; Meachem et al., 2001; Kangasniemi et al., 1990).* Great reductions/depletions of gonadotrophins in primates (treatment with GnRH-antagonists) reduced the number of type B spermatogonia to less than 10% of controls indicating that the spermatogonium is a major target for gonadotrophin stimulation *(Zhengwei et al., 1998).* Treatment with GnRH-antagonists provokes a pronounced inhibition of spermatogonial proliferation indicating that the first step of mitosis (at the level of A spermatogonia) is under hormonal influence *(Schlatt & Weinbauer, 1994). Meistrich et al. (1994)* provided compelling evidence that high testicular testosterone concentrations have a detrimental effect on spermatogonial development.

Stem cell factor (SCF) and its receptor c-kit play an important role in spermatogonial development. Mutation in the gene encoding the SCF or the c-kit results in infertility owing to defective migration, proliferation, and survival of primordial spermatogonia. SCF has been found to act as a mitogen *(Allard et al., 1996)* and survival factor *(Dirami et al., 1999).*

Inhibin inhibits the incorporation of tritiated thymidine into differentiated mouse and hamster spermatogonia *(van Furth & Van Dissel, 1989),* whereas, activine stimulates spermatogonial mitosis in vitro *(Mather et al., 1990).* Bcl-2 family plays a key role in integrating the positive and negative signals on spermatogonial survival. There are members of this family that promote cell survival (Bcl-2, BcL-xl, Bcl-w, A1/Bfl-1) and others promoting cell death (Bax, Bak, Bad, Bim) *(Adams & Cory, 1998).* Transgenic loss of the Sertoli cell-produced glial cell derived neurotrophic factor (GDNF) function results in depletion of spermatogonia stem cells reserves, whereas mice overexpressing GDNF accumulate undifferentiated spermatogonia within the seminiferous tubuli *(Meng et al., 2000).* Recolonization of a recipient testicle by transplanted donor germ cells offers an excellent approach to understand the mechanisms regulating the influence of the testicular microenvironment on spermatogonial development.

Spermiogenesis is a metamorphosis process where no cell division is involved. During spermiogenesis alterations can be seen in the male gamete nuclear proteins, the cellular size, the cellular shape, and position of pro-acrosomal granules and the localization of the centrioles, as well. A fascinating cascade of events results in the formation of sperm tail. Elongation of the round spermatid is observed in the steps 7-8 of spermiogenesis in the rat *(Sofikitis et al., 1999c). Sofikitis and co-workers (1999c)* have shown that a decrease in the intratesticular testosterone results in a premature detachment of round spermatids from the seminiferous tubuli and passage of the round spermatids through the epididymal lumen. The latter spermatids have an impaired fertilizing capacity.

THE IMMUNOLOGICAL TOLERANCE OF THE TESTICLE

The testicle is an immuno-privileged organ *(Selawry and Whittington, 1984; Selawry et al., 1989; Clouthier et al., 1990; Belgrau et al., 1995; Russell and Brinster, 1996; Clouthier et al., 1996). Selawry et al. (1985)* showed that pancreatic islets of MHC-

incompatible donors survived indefinitely in the abdominal testicles of diabetic BB/W rat recipients. Furthermore, it has been shown that the abdominal testicle rather than the testicle in its original scrotal position is the most suitable site for extended functional survival of pancreatic islet allo-and xenofrafts *(Selawry et al., 1989).* The colonization of the mouse testicle by rat germ cells *(Clouthier et al., 1996; Russell and Brinster, 1996; among others)* and the rat testicle by hamster germ cells *(Tanaka et al., 1997; Shimamoto et al., 1999; among others)* further confirm the testicular capacity to inhibit local immune responses. However, the mechanisms responsible for the unique immunologically privileged status of the testicle are, as yet, undefined. It has been suggested that elevated levels of intratesticular testosterone and /or progesterone may cause an inhibition of the local immune responses *(Grossman et al., 1985).* However, *Cameron et al. (1990)* provided strong evidence that Leydig cells and Leydig cell secretory products do not represent a prerequisite for the success of transplantation of pancreatic cells into testicles. On the other hand the testicle contains high levels of steroids, molecules that are immunosuppressive by nature *(Russell & Brinster, 1996)*. The most commonly recognized mechanism for the immunological privilege properties of the testicle is the physical isolation of cell surface antigens by the Sertoli cell-barrier. However, immune privilege in the testicle may not be simply a passive process involving physical barriers (Sertoli cell-barrier) but it may also be regarded as an active mechanism having the capacity to induce cell death (via apoptosis) on potentially dangerous lymphoid and myeloid cells *(Belgrau et al., 1995).* In fact *Belgrau et al. (1995)* have shown that expression of functional FasL by Sertoli cells has a major contribution to the immune privileged nature of the testicle. Fas and its ligand FasL are cell surface molecules known to interact in the regulation of immune responses. FasL protein is expressed by CD4+ T helper cells and by CD8+ cytotoxic T lymphocytes. Resting T cells normally express low levels of Fas. In contrast, during the first hours post-engagement of their antigen receptors, Fas expression increases significantly. Post-activation, Fas in T cells becomes functional and the T cells undergo apoptosis in response to crosslinking with FasL *(O`Connell et al., 1996).* Thus, FasL ligation onto its receptor Fas, on sensitized cells, induces programized cell death. *Griffith et al. (1995)* suggested that through the expression of FasL, the eye directly kills activated T cells that might invade the globe and destroy the vision. Similarly, considering that Sertoli cells constitutively express FasL transcripts *(Belgrau et al., 1995),* it may be suggested that FasL interacting with Fas of activated T cells induces apoptosis in the infiltrating T cells and subsequently contributes to the capacity of the testicular tissue to maintain an immune privilege environment. The latter thesis is additionally supported by findings in our laboratory showing enhancement of the immune privilege status of the rat seminiferous tubules during a) intratubular administration of interferone-γ (known to up-regulate FasL expression) *(Shimamoto et al., 1999),* or b) seminiferous intratubular transfer of anterior chamber eye cells (known to express FasL) from the same recipient animal *(Shimamoto et al., 1999).*

THESES AND CONTROVERSIES ON SYNGENEIC MALE GERM CELL TRANSPLANTATION TECHNIQUES

A. Development and evolution of syngeneic germ cell transplantation procedures

Brinster & Zimmermann (1994) showed

that germ cells isolated from testicles of donor male mice would repopulate immunologically compatible sterile testicles when injected into the seminiferous tubules of these recipient animals. Donor spermatogenesis in recipient testicles showed normal morphological features characteristic of the donor species. In that study, germ cells were transplanted into mice carrying the W, W_V and W_{44} mutant alleles in the homozygous or compound heterozygous conditions (spermatogenesis does not occur in the testicles of these mice; very few germ cells of the most primitive stages can be found in the testicles of W/W_V mice). At the end of the experimental period, donor germ cells were found in 18% of the recipients. In additional experiments immunodeficient male mice had been treated with 40 mg/kg of the chemotherapeutic agent busulfan approximately 4 weeks prior to the transplantation of donor germ cells. At this dose, busulfan destroys almost all endogenous spermatogonial stem cells, thereby space is created on the basal surface of the tubules for the transplanted spermatogonia to seed and develop. In addition, the lack of recipient germ cells facilitates the injection procedure due to a decrease in the resistance to the flow of the donor germ cell suspension through the seminiferous tubuli. In that study the transferred cells bore the LacZ gene (the expression of this gene is detectable histochemically in germ cells using the substrate X-gal). At the end of experimental period among the busulfan-treated recipients 37% were found to be positive for donor germ cells. In tubules colonized by transferred donor cells, the organization of the spermatogenic stages within the recipient seminiferous tubules was normal, characteristic of the donor species and mature donor spermatozoa were observed.

In another study *Brinster & Avarbock (1994)* demonstrated that mouse germ cells transplanted into the testicles of infertile mice colonized the recipient seminiferous tubules and initiated donor spermatogenesis in more than 70% of recipients. The most striking result of these experiments was the production of healthy offspring (by mating) from spermatozoa generated within the recipient testicles by donor germ cells. Increasing the number of Sertoli cells in the donor cell population did not increase the efficiency of colonization of the recipient testicles by donor germ cells. Some of the recipients that underwent transplantation passed on the LacZ gene to 80% of their progeny. The initiation and maintenance of donor spermatogenesis within the recipient testicles post-donor germ cell transplantation seems to be inconsistent with the fact that the donor spermatogonia have been injected into the lumen of the seminiferous tubules. It appears that donor spermatogonia have moved from the recipient adluminal to the basal compartment of the seminiferous tubulus, a translocation that does not occur naturally in adult mammalian species. The mechanisms regulating the translocation of the injected spermatogonia from the lumen towards the basal surface of the tubule are still unexplained. On the other hand, the success of the experiments of *Brinster & Avarbock (1994)* and additional studies (see Table I) suggest that the recipient Sertoli cell can regulate translocation of recipient and donor germ cells a) from the basement membrane to the lumen and b) from the lumen to the basement membrane, respectively.

Jiang and Short (1995) transferred primordial germ cells or gonocytes recovered from male Sprague-Dawley rat fetuses or neonates into the rete testicles of recipient adult Long Evans rats that had been treat-

Table 1: Evolution of male germ cell transplantation techniques.

Author	Year	Report
Brinster and Zimmermann	1994	First report of successful induction of donor mouse spermatogenesis within a recipient mouse testicle
Brinster and Avarbock	1994	Germ-line transmission of donor mouse haplotype in recipient mice
Jiang and Short	1995	Rat male germ cell transplantation in recipient rats
Clouthier et al.	1996	First report of xenogeneic transplantation of rat germ cells into recipient mice
Avarbock et al.	1996	Transplantation of frozen/thawed mouse spermatogonia into recipient mice
Ogawa and Brinster	1997	Detailed description of transplantation techniques
Franca et al.	1998	Germ cell genotype controls cell cycle during spermatogenesis in the rat
Ogawa et al.	1999	Transplantation of hamster germ cells into mouse testicles
Schlatt et al.	1999	First successful attempt of germ cell transfer into a primate testicle
Nagano et al.	1999	Pattern and kinetics of mouse spermatogonial stem cells into recipient testicles
Sofikitis et al.	1999	First report of successful production of human spermatozoa after transplantation of human germ cells into mouse and rat testicles
Schlatt et al.	1999	Magnetic cell sorting for enriching viable spermatogonia from rodent and primate testicles
Nagano et al.	2001	Primate spermatogonial stem cells colonize mouse testicles
Sofikitis et al.	2001	Successful hamster seminiferous tubuli xenogeneic transplantation

ed with busulfan. They suggested achievement of normal donor spermatogenesis in 62% of the recipients. However, due to a lack of a specific marker for the identification of donor cells it was difficult to conclude in that study that the donor cells had colonized the seminiferous tubules. In another study, *Jiang and Short (1998)* provided clear evidence that transplanted primordial germ cells could divide within the recipient seminiferous tubules. The authors concluded that the pattern of donor cell colonization and donor spermatogenesis following transplantation in terms of their spatial location and connection with the recipient seminiferous epithelium depend on the developmental stages of the donor cells at transfer.

Russell et al. (1996a) described the ultrastructural features of donor spermatogenesis in immunologically compatible sterile mice post-transplantation of mouse spermatogonia. Recipient animals carried mutant alleles and lacked endogenous testicular spermatogenesis. Testicular tissue of recipient animals was examined by light and electron microscopy 12 to 15 months following donor germ cell transplantation. Donor mouse germ cells were demonstrated to form cellular associations, characteristic of the donor species, within the recipient testicle. Donor spermatogonia were found exclusively in the basal compartment. Apparently they had been translocated from the recipient tubular lumen through the recipient Sertoli cell junctions eventually to reside on the basal lamina. In addition, some recipient seminiferous tubules exposed qualitative and quantitative defects in donor spermatogenesis. Most of the common abnormalities were seen at the elongation phase of donor spermatogenesis. There were regions in the recipient seminiferous tubules where donor germ cell colonization was absent. In these regions recipient Sertoli cells were observed that were actively phagocytosing donor spermatozoa. In the latter study, failure of elongation and chromatin condensation of donor spermatids was a major barrier for the completion of donor spermatogenesis. The authors hypothesized that the failure of achievement of donor spermatogenesis in these areas was due to dysfunction of the testicular somatic cells of the recipient mutant genetic model used rather than due to failure of donor germ cells to develop.

Tanemura et al. (1996) transplanted very thin seminiferous tubules of old (33 months of age) BDF1 mice into W/W_V mouse testes. Artificially cryptorchid younger BDF_1 mice were used as controls. Testicular tissue from the above cryptorchid animals was transplanted into the right testicles of W/W_V mice while testicular tissue from the old BDF_0 mice was transplanted into the left testicle of the same animal. Two weeks post-transplantation the most advanced donor spermatogenic cells on the left side were spermatocytes, whereas, on the right side both donor spermatocytes and round spermatids were detected. At four weeks post-transplantation, elongated spermatids were detected in tubules of the right testicles but spermatids were still undetectable in the left testicles. It appears that spermatogenic cells derived from old mice could not pass through meiosis. The authors concluded that either a defective extratubular environment or a defective intratubular environment in the donor seminiferous tubuli had caused the arrest of spermatogenesis (of the old mice) in the transplanted seminiferous tubules within the recipient left testicles.

Schlatt et al. (1999) injected bull, monkey, or human germ cells into resected bull, monkey or human testicles, respectively.

Introduction of donor germ cells to areas of recipient seminiferous tubuli distal to the sites of injection could not be achieved, probably because the endogenous recipient spermatogenesis had not been depleted and recipient germ cells and seminiferous tubular fluid impeded the movement of injected donor cells into the tubules. Distal movement of injected donor cells within the recipient seminiferous tubuli could be only achieved in immature or partially regressed testes. Rete testis injections were applied via ultrasonographically-guided technique in vivo on two cynomolgus monkeys that had been previously treated with a GnRH-antagonist. Ultrasonography allowed the localization of the rete testis and guidance of the injection needle into the rete testis. The authors suggested that this technique is the most promising one for transplantation of germ cells into large testicles. In one monkey transplanted donor germ cells were present in the recipient seminiferous tubules four weeks post-transfer. These cells had the morphological characteristics of B-spermatogonia and were demonstrated as single cells and as small aggregates of cells in both the interstitium near the rete testis and within the seminiferous tubules.

B. Cryopreservation of male germ cells

Although advances in cryobiology have resulted in the development of well defined media for cryopreservation of mature spermatozoa there is inadequate knowledge concerning the constituents of optimal chemical media for the maintenance of the viability and function of immature male germ cells. *Yamamoto et al., (1999a)* in our laboratory have shown that the seminal plasma, milk, and other specific media can serve as cryoprotectants for the maintenance of immature male germ cells during freezing or cryostorage. Furthermore, in the latter study, the authors proved that post-thawing haploid round male germ cells could fertilize oocytes.

Avarbock et al. (1996) have demonstrated that donor germ cells recovered from prepubertal or adult mice have the capacity post-freezing/thawing to generate donor spermatogenesis in recipient mouse seminiferous tubules. Among 30 recipient testicles, 22 (73%) testicles were found to be positive for donor spermatogenesis, post-transplantation of donor frozen/thawed germ cells. In the latter study, satisfactory colonization of recipient testicles by donor frozen/thawed germ cells was found when propanediol and glycerol had been used as cryoprotective agents. Cryopreservation of spermatogonia cells in men with primary testicular damage or men with oncological disease offers advantages over cryopreservation of spermatozoa, since the spermatozoon cannot replicate, and a single spermatozoon carries a defined set of genetic information. In contrast, cryopreservation of spermatogonia allows the whole genetic information/potential of an individual to be preserved. Furthermore, an additional number of donor spermatogonia and subsequently spermatids/spermatozoa can be produced post-transplantation of donor frozen/thawed spermatogonia within the recipient testicles (via mitotic divisions and via meiotic divisions, respectively) since subpopulations of the donor spermatogonia undergo mitoses and subsequently meioses within the recipient testicles. Frozen male germ lines could be considered as biologically immortal. The implications of this technology in basic sciences, medicine, and zoology are tremendous.

C. Germ cell transplantation techniques as a powerful tool to reconstitute spermatogenesis in animals with testicular damage and study the mechanisms of genetically caused male infertility.

Disruption of spermatogenesis is commonly caused by factors affecting either the germ cells or the Sertoli cells or both of them. In most cases of male infertility the pathogenesis is not well defined. Recently several reports have demonstrated the importance of male germ cell transplantation techniques for the investigation of mammalian male infertility that occurs due to a natural gene mutation or a targeted gene deletion. Gene knockout experiments often lead to male infertility. In most cases, the testicular cell types where the disrupted gene is phenotypically important cannot be easily identified. For the characterization of the latter cell types, the transplantation of germ cells from the evaluated infertile (donor) animals into seminiferous tubuli of animals with distinct genetic features *(Ogawa et al., 2000)* may offer important information. Thus, transplantation of germ cells carrying a disrupted gene into a wild-type recipient may indicate the testicular cell types where the disrupted gene is phenotypically important.

Mahato et al. (2000) transplanted male germ cells recovered from testicles of estrogen receptor-a (ERa) knockout infertile mice into the seminiferous tubules of germ cell-depleted wild-type mice. The donor germ cells carrying the knockout mutation underwent qualitatively normal mitoses and meioses within the recipient testicles, and the recipients became fertile. Offspring derived from the recipient mice were proven to have been generated from the fertilization of oocytes with spermatozoa that carried the disrupted gene for the ERa. This was the first application of the germ cell transplantation technique to demonstrate that a gene knockout that disrupts spermatogenesis has no direct detrimental action on the germ cell capacity to proliferate and differentiate. The authors used coat color differences in offspring to prove that some recipients post-transplantation produced spermatozoa (with fertilizing capacity) that carried the ERa knockout gene. Both of the estrogen receptors ERa and ERb have been reported in multiple cell types in the testicle and the epididymis including germ cells *(Hess et al., 1997).* Studying separately the ERa and ERb genes indicates a distinct functional role of ERa in spermatogenesis *(Krege et al., 1998).* These experiments have provided biological proof that a direct action of estrogens on the germ cells is not required for the completion of spermatogenesis.

Johnston et al. (2001) injected testicular cells from mice with testicular feminization pathophysiology into the seminiferous tubules of azoospermic mice expressing functional androgen receptors. Recipient testicles were analyzed 110 to 200 days following transplantation. Multiple colonies of complete and qualitatively normal donor-derived spermatogenesis were observed within the tubules of the recipient testicles demonstrating that male germ cells do not require functional androgen receptors and direct stimulation by the testosterone to complete spermatogenesis.

Ogawa et al. (2000) transplanted spermatogonia from an infertile mouse strain (Steel [Sl] mouse as donor of germ cells) to an infertile recipient mouse (dominant white spotting [W]) to determine if germ cells from that infertile male mouse were capable of generating spermatogenesis. Post-transplantation the recipient mice were shown to be fertile. Thus, fertility was restored after

transplantation of spermatogonia cells from an infertile donor into an infertile recipient that had a permissive testicular environment. That study indicates that both: a) male germ cells and b) supporting environment can retain full functional capability for a long period of time in the absence of normal spermatogenesis.

Another naturally occuring mutation in mice that affects spermatogenesis is the jouvenile spermatogonial depletion (jsd) mutation. The overall result of this mutation is a single wave of spermatogenesis followed by a failure of type A spermatogonial cells to repopulate the testis. When testicular germ cells from jsd animals were injected into W/W_V or busulfan-treated recipients *(Boetger-Tong et al., 2000)* no donor-derived spermatogenesis was observed in the recipients. In contrast, when *Boetger-Tong et al. (2000)* injected germ cells without jsd mutation into jsd recipients, demonstrated that jsd animals could support donor spermatogenesis for up to seven months. These data indicate that the jsd infertile phenotype is due to a defect in the germ cells and cannot be attributable to functional or anatomical alterations of the testicular somatic cells. Additionally, *Ohta et al. (2001)* injected into the seminiferous tubules of B6-jsd/jsd mutant mice donor germ cells derived from the wild type GFP transgenic mouse (B6-+/+GFP). The latter donor cells were able to undergo complete spermatogenesis within the recipients indicating that the jsd mouse possesses optimal testicular microenvironment for supporting germ cell differentiation. The conclusion of these experiments is that gene mutations that lead to germ cell inability to undergo meiosis do not preclude the probability that the respective testicular biochemical microenvironment allows differentiation of germ cells of a different gonotype.

Transplantation of spermatogonia from infertile mice carrying a mutation in the SCF gene into infertile mice with a mutated c-kit receptor gene leads to qualitatively normal spermatogonial repopulation of the recipient testicles with developing and differentiating donor germ cells due to the presentation of recipient Sertoli cells producing membrane-bound SCF and donor germ cells expressing the functional c-kit receptor. Thus, *Ohta et al. (2000)* showed that SCF stimulation is required for differentiation of germ cells but not for spermatogonial stem cell proliferation (mitoses). In additional experiments, *Ohta and co-workers* transplanted undifferentiated testicular germ cells of GFP transgenic mice into seminiferous tubules of mutant mice with male sterility, such as those dysfunctioned at the Steel (Sl) locus encoding the c-kit ligand or at the Dominant white spotting (W) locus encoding the c-kit receptor. In the seminiferous tubules of Sl/Sl^d or Sl^{17H}/Sl^{17H} mice, transplanted donor cells proliferated and formed colonies of undifferentiating spermatogonia but where unable to differentiate further. When these undifferentiated but proliferating spermatogonia were retransplanted into Sl (+) seminiferous tubules of W-locus-mutant animals, they resumed differentiation. These experiments have indicated very vividly that when germ cell transplantation techniques are used to reconstitute spermatogenesis in subjects with testicular damage, stimulation of c-kit receptor by its ligand is necessary for the achievement of full donor spermatogenesis within the recipient testicle.

D. Culture of spermatogonia cells prior to transplantation.

1. Optimal conditions for culturing donor

germ cells pre-transplantation.

Culture of spermatogonia cells in vitro increases their number and allows transplantation of a larger number of donor premeiotic germ cells within a recipient testicle. Meiotic differentiation of male germ cells in culture has been reported by *Rassoulzadegan et al. (1993).* The authors reported that mouse immature germ cells differentiated up to the spermatid stage in vitro when they had been co-cultured with a Sertoli cell line. However, in that study, in vitro derived spermatids never proceeded to form mature spermatozoa. Additionally that in vitro culture system could not be maintained beyond two weeks. *Kierszenbaum and co-workers (1994)* claimed that germ cells could not survive in vitro for longer than few weeks. However, the absence of an assay to evaluate spermatogonia cell function prevented an accurate evaluation of the function of the cells. The development of the spermatogonial transplantation technique has provided a functional assay to assess the potential for meiosis of any population of spermatogonia *(Brinster & Zimermann, 1994; Brinster & Avarbock, 1994).* It should be emphasized that the rationale to culture donor germ cellular populations prior to the performance of transplantation techniques is to increase the number of premeiotic spermatogonial cells rather than the number of spermatocytes and spermatids. Among the transplanted donor germ cells, the spermatogonia are the cells that will move towards the basement membrane of the recipient tubules and will organize the first colonies of donor spermatogenesis within the recipient tubules.

Nagano et al. (1998a) demonstrated that mouse testicular spermatogonia cells could be cultured into Dulbecco`s modified Eagle`s medium containing 10% fetal bovine serum on a feeder layer of STO cells for four months and could still generate donor spermatogenesis successfully when transplanted into recipient mice. Donor premeiotic germ cells pre-transplantation survived under relatively simple culture conditions without the addition of growth factors and/or hormones into the culture media. In this experiment all the successful donor germ cellular cultures that subsequently resulted in spermatogenesis following transplantation into recipients had been supported by STO feeder cells. STO feeder cells are known to have a beneficial effect on cultures of embryonic stem cells and primordial germ cells *(Matsui et al., 1992; Resnick et al., 1992).* This beneficial effect during culture (pre-transplantation) may be mediated by growth factors and cytokines secreted by the feeder layer or by cellular associations between the germ cells and the feeder cells *(Robertson 1987; Smith et al., 1992).* Under these conditions germ cells remained alive in culture up to 111 days.

It appears that nowadays culture of male germ cells pre-transplantation is possible and increasing the number of available for transplantation spermatogonia may have a beneficial effect on the transplantation outcome.

2. Human spermatogenesis in vitro

Numerous researchers have aimed to induce human male germ cell meiosis in vitro. *Aslam & Fishel (1998)* cultured in vitro cellular suspensions from men with obstructive and non-obstructive azoospermia for 96 hours. Culture medium was a modified Eagle`s minimum essential medium supplemented with 15 mM HEPES, pyruvic acid, lactic axid, glutamine, penicillin, streptomycine and 10% fetal bovine

serum. Some cells lost gradually their viability during the first 24 h period. Between 72 and 96 hours of culture all cells lost eventually their viability. However, during the culture period 22% of round spermatids developed some growth of flagella. *Sofikitis and co-workers (1998a and 1998b)* in our laboratory had developed a medium (SOF medium) for maintenance of the viability and function of spermatogonia, spermatocytes, and spermatids for relatively long periods. With SOF medium it could be possible to prolong the viability and maintain the function of round and elongating spermatids in vitro. SOF medium contains lactate and glucose as energy substrates. It should be emphasized that the preferable energy substrate for immature germ cells is the lactate rather than glucose *(Sofikitis et al., 1998a).* Protection of round spermatids against environmental shock and stabilization of the spermatid membrane was achieved by cholesterol that had been added to the SOF medium in a small concentration. Vitamins and ferric nitrate had been chosen as components of the SOF medium due to their positive influence in spermatid viability.

Tesarik et al. (1998) demonstrated that FSH stimulates both male germ cell meiosis and spermiogenesis during in vitro culture of human germ cells in GAMETE-100 medium in the presence of Sertoli cells. In addition, testosterone was found to potentiate the effect of FSH on meiosis and spermiogenesis. This effect was probably caused by the prevention of apoptosis of Sertoli cells. *Tesarik et al. (1998; 1999)* have suggested that human spermatogenesis can proceed in vitro with an unusual speed in the presence of high concentrations of FSH and testosterone. However, the generated gametes are often morphologically abnormal. *Tesarik et al. (1999)* reported that application of this method in patients with spermatogenetic arrest resulted in a birth after fertilization of oocytes with elongated spermatids obtained by in vitro meiosis of primary spermatocytes.

Cremades et al. (1999) cultured in vitro (co-culture with Vero cells) round spermatids recovered from: a) men with non-obstructive azoospermia with late maturation arrest and b) one patient with total globozoospermia. Mature spermatozoa with normal morphology were detected after five days of culture in one man with spermatogenetic arrest and from the patient with total globozoospermia. The authors suggested that co-culture with Vero cell monolayers could support full maturation of human round spermatids. In additional studies *Cremades et al. (2001)* provided evidence that few round spermatids could differentiate in vitro up to the elongated spermatid or spermatozoon stage after a seven to 12 day-culture with Vero cells. The latter spermatids were able to fertilize human oocytes and normal embryos were generated.

XENOGENEIC TRANSPLANTATION OF MAMMALIAN MALE GERM CELLS

Clouthier et al. (1996) transplanted rat germ cells into the seminiferous tubules of immunodeficient mice and generated rat spermatogenesis into mouse testicles. In all of 10 recipient mice (in 19 out of 20 testicles) rat spermatogenesis was achieved. Some recipients were found positive for donor spermatozoa within their epididymes. *Rusell & Brinster (1996)* transplanted rat germ cells into the seminiferous tubules of immunodeficent mice, as well. The success of rat to mouse transplants led to a reevaluation of the role of the Sertoli cell in terms of its requirement to support differentiation

of germ cells. Rat germ cell associations into the mouse seminiferous tubuli followed the characteristic patterns of rat spermatogenesis. The recipient Sertoli cells associated with rat spermatogenesis were identified ultrastructurally as being of mouse origin. However, donor spermatogenesis derived from the xenogeneic germ cells was not always qualitatively or quantitatively complete in the recipient animals as donor elongated spermatids were often missing or deformed. In recipient tubular regions lacking donor spermatogenesis, recipient Sertoli cells were phagocytosing donor elongated spermatids indicating that some recipient Sertoli cells do not support donor spermatogenesis and develop surface features that can recognize donor elongated spermatids and remove them.

Franca et al. (1998) transplanted rat germ cells into mouse testicles and the mice were killed 12.9-13 days after transplantation. The most advanced rat germ cell subtype labeled within the recipient testicle was the pachytene spermatocyte of stages VI-VIII of the rat spermatogenic cycle. Recipient animals had been treated with busulfan pre-transplantation. In the recipient animals that underwent transplantation of rat germ cells a degree of mouse endogenous spermatogenesis recovered. The most advanced labeled mouse germ cell types (in recipients killed 12.9-13 days post-transplantation) were meiotic cells or young spermatids. The same findings were observed if a mixture of rat and mouse germ cells were transplanted in mice recipients. Two separate timing regimens for germ cell development were found in the recipient mouse testicle: one of rat duration and one of mouse duration. Rat donor germ cells that were supported by recipient mouse Sertoli cells always differentiated with a cell cycle timing characteristic of the rat and generated the spermatogenic structural pattern of the rat. Thus, the length of the spermatogenic cycle of the rat remained the same in the mouse testicle as in the rat testicle indicating that the length of germ cell cycles was determined by the germ cells alone and was not influenced by Sertoli cells.

Ogawa et al. (1999a) used the rat as a recipient animal and transplanted rat and mouse germ cells into the seminiferous tubules of rats. They demonstrated that 55% of rat testicles were colonized by mouse cells. Depletion of recipient endogenous spermatogenesis before donor cell transplantation was more difficult in the rat than previously reported for mouse recipients. Transplantation of mouse testicular cells into rat seminiferous tubules was most successful in cryptorchid recipients pretreated with busulfan. Mouse donor cell-derived spermatogenesis within the recipient rat testicle was proven. Pretreatment of rat recipients with leuprolide had a beneficial effect on the colonization of the recipient testicle by donor mouse cells. The authors suggested that recipient testicular preparation prior to donor spermatogonial cell transplantation is critical and differs among species.

In our laboratory *Tanaka et al. (1997)* and *Sofikitis et al. (1998a)* transplanted testicular germ cells from cryptorchid hamsters into the seminiferous tubuli of recipient immunodeficient mice, immunodeficient rats, and non-immunodeficient Wistar rats. After mincing the recipient testicular tissue and epididymal tissue it was found that hamster spermatogonia had differentiated up to the spermatozoon stage within the recipient seminiferous tubuli. An interesting observation was that in some microscopical fields of the droplets prepared from the minced recipient tissue the number of

donor spermatozoa was larger than that of the recipient spermatozoa. Transmission electron microscopy revealed hamster round spermatids into recipient seminiferous tubuli. A novel characteristic of those studies was that as donors had been used animals with primary testicular damage due to induction of cryptorchidism. We have chosen donors with primary testicular damage considering that if the transplantation techniques are applied in the future for the management of non-obstructive azoospermia the candidates for such treatments will be men with primary testicular damage. The recipients had not been treated with busulfan. Immature animals with spermatogenic arrest at the spermatogonium or primary spermatocyte stage had been used as recipients in all experiments (see next paragraphs for the advantages and disadvantages of preparation of recipients with busulfan). The accomplishment of donor hamster spermatogenesis within a recipient Wistar rat testicle indicates that the immunological privilege properties of the testicle of a non-immunodeficient recipient animal may be occasionally sufficient for the survival of donor xenogeneic cells within the recipient testicle. Ooplasmic injections of hamster spermatozoa generated into xenogeneic testicles resulted in fertilization and adequate embryonic and fetal development up to the delivery of healthy offspring. The latter animals were proven later to be fertile *(Shimamoto et al., 1999; first post-transplantation offspring generation).* The second and third post-transplantation offspring generations were fertile, as well. The results of these experiments represent the first application of assisted reproductive technology using end haploid products of donor spermatogenesis within a xenogeneic recipient testicle.

Ogawa and co-workers (1999b) achieved hamster spermatogenesis into xenogeneic testicles, as well. The authors demonstrated that hamster spermatogenesis could be produced within immunodeficient mouse recipients. They transplanted fresh hamster donor testicular cells into the seminiferous tubules or the rete testis of immunodeficient mice that had been pretreated with busulfan before transplantation. Among 13 recipient mice, hamster spermatogenesis was identified in the testicles of all mice. Approximately 6% of the evaluated recipient seminiferous tubules demonstrated xenogeneic donor spermatogenesis. Additionally, frozen/thawed hamster testicular cells post-transplantation were differentiated within the xenogeneic recipients. However, the outcome of transplantation techniques using donor frozen/thawed germ cells appeared to be inferior to the fresh germ cell transplantation techniques. Some morphologically abnormal hamster spermatids were recognized into the recipient seminiferous tubuli. Specifically, within the recipient seminiferous tubules hamster elongated spermatids with abnormal head shapes were frequently observed. Within the recipient mouse epididymes the acrosomes of hamster spermatozoa were often absent or poorly formed. It appears that although transplantation techniques can be successful in xenogeneic recipients the derived haploid cells may have anatomical abnormalities.

Xenogeneic transplantation of rabbit or dog testicular germ cells into mouse seminiferous tubules *(Dobrinski et al., 1999b)* and of porcine, bovine, or equine testicular germ cells into mouse seminiferous tubules *(Dobrinski et al., 2000)* did not result in the production of donor spermatozoa within the recipient testicles. All the recipient mice were immunodeficient and had been treated with busulfan approximately four weeks

prior to donor germ cell transplantation to deplete endogenous spermatogenesis in the testicles. Post-transplantation donor rabbit germ cells were present in the testicles of all recipients and had proliferated to form chains of cells connected by intercellular bridges or formed more elaborate mesh structures. This pattern of colonization did not change during the 12 month-observation period. Dog testicular germ cells (fresh, cryopreserved, or cultured) were found located in pairs, short chains, or small mesh structures in the recipient seminiferous tubules post-transplantation. Colonization of recipient seminiferous tubuli appeared less extensive than that observed post-transplantation of rabbit germ cells. Porcine donor germ cells formed chains and networks of round cells connected by intercellular bridges but advanced stages of donor-derived spermatogenesis were not observed within the recipient seminiferous tubuli. Transplanted donor bovine testicular germ cells initially exposed a similar architecture but then developed predominantly into fibrous tissue within the recipient seminiferous tubules. Few equine donor germ cells proliferated within the recipient mouse testicles. Transplantation techniques of equine germ cells recovered from a scrotal testicle or from a cryptorchid testicle resulted in similar outcome. These results indicated that under the experimental conditions the authors followed, fresh or cryopreserved germ cells from a few mammalian species could colonize recipient mouse testicles but did not differentiate beyond the stage of spermatogonial expansion. The authors hypothesized that the longer the phylogenetic distance between the donor and the recipient becomes, the less likely is spermatogonial transplantation to result in complete donor spermatogenesis within the recipient testicle. The authors speculated that the mouse Sertoli cell may not be able to support donor spermatogenesis of animals phylogenetically further removed than the hamster. Additionally, since donor spermatogonia were found in the immunodeficient recipients, immunological incompatibility might not be responsible for the failure of achieving donor spermatogonia differentiation in the latter experiments. On the other hand, the recipients in the most of the latter studies were busulfan-treated animals. The administration of busulfan may have influenced detrimentally the recipient Sertoli cell secretory function with an overall result inability of the recipient Sertoli cells to support donor germ cell differentiation (see next paragraphs). In addition, no efforts were directed to enhance the immunological privilege properties of the recipient testicle *(as suggested in a study by Shimamoto et al., 1999).* Furthermore the recipients were not immature in all of the above experiments. Mature animals are considered as less appropriate recipients than immature animals *(Shinohara et al., 2001).* For all the above reasons the authors' conclusion concerning the presumed causes of failure to induce donor germ cell differentiation in the above experiments cannot be unequivocally adopted.

In 1999 *Sofikitis et al. (1999a; 1999b)* reported completion of human meiosis and spermiogenesis within recipient xenogeneic testicles. This was the first report of successful germ cell transplantation procedure with a given long phylogenetic distance between donor and recipient.

Nagano et al. (2001) used baboon testicles from both prepubertal and postpubertal animals and demonstrated that donor spermatogonial cells of the baboon, regardless of reproductive age, have the capacity to establish germ cell colonies in recipient mice. The latter colonies remained within the recipient seminiferous tubuli for periods

of at least six months. The long survival of baboon germ cells in the mouse testicle and the formation of clusters may represent *(according to the authors' speculation; Nagano et al., 2001)* a process of slow division of undifferentiated spermatogonia followed by apoptosis of early differentiation stages, as previously described by de *Rooij et al. (1999)* and *van Pelt et al. (1990).* The undifferentiated donor spermatogonia replicated but were unable to proceed to differentiating spermatogonial stages and finally underwent apoptosis. Recipient animals had been pretreated with busulfan to destroy the endogenous spermatogenesis. That may have affected recipient Sertoli cell secretory function and donor germ cell differentiation. The maintenance of primitive baboon germ cell clusters in mouse testicles clearly demonstrated that antigens, growth factors, and signaling molecules that interact between baboon germ cells and the mouse seminiferous tubular environment have been preserved for 100 million years in these widely divergent species *(for additional information see Kumar et al., 1998).* As it is described extensively below transfer of human germ cells into immunodeficient immature animals resulted in the completion of human meiosis and spermiogenesis within the recipient testicles. These latter studies *(Sofikitis et al., 1999a; Sofikitis et al., 1999b)* further support the preservation of interacting signaling molecules in the seminiferous tubular microenvironment in widely divergent species.

Sofikitis et al. (2001) reported generation of hamster spermatozoa within xenogeneic testicles after hamster testicular tissue transplantation (see for details next paragraphs). The latter study provided a novel and easy methodology for xenogeneic germ cell transplantation since transfer of donor seminiferous tubules under a recipient testicular tunica albuginea is easier and more rapid method than microsurgical puncture of recipient seminiferous tubuli.

TRANSPLANTATION OF HUMAN MALE GERM CELLS INTO XENOGENEIC TESTICLES

Sofikitis et al. (1999a; 1999b) evaluated the potential of spermatogonia cells of non-obstructed azoospermic men to differentiate to round spermatids or spermatozoa into the seminiferous tubules of immunodeficient immature recipient animals. Recipients were nude rats, which lack B cells, and severe combined immuno-deficient (SCID) mice, which lack both T and B cells. Recipient animals were immature and exposed endogenous spermatogenesis up to the spermatogonium or primary spermatocyte stage at the time of transplantation. Human spermatogonia cells had been recovered from 18 non-obstructed azoospermic men with premeiotic spermatogenic arrest who visited for infertility evaluation the Department of Urology of Tottori University, Yonago, Japan and the M.F.C. Clinic, Yonago, Japan. Spermatogonia cells were transferred microsurgically into the seminiferous tubules of ten nude rats and eight SCID mice *(Sofikitis et al., 1999a; Sofikitis et al., 1999b).* Ten seminiferous tubules per recipient testicle were microinjected with human testicular germ cells plus anterior chamber eye cells from the respective recipient. Recipient anterior chamber eye cells (they express Fas L) were co-transferred to enhance the immune privilege status of the recipient testicle *(Shimamoto et al., 1999).* Previous studies in our laboratory have suggested strongly that co-transfer of recipient anterior chamber eye cells together with the donor germ cells within the recipi-

ent seminiferous tubulus lumen at the time of transplantation enhancing the immunological privilege properties of the recipient testicle, increases the percentage of testicular infiltrating recipient lymphocytes that undergo apoptosis with a final overall result a beneficial influence on the survival of the donor germ cells within the recipient seminiferous tubulus and an improvement in the outcome of transplantation technique *(Shimamoto et al., 1999).* One hundred sixty six to 172 days post-transplantation, testicles, epididymes, and vas deferens of each recipient animal were evaluated for the presence of human spermatozoa. Transmission electron microscopy and fluorescent in -situ hybridization techniques using specific probes against human X-, 18-, and Y-chromosomes were also performed in: a) some of the round germ cells found in the testicles of the recipient animals postmincing and b) some of the recipient seminiferous tubuli. Prior to these experiments we confirmed that the probes used could not react with /label rat or mouse chromosomes.

Post-transplantation human spermatozoa were observed in minced samples from the testicles, epididymes, and vas deferens of three nude rats and two SCID mice. Within the group of the latter five animals the percentage of motile human spermatozoa liberated after recipient tissue mincing and filtration ranged from 0% to 19% in testicular samples, 0% to 83% in the epididymal caudal samples, and 0% to 88% in the vas deferens samples. Fluorescent in-situ hybridization (FISH) techniques in recipient dispersed round cells from minced testicular tissue demonstrated human spermatogonia/primary spermatocytes, human secondary spermatocytes, and human round spermatids (n-DNA haploid cells). In addition FISH procedures in fragments of recipient seminiferous tubuli revealed human spermatogonia in proximity with the recipient animal seminiferous tubulus basement membrane. Human primary spermatocytes, round spermatids, and elongating spermatids were observed, as well in the recipient seminiferous tubuli via FISH techniques. In a very limited number of recipient seminiferous tubuli recipient spermatogonia and donor spermatogonia could be seen at the basal compartment of the same seminiferous tubulus. In addition, recipient sperm heads and human spermatids could be seen in the lumen of the same seminiferous tubulus. This mixed donor-recipient spermatogenetic architectural pattern has not been observed in the rat-mouse transplantation model *(Russell et al., 1996b).* It should be emphasized that in contrast to the studies by *Russell`s group (1996a and 1996b)* and most other researchers' investigations, the recipient animals in our investigation were immature and had a certain degree of endogenous spermatogenesis (i.e., up to the spermatogonium or primary spermatocyte stage) because busulfan had not been administered. This may be the reason for the presence in our study of both donor and endogenous advanced spermatogenesis in the same recipient seminiferous tubulus post-transplantation. Furthermore, transmission electron microscopy demonstrated human round spermatids (mitochondria are scattered in the cytoplasm) in contact with recipient rat round spermatids (mitochondria are located peripherally only). This finding confirms the presence of mixed human-animal spermatogenesis in the same seminiferous tubulus. It appears that human germ cells post-transfer into animal seminiferous tubules can complete meiosis and differentiate up to the spermatozoon stage. Additionally, transmission electron microscopy revealed human spermatids in

the lumen of the rat seminiferous tubules. These results have indicated that the mouse or rat Sertoli cell can support human spermatogenesis. This is an interesting finding since there are differences in the duration of spermatogenesis among the mouse, rat, and human species. To explain the development of human spermatogenesis within the mouse or rat seminiferous tubules it may be suggested that the donor germ cells influence the recipient Sertoli cells to support/stimulate the survival and differentiation of the donor germ cells. This suggestion is supported by studies showing that humoral factors secreted by spermatogenic cells may affect the viability and presumambly modulate the function of Sertoli cells *(Chen et al., 1997).* In addition, the completion of human meiosis and spermiogenesis within a recipient testicle suggests that mouse or rat Sertoli cell secretory function influences positively the induction of human meiosis. This hypothesis is supported by a very recent study in our laboratory showing that co-culture in vitro (at 31^0 C) of rat Sertoli cells, rat germ cells, and rat human spermatogonia/primary spermatocytes (in the presence of human FSH, rat FSH, and testosterone) resulted in the completion of the human meiosis (formation of human round spermatids). In that study we prepared two in vitro culture systems. Type-1-system contained rat Sertoli cells (number of nuclei: $2x10^6$/ml), rat round germ cells (60-$70x10^6$/ml) (rat round germ cells could be separated from rat elongated spermatids, and spermatozoa by enzymatic treatment with pronase as we previously applied for mouse round germ cell purification; *Yamamoto et al., 1999b;* enzymatic treatment occasionally decreases the available for culture number of Sertoli cells and human spermatogonia/primary spermatocytes (60-$70x10^6$/ml). Human spermatogonia/primary spermatocytes had been collected after mincing and filtering (the sediment in the filter was kept and the filtrate was discarded) via a 13 μm-pore size filter testicular biopsy specimens recovered from non-obstructed azoospermic men with complete spermatogenic arrest at the primary spermatocyte stage (these purification procedures were similar to those we previously published for mouse spermatogonia/primary spermatocytes purification; *Yamamoto et al., 1999b).* Prior to culture, FISH techniques (with probes for human chromosomes 18, Y, and X) and confocal scaning laser microscopy *(Sofikitis et al., 1998a)* were applied in subfractions of cultured cells to distinguish between: a) human and rat germ cells and b) rat germ cells and rat Sertoli cells nuclei, respectively. Thus the number of each cell type preculture was evaluated. Type-2-system contained rat round germ cells (60-$70x10^6$/ml; prepared as previously described for mouse round germ cells; *Yamamoto et al., 1999b*) and human spermatogonia/primary spermatocytes (60-$70x10^6$/ml). Type-2-system contained less than 100.000 rat Sertoli cell nuclei/ml. In type-1-system, rat minced testicular material had not been filtered, prior to its treatment with pronase, therefore this culture system was positive for a significant number of rat Sertoli cells *(Yamamoto et al., 1999b).* In contrast, in type-2-system, the rat minced testicular material had been filtered repeatedly *(Yamamoto et al., 1999b)* and the vast majority of rat Sertoli cell nuclei were removed (they remained in the sediment of the respective filters; *Yamamoto et al., 1999b*). Rat FSH had been added in both culture systems to a final concentration of 15 ng/ml. Human recombinant FSH had been added in both systems to a final concentration of 50 IU/ml. Water soluble testosterone had been also added in both systems to a final con-

centration of 1 μmol/L. Prior to culture in both type-1-system and the type-2-system cellular subfractions were proven by FISH techniques and confocal scanning laser microscopy to be negative for human round spermatids and human Sertoli cell nuclei. Seventy-two hours post-culture 1.8-4.6 ($x10^6$/ml) and 0 human round spermatids were found in the type-1-system and type-2-system, respectively, by FISH techniques and confocal scanning laser microscopy *(Yamamoto et al., 2002).* Human elongated spermatids or spermatozoa could not be seen. These results represent the findings of 12 culture experiments of type-1-system and type-2-system performed as above described. Always human round spermatids were found post-culture in type-1-systems, whereas, no round spermatids were generated in type-2-systems. It appears that animal Sertoli cells can stimulate human meiosis in vitro, in other words, animal Sertoli cellular paracrine or endocrine factors can affect positively human meiosis.

The presence of human spermatozoa with potential for strong forward progression within the epididymal tail and vas deferens of one rat post-transplantation *(Sofikitis et al., 1999a; 1999b)* suggested that the rat epididymis could induce human sperm maturation. Thus, the thesis that epididymal sperm maturation process is strictly a species-dependent process may need to be reconsidered.

In contrast, *Reis et al. (2000)* failed to establish a complete human spermatogenic line in the testicles of mutant aspermatogenic (W/W_V) mice and severe combined immunodeficient mice (SCID). The latter species received germ cells from azoospermic men. Spermatogenic cells were obtained from testicular biopsy specimens of adult non-obstructed or obstructed azoospermic men undergoing infertility treatment. However, sections from the recipient testicles examined up to 150 days after transplantation showed recipient seminiferous tubules lined mainly with recipient Sertoli cells, whereas, xenogeneic germ cells were not found. The authors speculated that the donor germ cells did not survive and colonize the mouse testes because of non-compatible cellular interactions and immunological rejection resulting from interspecies differences. However, the conclusions of the study by *Reis et al. (2000)* cannot be unequivocally accepted for the following reasons: a) to identify human germ cells the authors relied on an antibody against proacrosin which appears only in late stages (mid-pachytene primary spermatocytes and spermatids) of germ cell differentiation *(see comment by Nagano et al., 2001);* thus, undifferentiated human spermatogonia and early primary spermatocytes could not be identified by the authors' methodology, b) the number of the transplanted donor germ cells was low, c) the authors did not use any method to enhance the immunological privilage properties of the recipient testicle as was done by *Sofikitis et al. (1999a; 1999b)* or *Shimamoto et al. (1999)* d) the recipients used were neither immature nor newborns (studies by *Shinohara et al., 2001*, support Sofikitis' choice to use as host testicles for donor germ cells those of immature animals rather than mature animals), and e) the recipients used had been treated with busulfan or were aspermatogenic (recipients carried a mutation disrupting spermatogenesis). Since a) busulfan may have affected recipient Sertoli cell secretory function and subsequently may have had a detrimental effect on recipient intratesticular profiles of androgen-binding protein impairing the differentiation of donor germ cells (see next paragraphs) and b) Sertoli cells from aspermatogenic animals may have

had a secretory dysfunction due to the lack of experience to interact with germ cells (see next paragraphs), the lack of positive findings in the study by *Reis et al. (2000)* may not be due to non-compatible cellular interactions between recipient and human cells but it may be attributable at least partially to the methods the authors followed and the preparation of the recipients pre-transplantation.

TECHNIQUES TO INCREASE RECIPIENT COLONIZATION BY DONOR CELLS

A. Selection/preparation of donor germ cells

Selecting donor germ cells for transplantation techniques those subpopulations of germ cells with specific surface markers helps to improve recipient colonization efficiency. *Shinohara et al. (1999)* studied the known association of stem cells with basement membranes to identify specific molecular markers on the stem cell surface that may influence recipient colonization efficiency. Selection of mouse testicular cells with anti-β_1 – or anti-α_6-integrin antibody resulted in the isolation of germ cell populations with significantly enhanced ability to colonize recipient testicles. Spermatogonial cells that preferentially adhered to laminin but not to collagen IV or fibronectin were proven to have the capacity to colonize recipient testicles. The ability of fractions of spermatogonial cells to adhere to laminin suggested that integrins may have a role in the identification of the respective fractions because β_1-integrin has the ability to adhere to laminin and α_6-integrin forms dimers with β_1-integrin *(Hynes, 1992).* Subpopulations of testicular cells can be isolated with a system of magnetic beads and anti-β_1 and anti-α_6-integrin antibodies *(Shinohara et al., 1999).* The colonization of recipient testicles by donor germ cells selected with the aid of anti-β_1 and anti-α_6-integrin antibodies is significantly enhanced compared with control donor germ cells. Additionally, donor germ cells selected by anti-α_6-integrin antibodies appear to be twice as effective in colonizing recipient testicles as those selected by anti-β_1-integrin antibodies. In contrast, spermatogenic cells selected with an anti-c-kit antibody did not show improved rates of recipient testicular colonization. β_1-and α_6-integrins could be used as markers to identify subpopulations of donor spermatogonial stem cells with a higher potential to colonize recipient testicles. The presence of surface integrins on spermatogonial stem cells suggests that these cells share elements of a common molecular machinery with stem cells in other tissues, including hematopoietic, intestinal epithelial, and epidermal *(Potten et al., 1992). Shinohara et al. (2000a, 2000b)* examined the colonization ability of a population of testicular cells enriched in stem cells. They demonstrated that Sl infertile mutant mice can provide an enriched source of testicular stem cells for transplantation techniques. In this mutant, the spermatogonia stem cells constitute a relatively high subpopulation of testicular cells (comparatively with the wild type mouse). The cryptorchid model provides another rich source of stem cells; one cell in 200 testicular cells is a spermatogonium stem cell. Using germ cell transplantation techniques as a functional assay to evaluate quantitatively the presence of spermatogonial stem cells, it was found that testicular cells recovered from cryptorchid mice were highly enriched in stem cells *(Shinohara et al., 2000c).* It appears that the function of spermatogonial stem cells is not adversely affected by the elevated temperature of a

cryptorchid testicle. Donor testicular germ cells from the Sl mutant mouse increased the number of donor germ cell colonies within recipient testicles post-transplantation to a much lower extent than was achieved by spermatogonial cells recovered from cryptorchid mice. Furthermore, *Shinohara et al., (2000c)* employed an in vitro fluorescence-activated cell shorting cellular analysis (FACS) in populations of spermatogonia from cryptorchid testicles to develop qualitative and quantitative cellular criteria that indicate spermatogonial stem cell subpopulations. The most effective stem cell enrichment strategy was the selection of testicular germ cells with proven expression of α_6-integrin and low side scatter. This strategy resulted in a 166-fold enrichment in spermatogonial stem cells.

Another way to increase the outcome of transplantation techniques is to enhance the immunological privilege properties of the recipient testicle by co-transferring recipient anterior chamber eye cells together with the donor germ cells *(Shimamoto et al., 1999)* within the recipient seminiferous tubuli. Co-transplantation of anterior chamber eye cells increases the percentage of infiltrating leukocytes that undergo apoptosis post-penetration of a recipient seminiferous tubule with a beneficial result on the survival of the transplanted donor germ cells and the transplantation technique outcome. Additionally, findings in our laboratory suggest that infusion of interferone-γ into a recipient seminiferous tubule at the time of donor germ cell transplantation upregulates the FasL expression by the recipient Sertoli cells, increases the percentage of infiltrating leukocytes that undergo apoptosis after the mechanical penetration/injury of the seminiferous tubule, and improves the outcome of transplantation techniques *(Shimamoto et al., 1999).*

B. Preparation of recipient animals

Tanaka et al. (1997) in our laboratory reported induction of meiosis of hamster spermatogonia not only within the tubules of immunodeficient animals but also within the tubules of a non-immunodeficient animal. That study was the first that suggested that the local immunological privilege properties of the testicle of a non-immunodeficient animal may be occasionally sufficient to allow survival and differentiation of donor germ cells.

A novel characteristic of three studies carried out in our laboratory *(Tanaka et al., 1997; Shimamoto et al., 1999; Sofikitis et al., 1999a)* was the presence of a certain degree of endogenous spermatogenesis in the recipient animals at the time of transplantation. Recipients were immature mice and rats with arrest at the spermatogonium or primary spermatocyte stage (in most of the cases). Endogenous recipient spermatogenesis had not been destroyed with toxic agents (i.e., busulfan) prior to transplantation. The presence of a degree of endogenous spermatogenesis in the recipients at the time of transplantation in our experiments may explain the presence of a mixed type of donor-recipient spermatogenesis within the same recipient seminiferous tubule post-transplantation. *Brinster & Zimmermann (1994), Brinster & Avarbock (1994), Clouthier et al., (1996). Russell et al. (1996), Russell and Brinster (1996), and Nagano et al. (2001)* used as recipients animals with endogenous spermatogenesis absent/disrupted due to genetic/toxic factors, respectively. This may explain the fact that in their studies post-transplantation recipient seminiferous tubuli showed only donor spermatogenesis or only recipient

spermatogenesis. Mixed type of donor and recipient spermatogenesis was not observed within the same recipient seminiferous tubule in the latter studies. Although the absence of endogenous recipient spermatogenesis in the recipients at the time of donor germ cell transplantation may a) facilitate the movement of the donor germ cells from the recipient seminiferous tubule lumen towards the basement membrane and b) be accompanied by a lower recipient intratubular pressure that decreases the resistance to the injection of donor germ cells, toxic agents administered for disruption of endogenous spermatogenesis may result in the development of an intratubular biochemical environment that is not optimal/ideal for colonization/differentiation of donor germ cells. In addition sterile mutant animals (used by other authors as recipients) may not be ideal recipients because their Sertoli cells do not have the "experience" to support meiosis and spermiogenesis and subsequently may not have optimal capacity to support the spermatogenesis of donor germ cells that enter the recipient seminiferous tubuli non-physiologically. In three studies *(Tanaka et al., 1997; Shimamoto et al., 1999; Sofikitis et al., 1999a)* in our laboratory most of the recipients were immature animals with arrest at the spermatogonium or primary spermatocyte stage. The choice of immature animals that neither had been treated with busulfan nor were genetically aspermatogenic in the latter three studies is supported by recent studies in our laboratory showing that androgen-binding protein profiles in testicular cytosols are significantly smaller ($p<0.05$) in a) sterile mutant $W^{w/v}$ mice compared with same age SCID mice or nude mice and b) busulfan treated (4, 8, and 12 weeks post-busulfan administration) nude rats compared with same age nude rats (non-treated with busulfan). The latter experiments showed that a) busulfan has a long time duration-detrimental effect on Sertoli cell secretory function and subsequently may impair (or decrease) the recipient Sertoli cell capacity to support donor germ cell differentiation, and b) sterile aspermatogenic mutant mice have diminished Sertoli cell secretory function compared with SCID mice or nude mice and subsequently may support to a less degree donor spermatogenesis.

Prolonged administration of GnRH-agonists inhibits the secretion of LH and FSH followed by suppression of testosterone production by the testicle and disruption of spermatogenesis *(Hadziselimovic et al., 1987; Ogawa et al., 1989; Meistrich and Kangasniemi, 1997).* Leuprolide reducing the intratesticular testosterone and subsequently disrupting spermatogenesis in the recipient animals may be used for suppressing endogenous recipient spermatogenesis in the recipient animals pre-transplantation. *Ogawa et al. (1998)* and *Dobrinski et al. (2001)* demonstrated that the efficiency of colonization of recipient testicles by donor cells was markedly higher after pre-treatment of recipients with leuprolide.

C. Methods for germ cell transplantation

Microsurgical puncture of seminiferous tubules was the initial approach to transfer donor germ cells into a recipient testicle. The standard method for donor germ cell transplantation in our laboratory *(Tanaka et al., 1997; Sofikitis et al., 1999a; Sofikitis et al., 1999d; Shimamoto et al., 1999)* is penetration of a seminiferous tubule by two needles guided by two micromanipulators. Several seminiferous tubuli are punctured in each recipient testicle. *Ogawa et al.,*

(1997) reported two additional methods for introducing donor germ cells into recipient seminiferous tubules. First, introduction of the donor germ cells into the recipient efferent ducts and second, introduction of the donor germ cells into the recipient rete testis. Direct injection of donor germ cells into the recipient seminiferous tubules is less invasive and allows entrance of donor germ cells into several tubules at various sites on the testicular surface. However, this approach is the most time consuming (compared with donor germ cell transfer into the efferent ducts or rete testicle) and may be inappropriate and ineffective for larger testicles (than the rodent testicle).

Injection of donor cellular suspensions into the recipient efferent ducts *(Ogawa et al., 1997)* is a more difficult technique and requires careful surgical dissection to expose the delicate efferent ducts. Post-transfer of donor germ cells into the recipient efferent ducts develops excessive intratesticular pressure which may result in intratesticular ischemia with a subsequent detrimental effect on donor cell viability and on the recipient tubular environment. However, this technique is faster than seminiferous tubular injection. An advantage of introducing donor cells into an efferent duct is that the injecting pipette can be held securely into the duct resulting in little donor cell suspension fluid leakage. Thus, smaller volumes of donor cell suspensions are needed to fill the recipient tubules.

Injection of donor cells into the recipient rete testicle requires less dissection than that needed to expose the recipient efferent ducts and for this reason is a faster approach. However, only one or two rete testicles penetrations can be performed before leakage prevents filling of the tubules. Rete testicle puncture is the most easy technique to transfer donor germ cells without the aid of a micro-manipulator or pressure injector. It may be considerably more difficult in some species, such as monkey, pig or sheep which have an axial rete testicle deeply located in the testicle.

It has been suggested *(Ogawa et al., 1997)* that microinjection of donor germ cell suspensions into the recipient seminiferous tubules, efferent ducts, or rete testicle are equally effective in generating donor germ cell-derived spermatogenesis in recipients. Using a microinjection apparatus, a volume of 10 to 50-100 µl can be easily injected into recipient rodent seminiferous tubuli, which is appropriate for the immature, mature, and post-mature rat and mouse testicle. However, in animals with larger testicles, larger volumes of donor cell suspensions (occasionally larger than 2 ml) are needed to fill the recipient testicles. The development of transplantation techniques into larger (than rodent) mammalian testicles is not an easy task. Microinjection into the seminiferous tubules of calf, bull, monkey and man is a difficult procedure due to a resistant lamina propria and a highly convoluted tubular mass. An alternative technique has been developed by *Schlatt et al. (1999)* for germ cell transfer into the primate testicle, using ultrasonographically guided injections into the rete testis. This technique allowed an efficient filling of up to 70% of the recipient seminiferous tubules when the secretory activity of the recipient testicle had been reduced.

Sofikitis et al. (2001) introduced a novel method for germ cell transplantation: transplantation of donor testicular tissue under the tunica albuginea of xenogeneic animals. This method resulted in the completion of hamster meiosis and spermiogenesis within hamster seminiferous tubuli under the rat tunical albuginea (within rat testicles). It is an easy and efficient method to

transfer donor genome within a recipient animal. In these experiments *(Sofikitis et al., 2001)* a piece of testicular tissue from cryptorchid hamsters was placed under the tunica albuginea of 13 nude rats. Vascular endothelial growth factor was administered three times a week intrascrotally in the recipients rats. Three nude rats, five months later, showed complete hamster spermatogenesis into the hamster seminiferous tubuli under the rat tunica albuginea. At that time transmission electron microscopy showed full hamster spermatogenesis into some transplanted hamster seminiferous tubules, whereas, arrest at the primary spermatocyte stage or spermatogonia plus Sertoli cell-only histology were demonstrated in other transplanted hamster seminiferous tubules. Vascular endothelial stain showed standard or extensive vascularization in a number of transplanted hamster seminiferous tubules.

PATTERNS, KINETICS, AND DIFFERENTIATION OF DONOR GERM CELLS WITHIN THE RECIPIENT SEMINIFEROUS TUBULI

An intriguing question concerning colonization of a recipient seminiferous tubule by donor germ cells is the pattern and timing of donor germ cell divisions post-transplantation. *Ogawa et al. (1997) and Nagano et al. (1998b)* used the transgenic mouse line B6, 129-TgR (ROSA 26) from the Jackson Laboratory (ROSA 26) (it was developed using gene trapping methods; it contains the E.coli LacZ [lacZ] structural gene) to evaluate the kinetics of donor germ cell post-transplantation. In the latter study it has been found that the donor germ cell suspension remains widely dispersed within the recipient seminiferous tubules during the first weeks following transplantation. Four weeks post-transplantation donor germ cells are divided on the basement membrane of the recipient seminiferous tubuli forming a network. Differentiation of donor germ cells to more mature germ cell stages appears to begin after this initial colonization and spreading on the recipient seminiferous tubulus basement membrane.

Nagano et al. (1999) observed the pattern and kinetics of mouse seminiferous tubular colonization by donor cells during a 4-month-period post-transplantation. The recipient testicles were fixed in 4% paraformaldehyde, washed, and stained with X-gal, as described by *Ogawa et al. (1997).* Using this system it was possible to observe that the recipient seminiferous tubular colonization process by the donor cells could be divided into three continuous phases. During the initial week transplanted donor cells were randomly distributed along the recipient tubules and a small number reached the basement membrane. In the period between the first to the fourth week post-transplantation, donor germ cells on the basement membrane divided and formed a monolayer network. Following the end of the first month, cells in the center of the network differentiated extensively and established a colony of spermatogenesis which expanded laterally by repeating the two previous phases. The number of colonized sites in the recipient testicle did not change in the period between the first and the fourth month. However, the average length of donor cellular colonies increased from 0.73 to 5.78 mm between the first and the fourth month. These experiments have offered an approach to study in a systematic and quantitative manner the pattern and kinetics of the donor germ cell colonization process. *Parreira et al. (1999)* evaluated by morphometric and ultrastructular studies (light and electron microscope) the devel-

opment of germ cells transplants. Ten minutes post- transplantation donor germ cells developed relationships with small recipient Sertoli cell processes. These relationships remained for one week post-transplantation. Clones of donor spermatocytes were found one month post-transplantation of mouse germ cells into mouse seminiferous tubules. Up to the end of the second month post-transplantation mature donor spermatozoa were produced and by the end of the third month an average of 30% of the recipient testicles contained donor-derived spermatogenesis. The cumulative lateral spread of donor spermatogenesis in a recipient tubule was rapid, moving at approximately 55-60 μm per day. However, to calculate the length of the colonies of donor cells the authors used an eyepiece micrometer on a stereo microscope. This technique should be considered subjective and extremely labor intensive, thereby limiting its application for detailed analysis of donor spermatogenic colonization after transplantation *(comment by Dobrinski et al., 1999a).*

Dobrinski et al. (1999a) used a computer-assisted image analysis system that evaluated quantitatively a wide range of information available by microscopic observation of specimens. Donor spermatogenesis in a recipient testicle was identified by blue staining of donor derived spermatogenic cells expressing the E. coli lacZ structural gene. Stained seminiferous tubules containing donor-derived spermatogenesis were selected for quantitative analysis taking into consideration their color by color thresholding (range of blue). Colonization was appreciated as number, area, and length of stained tubules. Interactive, operator-controlled color selection and sample preparation accounted for less than 10% variability for all collected parameters. This system quantified the degree of colonization of donor germ cells in recipient testicles following the injection of a known number of donor cells. The relationship between the number of transplanted cells and the colonization efficiency was investigated. The authors found a linear correlation between the number of injected cells and the degree of colonization. Transplantation of 10^4 donor germ cells per mouse testicle only rarely resulted in recipient testicular colonization whereas after transplantation of 10^5 and 10^6 donor germ cells per mouse testicle the extent of donor derived spermatogenesis in the recipient seminiferous tubuli was directly related to the number of transplanted donor cells. It appears that approximately 10% of the transplanted spermatogonial cells participate in colony formation in the recipient testicle. *Shinohara et al. (2001)* using this computer-assisted imaging system technology found a 39-fold increase in mouse male germ line stem cells during development from birth to the maturity. In addition, they found small differences in the size of the colonies of the donor cells within the recipient testicle when the donor germ cells had been recovered from young or adult mice. Transplantation of donor germ cells was more effective when as recipients were used immature pup testicles *(Shinohara et al., 2001).* This supports the methodology of the transplantation program in our laboratory employing immature animals exclusively as recipients *(Tanaka et al., 1997; Shimamoto et al., 1999; Sofikitis et al., 1999a; Sofikitis et al., 1999b).* The authors suggested that the microenvironment of the pup testicle represents a more hospitable biochemical environment for transplantation of male germ-line stem cells. The colonization area per donor spermatogonium stem cell was 4.0 times larger in immature pup recipient testicles than in adult recipient testicles.

POTENTIAL CLINICAL APPLICATIONS OF MALE GERM CELL TRANSPLANTATION TECHNIQUES

A. Preservation of the whole genome of patients with oncological disease in recipient animals (using an animal testicle as a surrogate organ)

Cryopreservation of spermatogonia (for transplantation purposes into host animal testicles) from young patients with oncological disease who are subsequently scheduled for chemotherapy treatment is of great clinical importance. Male germ cells prior to the performance of chemotherapy can be frozen and subsequently post-thawing can be transplanted into immunodeficient animals to maintain the whole genetic information of the donor for a certain period. Additionally, xenogenic transplantation of patients' germ cells into an animal testicle may increase the number of human spermatogonia with potential to enter meiosis because subpopulations of the transplanted human spermatogonia are anticipated to undergo mitosis within the animal testicle. This is an advantage of combined freezing and transplantating germ cells from men with oncological disease over cryopreservation techniques alone because after the former procedures the number of transplanted spermatogonial stem cells will increase within the recipient testicle allowing later (after the end of chemotherapy) a larger number of spermatogonial stem cells to be transplanted back from the surrogate host animals to the patients. Thus the recipient animal testicle can be used to maintain for a certain length of time the genome of a patient with oncological disease and after the end of chemotherapy a larger number of patients' spermatogonia cells (compared with the number of spermatogonia that was originally transplanted into the recipients) can be transferred back to patients' testicle.

Sofikitis et al. (1999b) and *Jahnukainen et al. (2001)* suggested that maintenance of donor germ cells within a recipient animal may be susceptible to risks concerning transmission of virus from the recipient animal to the donor cells.

Schlatt et al. (1999) demonstrated a rapid and effective method for the enrichment of spermatogonia from testicular cell suspensions with a purpose to avoid contamination of donor cell population with malignant cells. Receptors for c-kit are present in the plasma membrane of spermatogonia. A magnetic cell separation technique using c-kit antibodies for detection of spermatogonia allowed fractions of spermatogonia from rat, hamster and monkey testicles to be purified. This approach could be used to select only desirable cellular populations to transfer into recipients in order to avoid contamination of the donor cellular populations with malignant cells. However, until a) improved methods for purging testicular cancer cells or b) new techniques for the early detection of contaminating malignant cells will develop, this technique may not be unequivocally accepted for routine performance.

B. Autotransplantation of frozen/thawed testicular germ cells in men with oncological testicular disease (without using a recipient animal testicle as a surrogate organ)

Using different techniques frozen/thawed human testicular germ cells can be transplanted back to the patient's testicle without using a recipient animal testicle as a surrogate organ as proposed in the previous paragraph.

Eighteen men with unilateral testicular oncological disease underwent unilateral

radical orchiectomy in the Department of Urology of University of Ioannina. At the time of unilateral orchiectomy germ cells were isolated from the contralateral healthy testicle. Biopsies from the contralateral testicle demonstrated absence of neoplasia in all patients. Fractions of testicular germ cells from the healthy testicle were then frozen. Four out the above 18 patients received the same standard chemotherapy protocol (for clinical low-volume stage II non-seminomatous germ cell tumors) post-orchiectomy. Six months after the end of the protocol of the administration of the chemotherapeutic drugs (bleomycin, etoposide, cisplatin), all four men were considered to be free of oncological disease and were found to be azoospermic. At that time the respective frozen/thawed germ cells were transplanted back to the rete testicle of the contralateral to the neoplasia testicle in two patients. Thirteen months post-transplantation the latter two men showed sperm concentration $9x10^6$ /ml, and $4x10^6$ /ml, respectively, percentage of motile spermatozoa 13%, and 8%, respectively, and percentage of morphologically normal spermatozoa (W.H.O. criteria) equal to 22% and 11%, respectively. In contrast at that time the remaining two patients who had received chemotherapy but did not undergo autotransplantation were still azoospermic. It appears that autotransplantation of testicular frozen/thawed germ cells post-chemotherapy can result in colonization of the human testicle and production of semen samples of sufficient quality for assisted reproduction technology.

C. Germ cell transplantation techniques for preservation of endangered species.

Cryopreservation and germ cell transplantation procedures can be applied for preservation of the germ cell line of older animals unable to breed, economically valuable animals, and endangered species. Prior to cryopreservation and transplantation procedures, a germ cell culture system increasing the number of spermatogonia in vitro and will allow freezing of a larger number of spermatogonia cells. Then frozen/thawed spermatogonia can be transferred to host animal recipients. Thus it is possible to maintain and increase further the number of donor spermatogonia within the host animal recipients. Some of the donor spermatogonia will generate a number of donor spermatozoa within the recipient testicles. These donor spermatozoa can be recovered and processed for assisted reproduction.

D. Survival and differentiation of germ cells from non-obstructed azoospermic men into recipient human individuals (human to human transplantation techniques).

Combined secretory dysfunction of human Sertoli and Leydig cells (for example due to varicocele) *(Sofikitis et al., 1996; Sofikitis et al., 1998a)* occasionally results in azoospermia. Isolation of spermatogonia from testicular biopsy material from the latter azoospermic men and transplantation into the seminiferous tubuli of men with proven azoospermia and lack of testicular spermatozoa due to Y-chromosome microdeletions may result in production of donor human spermatozoa by the recipient human testicle. Since as recipient men are chosen individuals with lack of testicular spermatozoa and azoospermia due to Y-chromosome microdeletions, spermatozoa that will appear in the semen post-transplantation are anticipated to be of the donor

man origin. An attractive hypothesis is that the recipient human Sertoli cells and the intratubular biochemical environment will support the donor human germ cells to differentiate. The above hypothesis is supported by experiments in animals demonstrating that intratubular environment from infertile recipients can support differentiation of donor germ cells from infertile subjects *(Ogawa et al., 2000).* It is also supported by our recent studies showing that there are no significant differences in androgen-binding protein activity in testicular cytosols between five azoospermic men with deletions in the AZFc area of Y-chromosome and 11 obstructed azoospermic men (androgen-binding protein activity in human testicular cytosols was assayed as we previously described; *Yamamoto et al., 2002).* Thus it appears that the secretory activity of Sertoli cells of men with Y-chromosome deletions is within "normal" values probably indicating that the intratubular environment of these men can support donor spermatogenesis. The above described methodology may allow azoospermic men without a genetic cause of azoospermia to produce their own spermatozoa in a recipient human testicle with complete arrest in spermatogenesis due to genetic reasons. GnRH-antagonists may be administered to the recipient man prior to transplantation to further disrupt his arrested spermatogenesis (i.e., by eliminating any present spermatid or spermatocytes and some spermatogonia). Thus the former men (azoospermic men without a genetic cause of azoospermia) may be candidates for assisted reproduction programs. Of course several ethical issues should be considered prior to the performance of such techniques.

E. The role of human germ cell transplantation procedures in the therapeutic management of non-obstructive azoospermia (human to animal transplantation techniques).

A significant percentage of non-obstructed azoospermic men have testicular foci of active spermatogenesis up to the spermatid or spermatozoon stage *(Silbert et al., 1996; Amer et al., 1997; Silbert et al., 1997; Antinori et al., 1997; Sofikitis et al., 1998a; Sofikitis et al., 1998b; Schlegel et al., 1999).* Ooplasmic injections of spermatozoa *(Silbert et al., 1995; Palermo et al., 1998),* spermatids *(Antinori et al., 1997; Amer et al., 1997; Van der zwalmen et al., 1997; Sofikitis et al., 1998b)* or secondary spermatocytes *(Sofikitis et al., 1998c)* recovered from testicular foci of spermatogenesis of non-obstructed azoospermic men have resulted in delivery of healthy newborns. In contrast, non-obstructed azoospermic men with complete premeiotic block in spermatogenesis do not have foci of haploid cells in their testicular tissue and therefore cannot be candidates for assisted reproduction programs, nowadays. Transplantation of human germ cells into a host animal testicle may give the opportunity to men with premeiotic block (not caused by a genetic factor exerting a direct effect on germ cells) to produce haploid cells within a host animal testicle and subsequently be candidates for ooplasmic injections of spermatids or spermatozoa in the future. Candidates for such transplantation procedures may be non-obstructed azoospermic men with premeiotic block due to acquired testicular damage. Azoospermic men with an inherent inability of their germ cells (due to a genetic factor affecting directly the germ cells) to undergo meiosis will not have a benefit from such transplantation procedures. Transplantation techniques into animals represents the only

hope for men with incurable complete premeiotic block in spermatogenesis due to acquired testicular damage (such as varicocele, mumps-orchitis, trauma, and so on) to father their own children.

Considering that testicular foci of spermatogonia can be found in the vast majority of non-obstructed azoospermic men who are negative for haploid cells in the therapeutic testicular biopsy material *(Sofikitis et al., 1998a)* it appears that successful induction of human meiosis and spermiogenesis within a recipient animal testicle has the potential to increase the number of non-obstructed azoospermic men who can be candidates for assisted reproduction programs in the future.

F. Assisted reproduction techniques using human haploid cells generated into animal testicles; genetic and immunological risks.

It should be emphasized that application of assisted reproduction procedures using human spermatids or spermatozoa produced in an animal testicle is susceptible to ethical considerations and genetic, immunological, and infection-related risks. Considering that the cell nuclear DNA is protected by the nuclear envelop and surrounded by the respective cell cytoplasm it may be suggested that the nuclear genetic material of human spermatids/spermatozoa generated into a recipient testicle has not been contaminated by the recipient species-nuclear DNA. However, additional studies are necessary to confirm it. Furthermore, the prolonged contact of human germ cells with recipient Sertoli cells /germ cells during human spermatogenesis into the recipient testicle may result in binding on the human germ cell cytoplasmic membrane of molecules / antigens that are characteristic of the recipient species. Therefore, subsequent ooplasmic injections of human spermatozoa or spermatids recovered from animal testicles may lead to the production of human embryos positive for a recipient antigen molecule that is normally absent in the human species. Finally, the presence of a virus within the recipient host testicle may result in infection of the transplanted human germ cells and of the subsequently derived human haploid cells with an overall result transmission of the virus to the human embryonic cells and tissues after human assisted reproduction techniques.

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AROMATASE INHIBITORS IN MALE INFERTILITY

Kaan AYDOS

Önder YAMAN

Department of Urology and Research Center on Infertility,
School of Medicine, University of Ankara, Turkey.
ksaydos@superonline.com

Since its introduction as an estrogen suppressor agent, aromatase inhibitors have extensively been used to treat the women with breast carcinoma. The employment of aromatase inhibitors in the treatment of male infertility has been based on the theory that increased estrogen production or an inappropriate ratio of estrogen to testosterone may have an adverse direct or indirect effect on spermatogenesis. In this fashion, aromatase inhibition may improve testicular exocrine function by lowering the serum estradiol-to-testosterone ratio and/or increasing FSH. In this article, we first describe some of the basic elements of hormonal regulation of testicular functions and testosterone-estrogen interaction, which serve as background information for the ensuing sections. Specific factors affecting aromatization pathway are described in detail. Following this, clinical results of aromatase inhibitors in the treatment of infertile men are reviewed.

HORMONAL REGULATION OF TESTICULAR FUNCTIONS

Central regulation and testosterone

A large body of evidence available suggests that for quantitatively normal spermatogenesis to occur, the testis requires stimulation by the pituitary gonadotropins-follicle stimulating hormone (FSH) and luteinizing hormone (LH). While a wide variety of effects of FSH on the seminiferous epithelium has been described, LH exerts its influence on spermatogenesis by stimulating testosterone secretion from the Leydig cells *(for review see Mak et al., 2000)*. Although FSH and LH are the key factors for optimal germ cell survival, their removal from the circulation either by hypophysectomy or use of gonadotropin-releasing hormone (GnRH) antagonists does not prevent entry of stem cells into the spermatogenic cycle. In fact, the need for both FSH and testosterone has been emphasized in the restoration of spermatogenesis *(Matorras et al., 1997).* Receptors for both hormones can be found on the Sertoli cells, and testosterone receptors are also found on the peritubular cells (*McLachlan et a1., 1994; Mauras et al., 2000*). FSH stimulates the production of an androgen-binding protein (ABP) in the seminiferous epithelium by the Sertoli cell, which acts as a carrier protein for androgen within the lumen of the seminiferous tubules and within the epididymis. The action of FSH is thus seen as providing a concentration gradient for testosterone that is directed toward the tubular lumen and a transport mechanism for androgen to reach the developing germ cells and stimulate their maturation (*Moreno et al., 2000*).

Thus, the survival of testicular germ cells appears to be dependent on gonadotropins and intratesticular testosterone (*Bartke, 1995*).

Estrogens

Although testosterone is the principal sex steroid in testis, the occurrence of estrogen synthesis, the existence of abundance estrogen receptors (ER) in male reproductive tissues (*Schleicher et al., 1984*), as well as data from knock out animals (*Krege et al., 1998*) indicate that spermatogenesis regulation is also under the influence of estrogens. Approximately 20% of estrogens present in blood are produced by the testis (*de Jong FH 1973*). Recent studies have shown that testicular germ cells and sperm, as well as Leydig and Sertoli cells are major cell types that produce estrogen in the male (*Janulis et al., 1998*). ERalpha and ERbeta genes are reported to be coexpressed in germinal and nongerminal epithelia of the mature testis of channel catfish and seem to be developmentally regulated in spermatocytes. These observations are, as reportedly, consistent with the concept that estrogens, via interaction with ERalpha and ERbeta, participate in the regulation of male gamete development and fertility (*Bilinska et al., 2000; Vecino et al., 2001*). Elaborate sperm transplantation studies have shown that the altered sperm function characteristic of the ERalpha knockout male are the result of the loss of ERalpha actions in the supporting somatic cells of the testis and epididymis rather than in the germ cell (*Couse et al., 2001*).

The implication of estrogens in gonadal differentiation has further been confirmed in various studies. When given to embryos of emys orbicularis, the nonsteroidal aromatase inhibitor letrozole was found to induce gonads with different degrees of masculinization, from ovary-like to testis-like. There was a strong correlation among aromatase activity, gonadal structure, and Mullerian duct status; high levels of aromatase were found in ovary-like gonads, however, low levels were found in strongly masculinized gonads (*Richard-Mercier et al., 1995*). These results confirm the importance of estrogens in gonadal differentiation and testicular structure formation as well.

Although it has been known for many years that estrogen administration has deleterious effects on male fertility, data from transgenic mice deficient in estrogen receptors or aromatase indicate an essential physiological role for estrogen in male fertility. Data available lead to the interesting observation that estrogen receptor function is absolutely required for normal spermatogenesis (*Smith et al., 1994*) and its disruption causes alteration of spermatogenesis and infertility (*Eddy et al., 1996*). Supportingly, estrogen has been shown to regulate the reabsorption of luminal fluid in the head of the epididymis and disruption of this essential function causes sperm to enter the epididymis diluted, rather than concentrated, resulting in infertility (*Hess et al., 1997*). Additionally, spermatogonial stem cell renewal was promoted by estradiol implantation so that; estrogen is considered as an indispensable “male hormone” in the early spermatogenetic cycle (*Miura et al., 1999*). An alternative or complementary explanation, given the recent identification of estrogen receptors (ERalpha and ERbeta) and aromatase within various cell types in the testis, is that estrogens also exert paracrine actions within the testis to

promote spermatogenesis (*Ebling et al., 2000*).

Contrary to the spermatogenesis-regulating effect of estrogens, elevation of testicular estradiol level has also been reported to be possibly one of the causes of male infertility (*Akiyama., 1997*). In rats, high doses of estrogen cause tubular damage, disorganization of the cytoarchitecture in the seminiferous tubules, vacuolation, absence of lumen and compartmentalization of spermatogenesis (*Leon et al., 1987; Gill-Sharma et al., 2001*). Likewise, in men increase in the rate of aromatase activity and the concentration of estradiol were found to correlate with the decreased Johnsen's score count (*Ichikawa, 1995*).

AROMATASE INHIBITION AND TESTICULAR FUNCTIONS

Intricate relation between estrogens and testosterone

Normal spermatogenesis depends upon the proper interaction of the androgens and estrogens. The data available suggest an intricate relation between the testosterone and estrogen levels in the testis. This relationship may be complex and depend on a certain ratio between the two hormones. It is well known that estradiol synthesized in the testis plays a role in the regulation of testicular testosterone production in some species (*Tapanainen et al., 1989*). The Leydig cell aromatase activity is, in part, regulated by some certain testicular paracrine factors including estrogens. It is noteworthy that the Leydig cell testosterone synthesis is highly stimulated by LH and the addition of seminiferous tubular culture medium further increases testosterone outputs which brings about a high intratesticular testicular level necessary for development of spermatogenesis (*Carreau et al., 1988*). Testosterone secretion in turn regulated by estrogens. Experiments with intact and hypophysectomized animals have demonstrated that estrogens can inhibit testosterone secretion by acting directly on the testis (*for review see Moger, 1980*).

Complexity of estrogen-testosterone interaction has further been proved by a large body of evidences. In cases with elevated estradiol-to-testosterone ratio the suggestion that lowered testosterone is not a major contributor to impaired spermatogenesis was raised based on the investigation indicating that exogenous testosterone was not capable of restoring declined spermatogonial number in the estradiol-treated rats despite the partial or full restoration of testicular testosterone levels (*Meachem et al., 1997*). However, no evidence was found for the involvement of non-androgenic Leydig factors in the control of spermatogonial numbers. It is possible that both androgens and estrogens participate in the regulation and maintain of spermatogenesis, and that estrogen deficiency or abundance has an impact on the gonad's output of sperm. Also, prevention of disruption of spermatogenesis induced by high dose estrogen, by antiestrogens indicates crosstalk between androgen and estrogen receptors in Sertoli cells (*Gill-Sharma et al., 2001*).

Aromatase enzyme and its functional regulation

The biosynthesis of estrogens from androgens is catalyzed by the terminal enzyme aromatase, an enzyme localized in the endoplasmic reticulum that consists of

two components: a cytochrome P450 (P450 Arom, P450 19 product of the CYP19 gene) and the NADPH cytochrome P450 reductase. P450 19 is a unique form of the P450 superfamily and also the aromatase has been supposed to be a good target for the development of selective P450 inhibitors (*Vanden Bossche et al., 1994*). Aromatase cytochrome P450 is found in gonads as well as adipose tissue and many other tissues (*Simpson et al., 1997*). *Carreau et al. (2001)* have evidenced a positive correlation between a fully developed spermatogenesis and a strong immunoreactivity for both P450arom and ERbeta not only in Sertoli cells but also in pachytene spermatocytes and round spermatids. Indeed, Leydig cells, Sertoli cells and spermatozoa have recently been shown to express aromatase enzyme as reported in experimental studies (*Tsai-Morris et al., 1985; Janulis et al., 1996; Janulis et al., 1998*). Human spermatozoa, mainly the tail and midpiece are a potential site of P450-aromatase protein expression and estrogen biosynthesis (*Aquila et al., 2002*). But the major source of circulating estradiol in man is from peripheral conversion of androgens to estrogens by the aromatase enzyme in adipose tissue (*Longcope et al., 1978*). Nevertheless, the overall data confirm the presence of a functional cytochrome P450arom in the male testis and consequently, suggest a physiological role for androgens and estrogens in the regulation of spermatogenesis.

Testicular aromatization has been evaluated extensively (*Canick et al., 1979; Dalterio et al., 1983; Ichikawa, 1995*). But the mechanism playing role in the regulation of aromatase enzyme activity is more complex than known. Indeed, it has been demonstrated that the monkey testis and, to a lesser extent, the epididymis could aromatize androgens (*Pereyra-Martinez et al., 2001*). However, in the epididymis a discrepancy between the aromatase activity and the mRNA was found pointing out the fact that aromatase activity was not strictly regulated at the level of RNA expression and that other mechanisms for this regulation should be considered. Physiologic studies have shown that aromatization pathway is strikingly dependent on the surrounding hormonal milieu in the testis. In fact, the Sertoli cell secretes a number of compounds, some of which are postulated to serve as chemical signals to the neighboring cells (*Ritzen, 1983*). Interestingly, aromatase inhibitors consist an example of Sertoli cell secretory products whose production is dependent on the type of surrounding germ cells. Further, it is known that androgens are the major factor that stimulates the production of Sertoli cell inhibin. Sertoli cell aromatase activity that converts the androgens into the estrogens is also stimulated by FSH (*Verhoeven & Franchimont, 1983*). Supportingly, there exist influence of FSH in female gonads for the induction of aromatase enzyme and LH receptors (*Erickson et al., 1985*). Similarly, previous findings demonstrated a direct inhibitory influence of tri-iodothyronine (T3) on aromatase activity and estradiol production in peripuberal Sertoli cells. In this context, a possible role of T3 on the interplay between testicular steroids and Sertoli cells has been investigated and consequently, thyroid hormone was found to modulate up-regulation of androgen and down-regulation of estrogen receptor content in the Sertoli cells of peripubertal rats, which possibly influences the androgen responsiveness of the Sertoli cells during spermatogenesis (*Panno et al., 1996*).

These results clearly emphasize the importance and complexity of the aromatization pathway interacting with Sertoli cell functions. Otherwise, the role of the aromatase activity in the peritubular cell-Sertoli cell interactions controlling Sertoli cell function by androgens was further investigated and testicular peritubular cells were found to secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells (*Verhoeven et al., 1988a*). Coculture with peritubular cells was shown to increase the sensitivity and/or the responsiveness of a number of Sertoli cell parameters including aromatase activity to androgens. This effect is in part mediated by the secretion of one or more diffusible factors (P-Mod-S) by the peritubular cells (*Verhoeven et al., 1992*). Otherwise, PModS alone was not found to affect aromatase activity at any of the developmental stages as examined in rats at various stages of pubertal development (*Rosselli & Skinner, 1992*). Interestingly, PModS suppress' the ability of FSH to stimulate aromatase activity and estrogen production in midpubertal Sertoli cells.

On the other hand, aromatase activity of Leydig cells was also indicated based on the experimental studies demonstrating that Leydig cell tumor had an active autonomous aromatase system that is accompanied with elevated serum estradiol concentrations. So that, although has not been confirmed, in infertile men with Leydig cell hyperplasia, severely impaired sperm production could be resulted from excess estrogen (*Orczyk et al., 1987*). Further, the effects of fibroblast growth factor (FGF) on testicular aromatase activity was studied previously, and a role has been suggested for FGF as a paracrine/autocrine agent in the control of estrogen secretion by Leydig cells (*Raeside JI, 1988*). Furthermore, transforming growth factor beta 1 (TGF beta 1) has inhibitory effect on FSH-induced aromatase activity (*Morera et al., 1992*). Aromatization is further stimulated by activin, alpha-MSH, growth hormone as well as FSH but is inhibited by aromatase inhibitor, inhibin, FSHBI (FSH binding inhibitor) (*for review see Demoulin & Franchimont, 1989; Closset et al., 1991*). However, the physiologic significance of these factors in germ cell-Sertoli cell interaction throughout the seminiferous cycle is unclear.

From the point of the androgen therapy, aromatase activity is once again important. Because testicular androgens may be responsible for the decrease in FSH-inducible aromatase activity, prolonged exposure to androgens are expected to suppresses follicle-stimulating hormone-induced aromatase activity in Sertoli cells, as observed in rat trials (*Verhoeven et al., 1988b*). *Scaglia & Carrere (1991*) demonstrated that in some oligoasthenoteratozoospermic or azoospermic patients testicular hormone production is altered. According to their data, when serum biphasic pattern of hCG-stimulated testosterone was altered, aromatase inhibitors (aminoglutethimide or testolactone) induced an improvement of the acute testosterone response only in patients with high or normal 17 OH-progesterone response to hCG, whereas no effects were observed in patients with low 17 OH-progesterone response. However, in cases of normal pattern of hCG-stimulated testosterone, the aromatase inhibitors induced no changes in the testosterone response. Recently, a family with the aromatase excess syndrome is described, in which the condition was inherited in an autosomal dominant man-

ner, led to feminizing manifestations in both sexes, and was associated with the aberrant utilization of a novel transcript of the P450arom gene (*Stratakis et al., 1998*). Treatment with an aromatase inhibitor (testolactone) and a GnRH analog successfully delayed skeletal and pubertal development. Afterword, an endocrinopathy in men with severe male factor infertility is identified, that is characterized by a decreased serum testosterone-to-estradiol ratio (*Pavlovich et al., 2001*). Another observation emphasizing this intricate ratio is estrogens' dose-related effects on spermatogenesis. Indeed, low doses of estrogens appear to maintain whilst high doses reversibly disrupt spermatogenesis (*Gill-Sharma et al., 2001*). Disclosure of these mechanisms of intratesticular regulation permited to discover the aromatase inhibitors as a therapeutic agent capable of correcting certain male factor infertility cases. Indeed, aromatase inhibition may offer potential benefits in the establishment of the estrogen-to-testosterone balance, as reported recently (*Raman & Schlegel, 2002*).

However, aromatization pathway has an important effect not only in male infertility but also in some other clinical conditions. Elevated aromatase activation is thought to play a significant role in these diseases. For instance, a study of a large cell calcifying Sertoli cell tumor of the testis associated with bilateral gynecomastia in an 8-year-old boy postulated that tumoral cells might stimulate neighboring interstitial cells to differentiate into Leydig cells and to secrete androgens, which in turn might have been aromatized to estrogens by tumoral cells, which results in gynecomastia (*Berensztein et al., 1995*). Further, impaired spermatogenesis in patients with testicular germ cell tumor was shown to be caused by increased aromatization and in situ estrogen production in Leydig cells of the non-neoplastic testis and in interstitial or stromal cells of the tumor, as well as increased tumor size (*Nakazumi et al., 1996*). Increased aromatase activity and impaired androgen receptor functions as well was also encountered in a female patient with partial androgen insensitivity (*Isurugi et al., 1996*). Interestingly, stress seems to exert a modifying effect upon the aromatase and 5 alpha-reductase hypothalamic activity, as shown in rats (*Tarasenko et al., 1996*). Furthermore, excessive or inappropriate aromatase expression in adipose fibroblasts and endometriosis-derived stromal cells, as well as in testicular, hepatic, adrenal and uterine tumors, is associated with abnormally high circulating estrogen levels and/or with increased local estrogen concentrations in these tissues. Elevated estrogen levels will in turn promote the growth of hormone-responsive tissues. Regarding these data, a common metabolic abnormality associated with activation of a cyclic AMP-dependent signaling pathway that gives rise to activation of aromatase in all of the affected tissues is suggested, being a common features of excessive aromatase expression in these disease states (*for review see Bulun et al., 1997*).

Influence of aromatase inhibition on regulation of testicular functions

The theoretic basis of the aromatase inhibition in the treatment of infertile men is that increasing the serum testosterone-to-estradiol ratio and/or FSH may improve testicular exocrine function. Indeed these female hormones or the ratio androgens/estrogens do play a physiological role (either directly on germ cells or via

testicular somatic cells) in the maintenance of male gonadal functions and obviously, several steps are concerned particularly the spermatid production and the epididymal sperm maturation (*for review see Carreau et al., 2001*).

One of the proposed effects of aromatase inhibitors in the treatment of male factor infertility is on FSH secretion. Experimental studies showed that there is differential regulation of FSH and LH secretion in the adult male rat, such that FSH is more sensitive to the effect of aromatase inhibitor administration (*Turner et al., 2000*). Indeed, treatment with aromatase inhibitors resulted in a significant increase in plasma FSH concentrations (*Mauras et al., 2000; Trunet et al., 1993*). Estrogen is also involved in the regulation of FSH secretion (*Turner et al., 2000*). There is a general consensus on the need for FSH in the regulation of spermatogenesis in humans. Various attempts have been made to induce sperm production in infertile men with oligozoospermia by administering hMG or FSH, but the results are still controversial. Since gonadotropins participate in regulation of normal spermatogenesis and are effective in the treatment of hypogonadism (*Matsumoto et al., 1986; Mastrogiacomo et al., 1991*), they were applied in idiopathic male infertility based on the assumption that increase of gonadotropin concentrations may lead to improved function of the seminiferous epithelium with a further stimulation of spermatogenesis. The main premises of empirical stimulation therapy are that there may exist a relative disproportion between the levels of certain hormones and their target cells, and the radioimmunoassays used to measure serum gonadotropins may not accurately reflect levels of biologically active gonadotropins. In a group of men with a history of failed fertilization in previous IVF attempts, Acosta et al. administered pure FSH at least 3 months and observed that fertilization rate was significantly improved, giving 26% term pregnancy rates per transfer (*Acosta et al., 1992*). Their results emphasized the benefits of systemic FSH administration as an adjunct to assisted reproduction in selected cases of severe male factor infertility. These results gave rise to other studies aimed to stimulate testicular exocrine function by FSH. Although several studies suggested that patients with severe male factor infertility might benefit from FSH in terms of sperm parameters and pregnancy rates (*Dirnfeld et al., 2000; Ashkenazi et al., 1999*), other randomized and placebo-controlled studies did not show any significant improvement of seminal parameters (*Matorras et al., 1997; Kamischke et al., 1998*).

The mean drawback for hormone therapy in men with idiopathic infertility is improper patient selection. In fact, it has been demonstrated that highly purified FSH could be the appropriate treatment for a group of selected patients with oligozoospermia when FSH plasma level is in the normal range and the testicular tubular structure is characterized by hypospermatogenesis without maturation disturbances (*Foresta et al., 2000*). Also, the presence or absence of testicular FSH receptors is found to be an important predictive parameter for the responsiveness to the FSH treatment (*Okuyama et al., 1998*). Further, FSH stimulation in the GnRH test has also been proposed as one of the methods to select the patients who may benefit from the treatment with pure FSH (*Glander & Kratzsch, 1997*). These results clearly indicate the need of proven criteria

being able to discriminate the FSH-responder patients from FSH-nonresponder ones.

Nevertheless, empirical increasement of FSH has not been regarded as useful in the treatment of all infertile patients since conventional semen analysis is a relatively insufficient tool for the diagnosis of male infertility and determination of the treatment outcome. On the other hand, FSH administration has important impacts on the subcellular organizations of the germ cells, which may not be reflected by standard microscopic semen analyses. Electron microscopic examination has revealed an improved fine architectural pattern, mainly involving acrosome, head and chromatin and middle-piece, in accordance with the positive changes of functional data (*Arnaldi et al., 2000*). Other studies have also proved the FSH treatment to be effective in improving the ultrastructural features of semen (*Bartoov et al., 1994; Baccetti et al., 1997*). Additionally, FSH administration has been found to prevent apoptosis in a stage-specific fashion (*Henriksen et al., 1996*). Probably, the high efficiency of FSH before IVF in the fertilization rates with no significant changes in seminal parameters may be secondary to sperm quality instead of quantity.

In addition to FSH increasing effect of aromatase inhibitors, lowering of estrogen-to-testosterone ratio is another mechanism by which spermatogenesis is expected to be stimulated. Aromatase inhibitors lower serum estradiol levels in men through the inhibition of the aromatization of testosterone to estradiol and androstenedione to estrone, and does not have any intrinsic androgenic or estrogenic activity. Lowering of estrogen, subsequently, induces increase in FSH concentration. In men aromatization of testosterone to estrogens was reported to be a prerequisite for the central regulation of gonadotropin secretion (*Loveland & de Kretser, 1999*). In the human male, estrogen has dual sites of negative feedback, acting at the hypothalamus to decrease GnRH pulse frequency and at the pituitary to decrease responsiveness to GnRH (*Hayes et al., 2000*). It has been shown that chronic administration of an aromatase inhibitor to intact male dogs led to an increase in both serum testosterone and LH in the presence of complete inhibition of estrogen production in the testes, thus suggesting the aromatizing pathway through estrogens being a major factor in mediating the effects of testosterone on the regulation of LH secretion and that the rise in serum testosterone was a consequence of the elevated serum LH (*Lunenfeld, 1986*). In this fashion, aromatization of testosterone to estrogen locally within the hypothalamus/pituitary gland is responsible for this negative feedback loop (*Turner et al., 2000*).

During the embryonic development, aromatase inhibitors administered before sexual differentiation of the gonads can induce sex reversal *(Vaillant et al., 2001).* In treated females, masculinization of the genital system was characterized by the differentiation of the gonads into a testis or an ovotestis, and this transdifferentiation occurred all along embryonic and postnatal development; thus, new testicular cords/tubes were continuously formed while others degenerated. Indeed, studies examining the sex-reversing potential of aromatase inhibitors have confirmed the spermatogenesis and spermiogenesis by histological examination of the gonads (*Wennstrom et al., 1995*). Formation of

male-specific structures and regression of female primordia are regulated in early male embryogenesis by testis-determining gene (SRY) on the Y chromosome, and directly controls male development through sequence-specific regulation of target genes (*Haqq et al., 1993*). In mammals, this male-determining gene of the Y chromosome may code for an intrinsic aromatase inhibitor. Studies showed the gene's product had a binding domain that recognizes regulatory elements in the promoter of the aromatase gene. Specifically recognizing ability of the SRY DNA-binding domain the proximal upstream elements in the promoters of the sex-specific genes encoding P450 aromatase was presented in other studies (*Haqq et al., 1993*).

Consequently, for the cellular physiology of sex steroid sensitive cells, the androgen/estrogen ratio may be more important than only one hormone action per se (*for review see Seralini & Mostemi, 2001*). This ratio is controlled in vertebrates by aromatase; its gene expression can be inhibited in different ways, and this is crucial for the treatment of estrogen-dependent diseases such as in male infertility for instance. To reach this goal, non-steroidal inhibitors have recently been used as first line agents. So that, testosterone-to-estrogen ratio may be a satisfactory selection criterion, as indicated previously, to predict the possible outcome of the aromatase inhibition treatment. Nevertheless, aromatase inhibitors can modulate the estrogenic balance essential for male gonadal function and available clinical data suggest that they may be a useful tool with which to ameliorate the semen parameters. However, complete suppression of aromatase enzyme may have adverse effects, as is evident in postmenopausal women (increased osteoporosis, cardiovascular disease, and urogenital atrophy). Although in male trials such inverse effects have not been reported even in high doses, further studies are warranted to establish the confident dose-response efficacy.

AROMATASE INHIBITORS IN THE TREATMENT OF INFERTILE MEN

Clinical trials

Experimental studies suggested that specific inhibitors of estrogen biosynthesis might be suitable to some extent for the treatment of estrogen-induced impairment of spermato- and spermiogenesis (*Leschber et al., 1989*). In a pioneer study, despite nondetectable amounts of intratesticular estradiol and estrone and higher amounts of testosterone, androstenedione and dihydrotestosterone than testes of control dogs were obtained by chronic treatment of male dogs with an orally active nonsteroidal aromatase inhibitor, no evidence on any effect of aromatase inhibition upon spermatogenesis was reported (*Juniewicz et al., 1988*). Further, Turner et al evaluated the effect of anastrozole on testicular histology and found that the majority of treated rats had testes with normal spermatogenesis but, occasionally, seminiferous tubules showed abnormal loss of germ cells or contained only Sertoli cells (*Turner et al., 2000*). Because no significant change in the plasma estradiol concentrations was observed during the treatment period, excess serum estrogens may be supposed having significant pathological effects on testes. In fact, increase of the rate of testicular aromatase activity and the following elevation of testicular estradiol level has been proposed as one of the possible causes of male infertility (*Akiyama, 1997*), and with anastrozole treatment a

decrease in estrogen-to-testosterone ratio was obtained in association with increased semen parameters (*Raman & Schlegel, 2002*). However, whether the amelioration in spermatogenesis is a result of local suppression of intratesticular estrogen-to-testosterone ratio or systematic normalization of aromatase activity is a subject of continued debate. Nevertheless, in these cases inappropriate ratio of estrogen to testosterone is suggested to result in the pathophysiology of impaired spermatogenesis.

In several studies, alterations of spermatogenesis have been evaluated when serum estradiol levels were decreased by suppression of aromatase activity and many suggested a beneficial effect on sperm parameters and hormonal profiles in men with normal gonadotropins and idiopathic oligozoospermia. However, inhibitors of this enzyme with more favorable properties had been necessary since the most extensively utilized inhibitor, aminoglutethimide, lacks specificity and causes frequent side effects. Data available suggest that nonsteroidal compounds have more favorable properties as a specific and potent aromatase inhibitor.

They are generally described as type 1 inhibitors which are steroidal compounds (testolactone and aminoglutethimide) that may be purely competitive inhibitors, or type 2 inhibitors which are non-steroidal (anastrozole, letrozole and exemestane) and bind to the haem group of the aromatase enzyme by co-ordination fashion through a basic nitrogen atom (*for review see Dowsett, 1999*). They were originally used in the treatment of women with metastatic breast carcinoma and found to be safe and well-tolerated medication and subsequently has been tried in men with idiopathic infertility.

Testolactone has long been used in advanced female mammary cancer. Pharmacological properties were well documented in both animals and humans. Clinical implications of testolactone treatment have been evaluated in various conditions related with hormonal disturbances, such as carcinoma of the pancreas (*Waddell, 1973*), uterine sarcoma (*Tseng et al., 1986*), pubertal gynaecomastia (*Zachmann et al., 1986*), benign prostatic hyperplasia (*Schweikert et al., 1987*), precocious puberty (*Wheeler & Styne, 1990*), epilepsy (*reviewed by Herzog et al., 1991*), familial adenomatous polyposis (*Tsukada et al., 1992*), congenital adrenal hyperplasia (*Laue et al., 1996*), and male fertility disturbances as comprehensively documented below. It is one of the pioneer aromatase inhibitors that has been studied in men with impaired spermatogenesis. When used 500 mg twice daily for 4 weeks, in patients with idiopathic oligospermia lowered circulating estradiol levels by about 30%, enhanced the secretion of follicle-stimulating hormone, and testosterone (+ 30%), but did not affect serum luteinizing hormone levels (*Dony et al., 1985*).

Letrozole (CGS 20267) as well, had previously been tried in male rats and dose-dependent suppression of serum estradiol and an increase in serum testosterone and LH were seen (*Bhatnagar et al., 1992*). In men, the systemic and subjective tolerability was shown to be good at single doses ranging from 0.02-30 mg (*Trunet et al., 1993*). Further, when used in boys with constitutional delay of puberty, letrozole inhibit-

ed estrogen synthesis and delayed bone maturation (*Wickman & Dunkel, 2001*). In another study, 35 boys who were referred to because of suspicion of delayed puberty were treated by letrozole-plus testosterone for 5 months, and significant inhibition of endogenous estrogen synthesis and increased LH concentration as well were seen (*Wickman & Dunkel, 2001*). Although majority of the data about letrozole have been obtained from the female studies, there are marked gender differences concerning pharmacokinetics, at least as seen in rats (*Liu et al., 2000*). Plasma concentration in female rats is about 7.4-fold of that of male rats probably due to estimated terminal phase half-live differences being 10.5 in males and 40.4 h in females. Metabolically, letrozole absorption decreases slightly under fed conditions, however, in view of the half-life of about 2 d in human this small change in the absorption rate is not considered to be of clinical relevance for treatment with repeated administrations (*Sioufi et al., 1997*).

Anastrozole, which is another potent and highly selective aromatase inhibitor, has also long been employed in the treatment of postmenopausal women with advanced breast cancer. In experimental studies, anastrozole was found to be selective for the aromatase enzyme, having no detectable pharmacologic activity other than aromatase inhibition, and did not interfere with steroid hormones produced by the adrenal glands. Also in human studies, anastrozole had no clinically significant effects on key enzymes that regulate cortisol and aldosterone biosynthesis. 1 to 10 mg doses suppress estradiol to the maximum degree measurable. Its absorption is rapid and maximum plasma concentrations occur within 2 hours after oral administration (*Plourde et al., 1994; Dukes et al., 1996*).

Until now, anastrozole has been used in male subjects for various experimental and clinical purposes. Initially, anastrozole was administered to male canine and when given with a 5-alpha-reductase a significant increase in both prostate and testicular volume were observed (*Suzuki et al., 1998*). Then, *Turner et al. (2000)* evaluated the effects of anastrozole on male reproductive function in adult rats. In addition to plasma FSH and testosterone concentrations, testis weight also increased significantly. As stated previously, histological evaluation of the testes revealed that spermatogenesis was grossly normal. The metabolic effects of selective estrogen suppression in the male using anastrozole, was further investigated by *Mauras et al. (2002)* and 50% decrease in estrogen concentrations with 1 mg daily doses was obtained. After 10 days of treatment, there were no significant changes in body composition (body mass index, fat mass, and fat-free mass) or in rates of protein synthesis or degradation, oxidation, muscle strength, calcium kinetics, or bone growth factors concentrations. *Schnorr et al. (2001)* used oral anastrozole (loading dose of 30 mg on the first day, and then 5 mg twice daily) for 5 days in healthy young men; and in summary, anastrozole abolished testosterone-dependent inhibition of GnRH-stimulated LH and FSH release. Subsequently, *Hayes et al. (2002)* used 10 mg/day anastrozole for 7 days in normal and idiopathic hypogonadotropic hypogonadic men and 41% of suppression in serum estrogen was obtained. However, in this study no clinical finding was reported. Interestingly, *Faglia et al. (2002)* treated a boy previously operated on for a hamartoma causing precocious puberty and pre-

senting with advanced bone maturation and nearly fused epiphyseal cartilages, with 1 mg/day anastrozole for 3 yr. Treatment delayed the epiphyseal closure and improved the final height. In another study, fifteen men over 65 yr were treated for 9 weeks with 2.0 mg/day of anastrozole, and the rate of bone resorption secondary to estrogen depression was shown to be limited by endogenous estrogen derived from aromatization of testosterone (*Taxel et al., 2001*). Further, to assess whether or not anastrozole has any anticoagulant activity, 16 men were given 7 mg of anastrozole loading dose on day 1, followed by 1 mg daily for 10 day. Overall, anastrozole was found having no effect on clotting mechanisms or on the pharmacodynamic activity of warfarin (*Yates et al., 2001*). Anastrozole was also tried in men with prostat cancer refractory to androgen-related therapies, but no patient experienced an objective response or disease stabilization (*Santen et al., 2001*).

Treatment results in infertile men

All the data available have confirmed the tolerability and safety of aromatase inhibitors in male gender. Also taking into consideration the role of the estrogens and FSH in the regulation of testicular functions, further attempts were undertaken to treat male factor infertility by this kind of drugs. It was hoped that suppression of estrogen as well as increased testosterone would induce the spermatogenesis observed when these patients are treated with an aromatase inhibitor. Although based on seminal parameters there seem to be striking ameliorations, little information is available on pregnancy rates. In a pilot study with the aromatase inhibitor testolactone, *Vigersky & Glass (1981)* treated ten men with idiopathic oligozoospermia and reported a dramatic decrease in the estrogen-to-testosterone ratio. Basal and LHRH-stimulated serum gonadotropin levels were unaffected by these changes. Sperm density rose from 10.8 ± 2.5 to 19.8 ± 4.7 x 10^6/ml and total sperm count from 26.8 ± 6.5 to 60.6 ± 14.3 x 10^6. There was no significant change in motility. 30% of the wives became pregnant. Their data suggested that testolactone might be an effective treatment for men with idiopathic oligozoospermia and it's lowering effect of estrogen levels might had been responsible for the improvement in spermatogenesis. These ensuring clinical data resulted in the successful induction of testicular functions using aromatase inhibitors.

Dony et al. (1986) showed a gradually rise in sperm density from 8.1 ± 1.3 before to 21.3 ± 6.7 X 10^6/ml after 6 months of testolactone treatment, whereas the total sperm count almost threefold increased. Sperm concentrations exceeding 20 X 10^6/ml were achieved in 4 of the 9 patients. Two of these patients' wives became pregnant. The estradiol decrease was accompanied by a temporary increase in the levels of FSH, not of LH, but less of testosterone. The testosterone/estradiol ratio almost doubled until the end of the treatment period.

In a subsequent study, *Schill et al. (1987)* reported 17% pregnancy rate after treating men with idiopathic oligozoospermia for 3 to 6 months with 1 mg testolactone daily. A significant increase in the sperm count, total sperm output and the absolute number of motile and progressively motile spermatozoa was observed. There was a significant increase of testosterone and FSH after 1 and 3 months of treatment,

while the increase in testosterone/estradiol ratio was more than twice.

Maier & Hienert (1988) used 150 mg testolactone/day in combination with 30 mg tamoxifen/day and compared the results with those of only 30 mg tamoxifen/day. The increase in sperm density was significant in both groups, but no improvement of the other ejaculate parameters was seen in either groups. The incidence of gravidity was the same being 15%. This study confirmed that testolactone prevents elevation of estradiol levels during monotherapy with tamoxifen.

Itoh et al. (1991) suggested that an aromatase inhibitor might be effective to male infertile patients with high serum estradiol levels. In that study nine male infertile patients were treated with testolactone for 3 months. Four of them had an increase in sperm count (more than 10 x 10^6/ml relative to base line). Serum estradiol levels and estradiol/free testosterone ratio were significantly decreased after treatment. In particular four patients whose sperm counts were improved after testolactone treatment had high values of basal serum estradiol levels and estradiol/free testosterone ratio before treatment, and these values were normalized after treatment. Improvement in seminal parameters seemed to be due to the testolactone's aromatase inhibition effect.

In a more recent work, a subset of infertile men with severe infertility who had significantly lower testosterone and higher estradiol than fertile control reference subjects were identified (*Pavlovich et al., 2001*). According to the results, 71% of infertile men were found to have testosterone-to-estradiol ratios in the lowest 20th percentile of those of normal subjects. Aromatase inhibitor testolactone was effective in increasing serum testosterone and/or lowering serum estradiol in all treated men. Furthermore, oligozoospermic men with abnormally low ratio had significant increases in sperm concentration and motility while on testolactone. Thus, authors' data suggested that alteration of the hormonal milieu in these men with testolactone was responsible for increased sperm production. As these results were indicative of an endocrinopathy characterized by a decreased serum testosterone-to-estradiol ratio, in a further study a group of infertile men with this condition were treated with two different aromatase inhibitors, anastrozole and testolactone (*Raman & Schlegel, 2002*). Both medications revealed an increase in testosterone-to-estradiol ratio in association with increased semen parameters. So, the introduction of testosterone-to-estradiol ratio monitoring in infertile men may be expected to add safety as well as effectiveness for treatment with aromatase inhibitors in achieving a high pregnancy rate in their wives.

Regarding these promising results, in order to evaluate the role of aromatase inhibition in spermatogenesis and germ cell maturation, we analyzed the abnormal retention of residual sperm cytoplasm in infertile men before and after anastrozole administration. 64 patients with different degrees of oligoasthenoteratozoospermia were included in the study. According to our preliminary unpublished data, at the end of the 3 months, significant increase in serum FSH, LH and testosterone levels accompanied by significantly suppressed estradiol were seen (Table 1).

Table 1: Serum hormone values before the treatment and at the end of treatment

	Before treatment	12 weeks after treatment	Mean difference	95% CI
FSH (IU/l)	4.7 ± 0.9	10 ± 3.3	5.3 ± 3	4.5-6.1
LH (IU/l)	3.8 ± 0.6	7.9 ± 2.2	4 ± 2	3.5-4.6
Testosteron (nmol/l)	14.2 ± 5.2	25 ± 8	11 ± 6.4	9.4-12.6
Oestradiol (pmol/l)	59 ± 3	36.7 ± 9	22 ± 8.8	20-24.4

p<0.001

Significant improvements of basic sperm parameters, including concentration, progressive sperm motility and morphology were also found (Table 2). Moreover, the mean percentage of sperm with retained cytoplasm, a morphologic characteristic of impaired spermatozoa maturation, was significantly lower after the treatment than baseline values (Figure 1).

Although our data do not clearly elucidate the mechanism of this effect, they are consistent with the possibility that aromatase inhibition's lowering of serum estrogen levels as well as increased FSH, LH and testosterone, may have been responsible for the improvement in spermatogenesis.

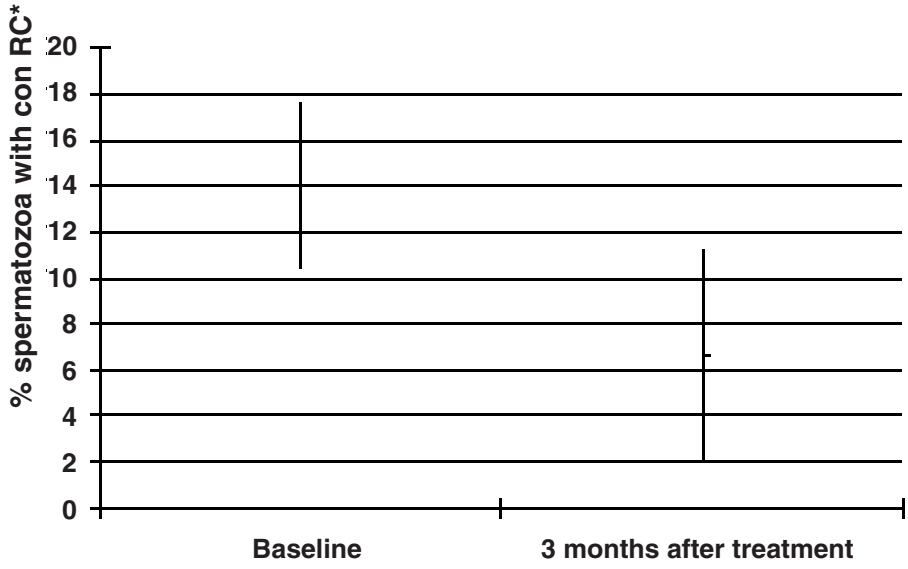
Although uncontrolled studies showed a positive response to aromatase inhibitors, in a randomized, placebo-controlled double-blind crossover trial sperm output and semen quality remained unchanged during either testolactone or placebo treatment,

Table 2. Semen parameters before and at the end of the treatment.

	Before treatment	12 weeks after treatment	Mean difference	95% CI
Ejaculate volume *(ml)*	3.1 ± 0.7	3.2 ± 0.8	0.1 ± 0.5	0.2-5.7
Sperm concentration *(10^6/ml)*	26 ± 7.5	36 ± 10	9.9 ± 7.4	8.0-11.7
Sperm motility *(%) (grade a + b)*	29 ± 6.9	37 ± 10	8.7 ± 7.3	6.8-10.5
Normal forms *(%)*	18 ± 4.9	33 ± 7.4	15 ± 6.8	13.4-16.8
Spermatozoa with RC* *(%)*	14 ± 3.6	6.6 ± 4.6	7.3 ± 4.1	6.3-8.3

**RC = retained cytoplasm.* *p<0.001*

Figure 1. Graphical representation of baseline and at the end of treatment percentage of spermatozoa with retained cytoplasm.



and no pregnancy occurred during the 16-month study (*Clark & Sherins, 1989*). Contrary to the usual reports, however, serum estradiol and testosterone levels during testolactone exposure did not change from basal and placebo values. Nevertheless, these data have been interpreted to indicate that chronic administration of testolactone fails to maintain aromatase inhibition and is not efficacious in the treatment of idiopathic oligozoospermic infertility.

In conclusion, most clinical trials with aromatase inhibitors have in fact resulted in a tendency to improved seminal parameters. It is thought that this improvement is dependent on suppression of estrogen-to-testosterone ratio, with an associated increase of FSH. However, placebo-controlled, double-blind, randomized additional studies are needed to determine if there is a subpopulation of infertile men who will benefit from aromatase inhibitors.

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MAMMALIAN TESTICULAR DESCENT AND MALDESCENT; IMPLICATIONS IN FERTILITY POTENTIAL

Charalampos Mamoulakis, Apostolos Kaponis, John Georgiou, Dimitrios Giannakis Spyros Antypas, Stavros Tsambalas, Xenophon Giannakopoulos, Ikuo Miyagawa and Nikolaos Sofikitis

Laboratory of Molecular Urology and Genetics of Human Reproduction, Department of Urology, Ioannina University, Ioannina, Greece

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ABSTRACT

Mammalian male sex determination is an active process involving complex interactions among several genes. SRY and SOX9 are both responsible for testis formation by initializing and maintaining respectively Sertoli cell differentiation. Male sexual differentiation is governed by testicular hormones. Testicular descent (TD) and scrotal evolution occurs exclusively but not universally in mammals. Although still debatable, this evolutionarily costly process aims at least to secure lower than core body testicular temperatures essential for viable sperm production and storage. TD in scrotal mammals is a multistaged process involving interplay of several anatomical structures and hormonal factors. The gubernaculum seems to posess a key role, especially during transabdominal TD (TTD). Androgens and Mullerian inhibiting substance have a rather limited, if any, role during TTD. Leydig cell-derived insulin-like 3 hormone acting directly upon the gubernaculum and homeobox genes represent good candidate controllers of TTD. Inguinoscrotal TD is mediated by androgens possibly acting indirectly upon the gubernaculum, in conjunction with mechanical (abdominal pressure) factors. Reports on secular trends of human testicular maldescent (TMD) are controversial. However, there is a general agreement on the seasonality of TMD at least in the northern hemisphere. Epididymal malformations, impaired testicular histology due to intrinsic testicular defects, mild hypogonadal state or increased germ cell apoptotic rate mainly due to abnormal testicular temperature may account for impaired fertility in TMD. The theory of an intrinsic testicular pathologic process might plausibly explain the association with testicular cancer. Management of human TMD aims to preserve future fertility. Surgery is currently the only effective measure to bring the testis into the scrotum. It should take place before the first year. Hormonal treatment may serve as an ancillary treatment to stimulate germ cell maturation and improve long term results.

PHYSIOLOGY OF MAMMALIAN SEX DETERMINATION AND SEXUAL DIFFERENTIATION IN THE MALE

During mammalian embryogenesis each individual, regardless of sex chromosome genotype, has the potential to develop both male and female reproductive systems. In order a normal male or female development to occur, a selective mechanism is required to ensure the development of only one of the two genital systems while the other must regress. Development of the male

phenotype is dependent by the presence or absence of the Y chromosome inherited by the father at fertilization (genetic sex establishment of the mammalian embryo at the time of fusion of the gametes) and can be divided into two distinct stages: sex determination and sexual differentiation. The sex-determining process is set in motion only during the period of organogenesis when the gonads develop within the developing urogenital system resulting in either ovary or testis formation, whereas sexual differentiation (controlled by the presence or absence of hormones produced by the gonads during fetal development) is the subsequent process that ultimately results in either the female or the male phenotype.

THE GENITAL SYSTEM DURING THE PRE-SEX DETERMINATION PERIOD

Prior to sex determination the mammalian gonadal anlage is formed as a bipotential primordium (derived from the intermediate mesoderm) with the capacity to differentiate into either testes or ovaries depending on the presence of the Sry gene (sex-determining region on Y; proximal to the pseudoautosomal region on the short arm). Knockout experiments in mice have implicated five genes in the formation or survival of the gonadal primordium: Wt1, Sf1, Lhx1 (Lim1), Lhx9, and Emx2 *(Koopman, 2001)*. During the initial stage of sexual development (indifferent or bipotential stage), genetically XX and XY embryos both develop two pairs of genital ducts, starting with the male or mesonephric or Wolffian ducts (which have the potential to differentiate into the vas deferens, epididymides, seminal vesicles and ejaculatory ducts), followed by the female or paramesonephric or Mullerian ducts (which have the potential to differentiate into the oviducts, uterus, and upper vagina).

The intermediate mesoderm is found on either side of the embryo filling much of the coelomic or body cavity. During development it forms into three structures which develop anteroposteriorly along the Wolffian duct: pronephros, mesonephros and metanephros. The pronephros is vestigial in mammals but mesonephros can serve as a primitive kidney during embryogenesis in some species. The definitive kidney is the product of the interaction between the metanephric mesenchyme at the posterior end of the urogenital system and the ureteric bud, which grows out of the Wolffian duct. The Mullerian duct originates within each mesonephros by vagination of the coelomic epithelium. It runs parallel to the Wolffian duct but turns toward the midline at the posterior end of the mesonephros and fuses with the companion duct. The Wolffian duct produces branching tubules, which extend through the mesonephros towards the coelomic epithelium. Cells in the coelomic epithelium proliferate, delaminate and join the gonadal cell population, accumulating on the surface of the mesonephros. During the bipotential stage (4^{th}-6^{th} gestational week in human (GW)), primordial germ cells derived from the extraembryonic mesoderm at the base of the allantois (caudal wall of the embryonic yolk sac) (4^{th} GW) migrate via the hindgut and mesonephros and populate the gonads (5^{th}-6^{th} GW) which arise as a thickening of the ventrolateral surface of each mesonephros and are first visible at ~10 days post-coitum in the mouse (dpc). This process causes mesonephros to bulge forming the genital ridges composed therefore of somatic cells derived from mesonephros and the primordial germ cells that have migrated and populated the indifferent gonad *(Ginsburg et al., 1990)*. Germ cells differentiate into gonocytes upon entering the testicular cords (see below) to become fetal spermatogonia by 15^{th} GW.

MOLECULAR BASIS OF MALE SEX DETERMINING PROCESS

The gonad initially develops in a non-specific manner, being morphologically identical up to ~12 dpc, and the 7th GW in XX or XY mouse and human embryos respectively. In normal XY embryos, expression of the Y chromosome gene Sry in somatic cells (pre-Sertoli cells) *(Hacker et al., 1995; Jeske et al., 1995)* of the genital ridge at the time of male sex determination initiates the differentiation of Sertoli cells *(Lovell Badge et al., 2002)* and determines the fate of the indifferent gonad to testis rather than ovary *(Gubbay et al., 1990; Sinclair et al., 1990; Palmer & Burgoyne, 1991)*. The human SRY gene encodes a DNA-binding motif, referred to as the high mobility group (HMG), which is relatively conserved among different mammalian species unlike the rest of the protein *(Tucker & Lundrigan, 1993; Whitfield et al., 1993)*. More than 100 genes have been identified containing this domain referred to as an (HMG) box, and those with the most extensive homology (>60%) to the SRYHMG box gene are collectively termed SOX (SRY-type HMG box) genes. The transient nature of Sry expression in the genital ridge of the mouse (~1.5 days) suggests that Sry acts as a switch toward Sertoli cell fate rather than involved in the maintenance of cell identity or function. Therefore, Sry must in some way activate other genes that are involved in defining and maintaining Sertoli cell identity. The autosomal gene Sox9 is a strong candidate for this type of downstream gene because it is upregulated in Sertoli cells just after Sry expression *(De Santa Barbara et al., 2000)* (~11.5 dpc) and persists throughout life *(Kent et al., 1996; Morais da Silva et al., 1996)*. The importance of both SRY and SOX9 proteins in sex determination has been substantiated in experiments with transgenic mice (Koopman et al., 1991; Vidal et al., 2001) and in mutational analysis of mice and humans with sex reversal *(Foster et al., 1994; Gubbay et al., 1992; Hawkins et al., 1992; Wagner et al., 1994)*. Almost all SRY mutations responsible for 46, XY sex reversal cluster in the HMG domain *(Harley et al., 1992)* which represents therefore the most critical part of the SRY protein. Thus, SRY and SOX9 proteins are both necessary and sufficient for male sex determination in humans and mice. However, their requirement and functions might not be conserved in all mammalian species *(Bowles et al., 2001; Nagai, 2001)*. All species of both subclasses of the Mammalian class (Simpson's classification) *(Novacek, 1992)* possess SRY gene homologs (*Foster et al., 1992; Gubbay et al., 1990; Sinclair et al., 1990)*: Prototheria (Monotremata: duck-billed platypus and spiny ant-eater or echidna), and Theria (Metatheria: Marsupialia: opossums, kangaroos etc and Eutheria or placental mammals). However, SRY gene homologs are absent from two vole species and the spiny rat *(Just et al., 1995; Soullier et al., 1998)*. Therefore, SRY is not probably the original mammal sex-determining gene that defined the Y chromosome and can be replaced as a trigger and get lost in the future, as have many other Y-borne genes in recent evolutionary history *(Marshall Graves, 2002)*.

Although the SRY and SOX9 genes appear to be primarily responsible for male sex determination, the regulation of their expression as well as their mode of function still remain elusive. The expression of SRY in the gonad is followed by complex interactions involving activation and repression of other male-specific genes, cell migration and cell proliferation in the developing male gonad *(Clarkson & Harley, 2002)*. It has been proposed *(Hammes et al., 2001)* that

the Wt1 gene must have a role in the activation or transcript stability of Sry (+KTS isoforms of Wt1 gene; specification of male gonad formation). (-KTS) isoforms of Wt1 gene are also required for the development of both the male and female gonad but they are not essential for sex determination *(Clarkson & Harley, 2002)*. The (+KTS) and (–KTS) isoforms arise from an alternative splicing event that incorporates or omits respectively three amino acids-Lys, Thr, Ser-between zink fingers 3 and 4 *(Hastie, 2001; Little et al., 1999)*. It has been suggested that SRY acting as a transcription factor antagonizes the function of repressors of male development (anti-testis genes) such as WNT4 and DAX1 *(Jordan et al., 2001; Swain et al., 1998)*. WNT4 is known to be involved in gonad differentiation, however, it remains unclear which gene(s) is directly downstream of its signal. It is likely that DAX1 gene transcription is upregulated by WNT4 *(Suzuki et al., 2002)*. DAX1 (an orphan nuclear receptor) may antagonize the function of SRY by several ways *(Clarkson & Harley, 2002)*: indirect inhibition of SRY action by binding to another orphan nuclear receptor, SF1 *(Ito et al., 1997)*, which is involved in the activation of male-specific genes such as the anti-Mullerian hormone gene/Mulerian inhibiting substance gene (AMH/MIS) *(Arango et al., 1999; de Santa Barbara et al., 1998)*, direct competition with SRY for similar DNA structures involved in the regulation of male-specific genes expression (inhibition of SRY-induced SOX9 activation), antagonization of WT1 (+KTS) function. Sry is required for proliferation of the coelomic epithelium (Sertoli precursors) *(Schmahl et al., 2000)* and migration of cells from the mesonephros into the gonad (peritubular myoid and vascular cells) *(Capel et al., 1999)*, two processes that are crucial for testis development. SRY might direct cellular changes in the developing male gonad through the activation of the intracellular signaling molecule fibroblast growth factor 9 (Fgf9) *(Colvin et al., 2001)*. On the other hand, SOX9 appears to be primarily necessary for the activation of AMH/MIS gene in conjunction with other genes such as SF1 and Wt1 (-KTS), GATA4 and HSP70 *(Arango et al., 1999; Clarkson & Harley, 2002; Swain & Lovell Badge, 1999).*

STRUCTURAL ORGANIZATION OF THE TESTIS, HORMONE PRODUCTION, AND MALE SEXUAL DIFFERENTIATION

Once Sertoli cell differentiation is triggered by SRY the fate of the gonad has been decided, a number of genes associated with these cells, such as the Desert hedgehog (Dhh) and the Mis, become activated (11.5 and 12 dpc respectivelly) *(Bitgood et al., 1996; Munsterberg & Lovell Badge, 1991)* and a number of events follow leading to the structural/functional organization of the testis and finally the sexual differentiation (*Swain & Lovell Badge, 1999)*. The testis starts organizing into two distinct compartments: the testicular cords (progenitors of the seminiferous tubules) and the interstitial region. Initially, Sertoli cells form clusters around germ cells and produce, as yet, unknown signal(s) that trigger the movement of cells from the mesonephros to the gonad which give rise to the endothelial and peritubular myoid cells that surround these clusters (cords formation) *(Buehr et al., 1993; Martineau et al., 1997; Merchant Larios et al., 1993)*. The peritubular myoid cells deposit a basal lamina in collaboration with Sertoli cells, sequestering germ cells inside testis cords and arrest them in mitosis possibly via a DHH-induced signal *(Bitgood et al., 1996)*. As a result germ cells entry into meiosis (believed to initiate the ovarian

pathway and block testis formation) is opposed (*Hung Chang Yao et al., 2002*). The androgen producing Leydig cells are excluded from the cords and remain in the interstitial region in close proximity to arterial and lymphatic vasculature. They start producing testosterone once the testicular cords have been formatted (~12.5-13 dpc, during the 8th GW). It is suggested that testosterone production is repressed by the presence of Wnt4 which is downregulated by Sry and steroidogenesis is allowed to proceed *(Vainio et al., 1999)*.

Jost's pioneering experiments investigated the influence of fetal hormones during development *(Jost, 1947; Jost, 1953)*. He was the first to suggest a model according to which, two fetal testicular hormones regulate sexual differentiation in eutherian mammals, one that stimulates Wolfian duct differentiation and masculinization of the external genitalia and one that inhibits the development of the Mullerian ducts. Subsequently, testosterone (converted by 5a-reductase in target tissues to DHT) induces differentiation of the Wolffian ducts into vas deferens, epididymides, seminal vesicles and ejaculatory ducts, while MIS (detectable at Sertoli cells by the 7th GW) is secreted by Sertoli cells after the 8th GW and actively causes regression of the Mullerian ducts, most likely by interactions with membrane-bound serine/threonine kinase receptors on mesenchymal cells surrounding the Mullerian ducts *(Baarends et al., 1994; di Clemente et al., 1994; Lee & Donahoe, 1993)*. The indifferent gonads of XX individuals will differentiate into ovaries that do not produce MIS during fetal development, thereby creating permissive environment for Mullerian duct differentiation. The lack of testosterone results in the passive regression of the Wolffian duct system. Thus, MIS and testosterone produced by the fetal testes impose a male pattern of differentiation upon a program that is inherently female.

2. TESTICULAR DESCENT AMONG DIFFERENT MAMMALIAN SPECIES: EVOLUTIONARY IMPLICATIONS

Testicular descent (TD) from the initial position on the urogenital ridge (which in most species is revealed in the adult by the position of the ovary) occurs exclusively in mammals (Theria). However, the degree of TD is highly variable, and abdominal testes are found in a wide variety and large number of taxa. Furthermore, genera within mammalian families often show variations in testes position *(Carrick & Setchell, 1977; Harrison & Lewis, 1986; Kinzey, 1971)*. Six mammalian orders contain species with internal testes as well as species with external testes (Marsupialia: kangaroo etc, Chiroptera: bats, Rodentia: rats, mice, porcupines, prairie dog, chinchilla etc, Carnivora, Perissodactyla (odd-toed ungulates): horses, rhinos etc and Artiodactyla (even-toed ungulates): pigs, hippos, camels, deer, cows etc) *(Kinzey, 1971; Pfeifer, 1956; Weir, 1974)*. Testes position in mammals can be generally classified (*Williams & Hutson, 1991a*) as transabdominal and inguino-scrotal. Each category can be schematically further divided into three groups respectively: embryonic (no descent from the ancestral and embryonic position; (position 1), intermediate (partially descended, intra-abdominal position; position 2), internal inguinal (position 3) and emergent (some part protrudes through the inguinal ring; position 4), beyond the inguinal ring but not within a true scrotum (position 5), scrotal (into a true scrotum; position 6). Non-mammalian species such as fish, birds, turtles, crocodiles have testes located in position 1 *(Freeman, 1990)*.

MAMMALIAN SPECIES WITH TRUE INTRA-ABDOMINAL TESTES: PRIMARY AND SECONDARY TESTICONDA

There is a number of mammalian species whose testes do not descend at all like non-mammalian vertebrates (position 1). These animals are called primary testiconda and the phenomenon is called testicondia *(Van der Schoot, 1996)*. Prototheria (Monotremata: the duck-billed platypus and the spiny ant-eater or echidna) *(Grant, 1984)* as well as some Eutheria (Hyracoidea: the rock hyrax (Procavia and Heterohyrax) *(Glover & Sale, 1968; Van der Schoot, 1996)*, a small herbivore living in rocky outcrops throughout much of Africa; Proboscidea: both extant elephant species (the Asiatic (Elephas maximus) *(Short, 1972)* and the African elephant (Loxodonta Africana)) *(Gaeth et al., 1999; Short et al., 1967)* as well as Sirenia: the sea cow Dugong dugon (*Marsh et al., 1984)*) are classified to primary testiconda.

Aquatic mammals (Catacea: porpoise, dolphin, whale), have testis which are partially descended within the abdomen (position 2) *(Meek, 1918)*. However, testicular position varies in different families. The testes of the common porpoise (Phocaena phocaena) are latero-caudal to the kidneys *(Ping, 1926)*. In the fin whale (Balaenopter physalus) the testes lie slightly below the kidney and a genital cord tethers the cranial portion of the vas deferens to the inguinal region *(Ommaney, 1932)*. The narwhale (Monodon monoceros) has its testes in a pelvic position adjacent to the bladder neck just inside the inguinal region (position 3) *(Meek, 1918)*. Testicondia in Catacea is considered secondary as they are judged, evolutionarily, the descendants of terrestrial mammals (ungulates) with TD *(Van der Schoot, 1995)*. It has been speculated that Catacea had scrotal testes during an earlier epoch, but this feature has been lost to a variable extent in subsequent millennia *(Meek, 1918)* to protect them from getting too cold, as even a brief period of extreme testicular cooling can render an animal permanently sterile *(Young et al., 1988)*. It has been suggested that the absence of the pelvic girdle together with the development of structures in and beyond the caudal abdominal region, particularily the caudal hypaxial musculature, precludes the outgrowth into caudal direction of hollow organs (such as the processus vaginalis) from the abdominal cavity (*Van der Schoot, 1995)*.

MAMMALIAN SPECIES WITH PARTIALLY DESCENDED TESTES

The testes of the prairie dog (Rodentia: Cynomys) remain in a similar position as those of the narwhale *(Anthony, 1953)*. This is the case also as far as most of Insectivora are concerned (Erinaceidae: hedgehog, Talpidae: mole, Soricidae: shrew, and Macroscelididae: elephant shrew) with the difference that these ascrotal mammalian species demonstrate seasonal changes in the position and the size of the gonad (enlargement and protrusion of the testes during spring (breeding season) into sacs near the base of the tail) *(Marshall, 1911; Pearson, 1944)*. The testes of armadillios and sloths (Edentata) lie between the bladder and the rectum *(Wislocki, 1928)*.

Chinchilla (Rodentia) is a representative mammalian species in which TD arrests just outside the inguinal region (position 4). Instead of a true scrotum, these animals present thin-walled diverticulae cauda-lateral to the base of the penis. Although testes are inside the inguinal apertures, the caudal epididymis protrudes into the diverticulum *(Roos & Schackelford, 1955)*. The

southern elephant seal (Carnivora: Pinnipedia: Mirounga leonine Linnaeus) is a typical example of a mammal with testes in position 5 (extra-abdominal testes in separate inguinal pouches rather than a single scrotum, no dartos present, rudimentary cremaster muscle) *(Bryden, 1967)*.

MAMMALIAN SPECIES WITH DESCENDED TESTES INTO A TRUE SCROTUM

Even in cases where TD within a true scrotum is present (position 6); the exact site and degree of development of the scrotal sac may vary widely among mammalian species. The hyaena's testes (Carnivora) for example usually complete their descent into two posterior shallow scrotal pouches but may occasionally be concealed subcoutaneously in the perineum *(Mathews, 1941)*. On the other hand, the kangaroo (subclass Theria: infraclass Metatheria: order Marsupialia) as well as most of the marsupials (e.g. the opossum (Didelphis)) has permanently descended testes within a large pendulous pre-penile scrotum *(Chase, 1939; Renfree, 1992)* (in contrast to most other mammals with permanent TD that develop post-penile scrota) *(Le Gros Clark, 1962)*. The testicular position relative to the scrotum may also vary with (breeding) season and/or sexual maturity. Such varieties are observed in some Carnivora, Rodentia and only in three species of the Primate order (see below) *(Miller, 1918; Rau & Hiriyannaiya, 1930)*. The fox, ferret, weasel, stoat, cat, and dog (Carnivora), as well as the mouse and rat (Rodentia: Muridae) possess a global scrotum between the inguinal region and the anus and exhibit permanent TD, irrespective of the breading season *(Hill, 1939; Sisson, 1940)*. On the contrary, in the bear (Carnivora) the testes are scrotal from infancy and are held close to the body except in the adult during the breeding season, when they are pendulous because of scrotal relaxation *(Erickson et al., 1968)*. Similarly, the woodchuck (Rodentia: Marmota Monax), has pouches beside the tail into which the testes descend seasonally *(Rasmussen, 1917)*, while the testes of Aplodontia rufa (Rodentia) are in a semi-scrotal position during the breeding season *(Pfeifer 1956)*. In young squirrels (Rodentia: Squiridae), the testes are either scrotal or inguinal but after sexual maturity they are never more than temporarily retracted *(Mossman, et al., 1932)*. In all living primates the testes descend into the scrotal sac and do not re-enter the abdomen *(Martin, 1969)*. It is worth noting however, that in loris and potto (Lemuroidea) the testes descend seasonally into the scrotum *(Rau & Hiriyannaiya, 1930)*. The testes of Rhesus macaque monkey descend to the scrotum before birth like in human but then re-ascend to the inguinal canal after birth to re-enter the scrotum finally at puberty *(Miller, 1918)*.

EVOLUTIONARY HYPOTHESES ON TESTICULAR DESCENT AND SCROTAL FORMATION IN MAMMALIAN SPECIES

The reason for the developmental fate and ultimate position of the testis and epididymis has been the subject of considerable contention among developmental biologists, mammalogists, evolutionary biologists and reproductive physiologists. TD is developmentally, physiologically and evolutionarily costly process *(Moore & Persaud, 1993)*. Therefore its benefits must be great to outweigh the cost. What the benefits of TD may be is poorly understood and the question only partially overlaps that of the adaptive value and evolution of the scrotum, since the development of the latter is not a prerequisite for the former. Several

hypotheses have been put forward to explain the evolution of the scrotum and TD. The most important of these are: a) the hypothesis of temperature dependency of spermatogenesis *(Moore, 1926)*; b) the mutation rates hypothesis *(Short, 1997)*; c) the display hypothesis *(Portman, 1952)*; d) the cold storage hypothesis *(Bedford, 1977)*; and e) the training hypothesis *(Freeman, 1990)*.

The oldest and classical hypothesis to explain TD and scrotal evolution originated with *Moore (1926)* states that testes descend into a highly specialized low-temperature environment (the scrotum) because such an environment is necessary for viable sperm production. It is well known that in many mammals normal spermatogenesis and epididymal storage require below core body temperatures *(Carrick & Setchell, 1977, Cowles, 1958; Cowles, 1965; Jameson, 1988; Setchell, 1998; VanDemark & Free, 1970; Waites, 1970)*. Spermatogenesis can be hampered at temperatures between 35-38°C (*Cowles, 1958; VanDemark & Free, 1970)*, while abdominal temperatures can detrimentally affect long-term spermatozoa storage of many species *(Bedford, 1977)*. Most mammals have acquired a large number of sophisticated anatomical and physiological adaptations of the scrotum (Table 1) *(Bedford, 1977; Freeman, 1990; Hutson & Beasley, 1992; Jameson, 1988; Kinzey, 1971; Setchell, 1978; Waites & Moule, 1961; Williams & Hutson 1991a)* many of which are also present in the human, to keep the epididymis and testis cool (~3-5°C below core body temperature). This hypothesis has been seriously criticized from a number of authors *(Bedford, 1977; Bedford, 1978; Carrick & Setchell, 1977; Freeman, 1990;)* merely on the basis of the relationship between core body temperature vs. testicular temperature in scrotal vs. ascrotal mammalian species, as well as for its failure to explain the observed variability in the degree of TD *(Werdelin & Nilsonne, 1999).* It is well known that special mechanisms exist for cooling the testis (venous plexuses carrying cool blood to the arteries supplying the testis: countercurrent heat exchangers) in a number of ascrotal taxa (southern elephant seal like other true seals *(Blix et al., 1983; Rommel et al., 1995)*, Cataceans *(Pabst et al., 1995; Rommel et al., 1992;)*) while others have core body temperatures (possibly evolved to accommodate ascrotal condition) that obviate the need for such mechanisms (hedgehog, *(Carrick & Setchell, 1977)* primary testiconda such as Monotremata *(Macdonald, 1984)*). Hyracoidea and Proboscidea (elephants) on the other hand, represent an exception to a rather distinct pattern of primary testiconda (characterized by low body temperature) *(Hanks, 1977)*. Hyraxes are known to have poor internal thermoregulation, controlling their body temperature to a large extent by behavioral means. *(Macdonald, 1984)* This together with their small size, testes placed close to the dorsal body wall and their seasonal reproductive activity *(Glover & Sale, 1968)* would make it relatively easy for them to regulate behaviorally their testicular temperature to the proper one for spermatogenesis. The elephants have a testicular temperature equal to their core body temperature (>36°C) *(Carrick & Setchell, 1977)* and their testicular veins ran straight into the posterior vena cava with no sign of a pampiniform plexus that could cool the testis *(Gaeth et al., 1999)*. These animals are regarded as “immune” to high temperature effects on sperm viability *(Hanks, 1977)*. However, a temperature range of 36-38°C is not so high to cause aspermia, though temperatures above 38°C do seem to have this effect *(Waites, 1970)*, and the fact that elephants belong to pri-

Table 1. Scrotum as a low-temperature environment: Thermoregulation at testicular/epididymal level

Anatomical characteristic	**Physiological role**
Pendulous scrotum (e.g. horse, bull, howler monkey)	Physical separation from the body core
Obliteration of processus vaginalis (e.g. chmpanzee, gorilla, capuchin, spider monkey, human)	Keeps testis outside abdominal cavity
Thin and pigmented scin	Convection/radiation of heat
Large surface area	Convection/radiation of heat
Absence of subcoutaneous fat	Convection of heat
Total absence of hair fur (e.g. rat)/or at least at the distal end	Heat loss (over caudal epididymis)
Cremaster muscle	Controling of testicular dependency in response to external temperature
Dartos muscle	Controling of scrotal dependency in response to external temperature
Scrotal veins located against the skin	Convection of heat
Pampiniform plexus formed by testicular veins around internal spermatic artery	Counter-current cooling of incoming blood within the spermatic cord
Testicular artery (sometimes) winds around the testis (up to three times in rams)	Heat loss before breaking up to supply the testis
Fat pad between testis and epididymis	Insulation of caudal epididymis from the testis
Fat pad in inguinal canal (e.g. rat)	Testicular insulation from abdominal cavity

mary testiconda is therefore not by itself sufficient to reject the temperature hypothesis for TD and evolution of the scrotum *(Werdelin & Nilsonne, 1999)*. The hypothesis is further supported by the phylogenetic approach *(Werdelin & Nilsonne, 1999)* which provides strong evidence towards the selective passage from the presence of a scrotum to the absence of one. Thus, the variation in testicular position could be seen as the result of the developmental and physiological difficulties encountered during the evolutionary pressure towards doing away with the scrotum and descensus.

A modification of this hypothesis has recently been proposed by *Short (1997)*. According to this theory, the causal explanation for the evolution of the scrotum and TD is not the disruption of spermatogenesis per se, but the need to keep the mutation rate in the male germ line under control. In humans it is known that mutation rates are higher in males than in females *(Shimmin et al., 1993; Shimmin et al., 1994)*. This, coupled with the fact that elevated temperatures have long been known to be associated with increased mutation rates, suggests that cooling the testes will assist in keeping male germ line mutation rates at acceptable levels *(Cowles, 1965; Ehrenberg et al., 1957)*.

The “display” hypothesis has been suggested by *Portman (1952)*. It is based on the assumption that the testes descend to

an external position (scrotum) because of their importance in social competition, either as a character in female choice of mates or as a signal of dominance in male-male competition. In some taxa the scrotum is brightly or at least distinctively colored, which serves to call attention to the sexual organs in the taxa involved. This is the case in primates for example, where the presence of a pigmented scrotum is associated with polygynous or promiscuous breeding systems characterized by intense male-male competition *(Freeman, 1990)*. Although there is a number of observations consistent with this hypothesis, its failure to explain the variable location of the testes among ascrotal species combined with the fact that pigmented scrota are not always readily presented (nocturnal animals, furred pigmented scrota) by the relatively small percentage of genera-owners, weakens its explanatory power *(Freeman, 1990)*. It is more possible that scrotum has evolved for some other function first and subsequently has been retained and furthermore, co-opted as a signaling device by some species (most notably primates) due to their particular sexual behavior systems *(Harrison & Lewis, 1986)*. In this way, social behavior can act as a constraint against the adaptive pressure towards loss of the scrotum rather than an evolutionary mechanism towards scrotal developmetal *(Werdelin & Nilsonne, 1999)*.

The "cold storage" hypothesis originated with *Bedford (1977)*. It was suggested that TD into a scrotum (whose primary function is to store sperm in a cool environment) is a secondary phenomenon, subsequent to the epididymal descent (evolutionarily "prime mover" in scrotal evolution), serving merely as a mechanism for providing structural support for the epididymides in some species. It was pointed out that the sperm storage organ in mammals (usually the epididymis but sometimes the seminiferous vesicles or vas deferens) always precedes the testes in descent, descending to the body wall or to a scrotal-like out-pocketing in most species with abdominal testes (even in non-mammalians e.g. in passerine birds sperm is stored in a cloacal protruberance lower-than-body core temperature by several degrees *(Wolfson, 1954) (Bedford, 1978; Glover, 1973; Glover & Sale, 1968; Harrison, 1969)*. However, the main flaw of this hypothesis is its incompatibility with the fact that in a few ascrotal taxa, the epididymis lays subcoutaneously or intra-abdominally (e.g. hyraxes *(Glover & Sale, 1968)*, elephant shrew *(Woodal, 1995)*). From the phylogenetic point of view (see above) *(Werdelin & Nilsonne, 1999)* Bedford's hypothesis cannot be justified although it explains why the epididymis remains in a distal position in some taxa, while the testes have migrated out of the scrotum during the process of scrotal loss.

The "training" hypothesis was formulated by Zahavi *(Freeman, 1990)*. It represents a radical departure from the other hypotheses presented above since it views the scrotum as a purposely hostile environment for sperm rather than an optimized one. The main idea is that, TD into a scrotum occurs because spermatozoa must get "trained" for their future endeavor in the physiologically rigorous female environment. The physiological basis for this hypothesis is that the degree of TD is inversely correlated to the testicular blood flow *(Setchell, 1978; Waites & Sethell, 1969)* directly correlated to hemoglobin affinity for oxygen (under lower scrotal temperatures*) (Eckert & Randall, 1983)* and therefore directly correlated to the degree of oxygen and pH stress on maturing sperm. Direct consequence of spermatogenesis under episodes of physiological oxygen and pH stress, is an increase in size, number and efficiency of

maturing spermatocyte mitochondria in a way that parallels the response of striated muscle cells during anaerobic training. If mitochondria are trained more efficiently in descended testes and perform better during active sperm transport in the female reproductive tract (better swimming and/or longer survival of spermatozoa), TD will enable a male to produce a physiologically-superior ejaculate which would then have an advantage in the between-ejaculate competition characterizing many mammalian mating systems *(Ginsberg & Huck, 1989)*. The main strength of this hypothesis is that it can account for the entire range of variation in testicular position among mammalia by providing an explanation for the strategies behind both scrotal and ascrotal testes: scrotal testes produce fewer sperm of higher quality, while ascrotal testes produce more sperm of lower quality. Zahavi's hypothesis has been criticized on the base that the blood supply to the testes could be modified without resorting to an elaborate and costly complex such as the TD and scrotal development *(Werdelin & Nilsonne, 1999)*.

3. PHYSIOLOGY OF TESTICULAR DESCENT AND PATHOPHYSIOLOGY OF TESTICULAR MALDESCENT IN SCROTAL MAMMALIA

In ancient times investigators new from animal studies that testes descend from the abdominal cavity to the scrotum but it was first observed in the 17th century that the same process occurs in the human fetus as well *(Weil, 1885)*. The study of TD really originated with *John Hunter (1786)*, an English anatomist who published the first important and highly accurate description of TD in human. In 1762 and 1786, he described the fetal testis and epididymis in the abdomen and he was the first to introduce the term "gubernaculum" (derived from the Latin word meaning helm or rudder) for the unique structure attached to the testis (caudal ligament), which he believed to be responsible for steering the male gonad into the scrotum. Since Hunter's first description, the gubernaculum has been described by many workers and numerous theories have been proposed up to date to explain the cause and mechanism of TD *(Heyns & Hutson, 1995; Williams & Hutson, 1991b)*. Among these theories, the role of the gubernaculum has indisputably occupied a central place. Although it is not universally accepted that the gubernaculum "holds the key to the mystery of "descent", the development of this structure is unique to the male fetus and offers the most obvious explanation of why the fetal testis descends while the ovary does not. Furthermore, there is strong evidence derived from the mammalian evolutionary history suggesting that the gubernaculum has an essential role on TD. Reptiles as well as primary testiconda mammals such as hyraxes *(Van der Schoot, 1996)* and elephants *(Gaeth et al., 1999)* show either no sign of a gubernaculum at any stage of their development, or at least partial (only gubernacular cord) gubernacular development (e.g. Hyrax capensis) *(Van der Schoot, 1996)*. On the other hand secondary testiconda such as Catacea (e.g. the common dolphin (Delphinus delphis), the Black sea harbour porpoise (Phocoena phocoena), the gray whale (Eschrichtius robustus), the sperm whale (Physeter catodon)) present gubernaculae primordia ever since the time of oncet of their sexual differentiation *(Van der Schoot, 1995)*. In theses mammals, gubernaculuae develop further into large masses of dense connective tissue in the ventral-caudal abdominal region at the site of the insertion of the mesonephric inguinal ligament. This is strong evidence that Catacea are the

descendants of terrestrial mammals (ungulate) with TD (see above).

Despite the extensive work on TD during the last centuries, the matter still remains controversial since no unified theory does exist as yet. This is probably due to the incorrect or incomplete observations many of these theories are based on, the kind of material used (e.g. histo-anatomical studies on autopsy material cannot determine the forces responsible for descent) as well as (mainly) the type of animal model used and the indiscriminate extrapolation of findings among different spiecies *(Heyns & Hutson, 1995)*. In fact there is no entirely satisfactory animal model for TD. Therefore, confusion has been produced by comparing TD of different species due to the marked morphological and topographical differences among the structures involved *(Wensing, 1986)*. Although it has been noticed as long as 1856 that TD should be studied primarily in the human in order to avoid false analogies drawn from animals *(Cleland, 1856)*, the difficulties and limitations of research on fetal processes in human or even in large mammals (which serve as much better models for the study of TD), account for the ample use of Glires (especially Rodentia: e.g. rat, mice but also Lagomorpha: e.g rabbit) as experimental models to study TD *(Heyns & Hutson, 1995; Wensing, 1986)*.

TD is a profound example of sexual dimorphism. It is a complex process that appears to be multi-staged involving an interplay of different anatomical structures and hormonal factors. TD has been accepted as being under hormonal control ever since *Engle (1932)* showed that pregnancy urine or anterior pituitary extracts caused premature TD in prepubertal monkeys. However the relation of this effect to the normal prenatal descent in man has remained unclear, since descent in the monkey is not complete until puberty *(Kinzey, 1971)*. The exact etiology of testicular maldescent (TMD) is not known but normal hypothalamo-pituitary-gonadal axis is usually a prerequisite for normal TD. Abnormal sexual differentiation is associated with TMD. Even though hormones cause TD, hormonal deficiency does not appear to be a common cause of TMD. Where true hormonal deficiencies are recognizable (i.e. abnormalities of the hypothalamic-pituitary axis) the testes are undescended. However, TMD in terms of defined hormonal syndromes comprise a very small subpopulation of the total number of children with undescended testes (UT), since the majority of boys with UT show no endocrine abnormalities after birth.

EMBRYOLOGY OF TESTICULAR DESCENT

Multi-staged TD was proposed first by *Gier & Marion (1969)* as initial nephric displacement by degeneration of the mesonephros at 7^{th}-8^{th} GW, transabdominal passage of the testis from the metanephros to the inguinal ring by 21^{st} GW and finally inguinal transit of the testis from the peritoneal cavity along the processus vaginalis at 28 GW. The fact that the first stage of TD proposed by this model has been completed very early during embryonic development precludes a major role of this step in sexual dimorphism *(Hutson et al., 1997)*. More recently, a biphasic model, with two morphologically and hormonally distinct phases, has been suggested to explain TD in normal males *(Hutson, 1985)*. According to this hypothesis the first (transabdominal) phase of TD (TTD), comprising the relative movement of the testis (compared with the ovary) from its initial posterior abdominal position (adjacent to the kidney by the 8^{th} GW) to the internal inguinal ring, is regulated by a non-

androgenic hormone/factor and a possible role of MIS was suggested (see below). The second (inguinoscrotal) phase of TD (ISTD) consists of the androgen-dependent descent of the testis from the internal inguinal ring to the scrotum. This phase begins at the 26th GW *(Backhouse, 1982; Heyns, 1987)*. Between 26th-28th GW, the testis descends rapidly through the inguinal canal and then moves slowly towards the scrotum reaching it at the 35th-40th GW *(Backhouse, 1982)*. In the human, migration of the gubernaculum and the testis occur simultaneously while in rodents, migration of the gubernaculum precedes TD (inguinoscrotal gubernacular migration: rat at 3-5 days post-natally, mouse at ~7 days post-natally; ISTD: rat at 3-4 weeks post-natally, mouse at ~2 weeks post-natally) *(Hadziselimovic et al., 1979; Wensing, 1986)*.

Between 10th-15th GW (TTD), the cranial suspensory ligament (CSL) that holds the urogenital tract near the developing diaphragm regresses (see below) while, simultaneously (8th-15thGW), the gubernaculum (that has appeared by the 7th GW *(Schneck & Bellinger, 2002)* as a condensation of mesenchymal tissue within the sub-ferous fascia on either side of the vertebral column that extends from the gonad to the fascia between the developing external and internal oblique muscles) enlarges caudally (see below) *(Hutson et al., 1997)*. Gubernacular caudal (bulb) enlargement keeps the testis anchored close to the future inguinal region via traction applied through the gubernacular cord which shortens as it becomes incorporated into the bulb *(Wensing, 1968; Wensing, 1973a)*. The net effect of these processes is the tethering of the testis near the groin and permission of its relative descent while the kidney migrates cranially during enlargement of the abdominal cavity *(Hutson et al., 1997)*. TTD takes place between 15.5-17.5 dpc in the mouse *(Hadziselimovic et al., 1980)* and between 16-20 dpc in the rat *(Van der Schoot, 1993a)*. ISTD needs development of the processus vaginalis, prior dilation of the canal by the bulb and some abdominal pressure to force the testis through the canal *(Hutson et al., 1997)*. Distal attachment of the gubernaculum has been experimentally shown to be important in the normal development of the processus vaginalis *(Clarnette, et al., 1996)*. During the 8th GW, the inguinal canal begins development as a caudal envagination of the abdominal wall that forms in conjunction with caudal elongation of the processus vaginalis *(Schneck & Bellinger, 2002)*. The processus vaginalis develops as a herniation of peritoneum at the deep internal inguinal ring and anterior aspect of the gubernaculum. This peritoneal diverticulum later elongates within the gubernaculum to form an annular cavity, dividing the gubernaculum into a central mesenchymal column and an outer parietal layer. Following complete descent of the testis, the central gubernacular column involutes by dissolution of the extra-cellular matrix. The residual tissue of the column forms the fibrous attachment of the testis to the scrotum. The cremaster muscle develops within the outer parietal layer of the gubernaculum forming a bilaminar sac in rodents, and a strip of muscle in ungulates and primates *(Wensing et al., 1980)*. The attachment of the cremasteric muscle to the inguinal abdominal wall varies from species to species *(Wensing, 1986)*. Its own nerve supplies the gubernaculum: the genital branch of the genitofemoral nerve (GFN) *(Hutson, 1970; Tayakkanonta, 1963)*. Careful dissection of the distal gubernacular end in humans and pigs shows that the lower end of the gubernaculum is free with no firm attachment to the scrotum *(Backhouse, 1982; Heyns, 1982)*. This is

strong evidence against the concept of the gubernaculum pulling the testis down to the scrotum, since it is not anchored inferiorly. The force of movement may come from the inta-abdominal pressure *(Elder et al., 1982; Frey et al., 1983; Frey & Rajfer, 1984; Gier & Marion, 1969; Hadziselimovic et al., 1979; Heyns & Hutson, 1995; Quinlan et al., 1988)* transmitted directly or indirectly to the testis via the lumen of the processus vaginalis and the gubernacular cord, respectively *(Hutson et al., 1995)*.

TRANS-ABDOMINAL PHASE OF TESTICULAR DESCENT

1. Regression of the cranial suspensory ligament under the influence of androgens

The CSL (derived from mesonephric mesenchyma) is a muscular cord-like structure, which borders the cranial part of the mesonephric mesentery, attaching the ovary and genital duct to the cranio-lateral surface of the dorsal abdominal wall, near the ventral aspect of the last rib. The primordium of the CSL is present in both sexes of, among other mammalian species, the rat, pig, dog, cattle and human *(Kersten et al., 1996; Van der Schoot & Emmen, 1996)*. The prevention of outgrowth of the fetal CSL in male rodents is an androgen-dependent process (prenatal exposure of females to androgens prevents development of the CSL, whereas males prenatally exposed to anti-androgens show CSL development in a female-like fashion) *(Barthold et al., 1994; Van der Schoot & Elger, 1992)* as well as in human (patients with complete or partial androgen insensitivity syndrome (CAIS/PAIS) preserve CSL), pig and cattle *(Clarnette et al., 1997; Van der Schoot & Emmen, 1996)*. However, androgen dependency of CSL regression has been disputed in the dog (*Kersten et al., 1996)*.

There is a critical period (~17-18 post-conceptional day) during which CSL development can be abolished by androgens in the male rat *(Van der Schoot P & Emmen, 1996)*. Since the rat testis starts to produce testosterone on 15.5-16.5 post-conceptional day *(Warren et al., 1975)*, this androgen effect is an early phenomenon during male sex differentiation, with an onset well before fetal testosterone production reaches its maximum on 18.5 post-conceptional day *(Habert & Picon, 1984)*. Furthermore, androgen receptor (AR) is expressed in the primordial cells of the CSL of both males and females, during this critical period (initially hormone-independent expression of AR) *(Emmen et al., 1998)*. However after 18 post-conceptional day AR is expressed intensively only in the male CSL primordium despite the loss of its androgen responsiveness. Thus the mesenchymal cells of the CSL can be considered as a direct target for fetal testicular androgens which have a strict time-specific morphogenic effect upon CSL. The relative absence of smooth muscle cells in the CSL of male fetuses, as compared to the female, indicates that androgens suppress smooth muscle cell differentiation in the CSL by possibly initiating a complex interaction among macromolecules (collagen, fibronectin, laminin) of the extra-cellular matrix and peptide growth factors (e.g. TGF?1) *(Emmen et al., 1998)*. This critical period seems to correlate with the precise time of androgen action on TD *(Husmann & McPhaul, 1991a; Spencer, er al., 1991)*.

Whether or not CSL regression is the key factor in TD is disputed. CSL regression is probably a prerequisite of TD *(Emmen, et al., 1998)*. However, it has been shown that regression of CSL alone is insufficient to cause gonadal descent (46,XX individuals

exposed prenatally to androgens retained their ovaries in a normal position) *(Scott, 1987)*. TD does not seem to be interrupted in cases with CSL retention in testicular feminized mouse (Tfm) (testes descended at the internal inguinal ring) and human with CAIS or PAIS (testes descended at the internal inguinal ring or beyond) *(Hutson, 1986)*. However, bilateral intra-abdominal testes associated with bilaterally retained CSL have been described in the dog *(Kersten et al., 1996)*. Furthermore, the testes of testicular feminization rat are intra-abdominal, inguinal and scrotal in 20%, 67% and 13% respectively, suggesting that CSL retention possibly has a role in TTD of the rat. Most authors generally agree that CSL has a limited role in at least some species *(Barthold et al., 1994; Husmann & Levy, 1995; Hutson et al., 1995)*.

2. The "gubernacular swelling reaction"

Caudal enlargement of the gubernaculum during relative TTD is known as the "gubernacular swelling reaction" (GSR) and it is caused by rapid cell proliferation and an increase in glycosaminoglycans and hyalouronic acid *(Backhouse, 1982; Backhouse & Butler, 1960; Heyns et al., 1986; Heyns et al., 1990)*. The hydrophilic nature of hyaluronic acid makes the end of the gubernaculum bulky and gelatinous similar to the Wharton's jelly found in the umbilical cord. GSR has been linked closely to trans-abdominal migration of the testis *(Wensing, 1973a)*. The (hormonal) control of GSR (and therefore TTD) remains controversial. Development of a male-like gubernaculum (and partial ovarian descent) in bovine freemartin (a female fetus exposed to the blood of a male twin by chorioallantoic anastomosis) *(Lillie, 1917; Van der Schoot et al., 1995)* and in female rabbit fetuses that had been grafted with a fetal testis, *(Van der Schoot, 1993b)* demonstrated the participation of a fetal testicular factor in gubernacular development and gonadal descent. However, it is less clear which testicular hormone/factor induces gubernacular outgrowth and regulates TTD. The testicular hormone/factor promoting TTD via outgrowth of the gubernaculum has remained unknown for decades. It received names such as "factor X", *(Habenicht & Neumann, 1983)* and "third hormone" *(Van der Schoot et al., 1995)*.

2.1. Evidence against androgenic control of the trans-abdominal phase of testicular descent

There is strong evidence that both GSR and TTD are not under androgenic control in a great number of mammalian species. Although AR-positive cells have been identified in the gubernacular mesenchymal core both in the rat *(Husmann & McPhaul, 1991b)* and the pig *(Heyns & Pape, 1991)*, their maximum expression in the rat is seen *(Husmann & McPhaul, 1991b)* after GSR becomes almost complete in this animal and both the receptor binding affinity and capacity are lower than in known androgen-sensitive target tissues (e.g. prostate) in the pig *(Heyns & Pape, 1991)*. Clinical examples supporting this theory include patients with CAIS/PAIS who have bilateral UT. In the majority of these cases testes are palpable bilaterally in the labioscrotal folds or inguinal regions, respectively *(Ahmed et al., 2000; Hutson, 1986)*. In addition there is a great number of experimental studies supporting this hypothesis. In the fetal pig, raccoon, dog and mouse with testicular feminization syndrome, GSR and TTD remain normal *(Fentener van Vlissingen et al., 1984; Hutson, 1986; Wensing et al., 1975)*. Furthermore, GSR and TTD is not impaired after exposure of monkeys, rats, mice and rabbits prenatally or dogs postnatally to

cryproterone *(Elger et al., 1977; Hutson & Beasley, 1988; Wensing, 1973b;)* or rats (prenatally) to flutamide *(Shono et al., 1994)*. In addition, injection of androgens into the rat or pig fails to stimulate GSR in fetal females *(Wensing, 1973a)*, while androgen replacemet after orchidectomy does not prevent gubernacular atrophy in the fetal dog *(Baumans, et al., 1982; Baumans, et al., 1983)*. However, in vitro studies of androgen effect upon gubernacular cell proliferation conclude to inconsistent results *(Emmen et al., 2000a; Fentener van Vlissingen et al., 1988; Kubota et al., 2002; Visser & Heyns, 1995)*. This is probably due to anatomical/ structural differences of gubernaculae among species (e.g porcine gubernaculum proper consist of mesenchymal cells only, in contrast to the gubernaculum of the rat that possesses a muscular outer layer-anatomical equivalent of the porcine cremaster muscle) *(Wensing, 1986)*.

2.2. Evidence against a significant role of the Mullerian inhibiting substance

A biphasic model of TD was proposed by *Hutson (1985)*, suggesting that a non-androgenic hormone, possibly MIS, regulates GSR and TTD. The conception is based on experiments showing that exogenous estrogens in the fetal mouse cause retention of the Müllerian ducts *(Hadziselimovic, 1983)*, inhibition of GSR *(Raynaud, 1958; Shono et al., 1996)*, and complete TTD blockage *(Hutson, 1987)* which is not reversed by simultaneous exogenous exposure to androgens *(Hutson & Watts, 1990)*. These results led to the hypothesis that estrogens have a primary effect of inhibiting Mullerian duct regression, as well as a second primary effect of inhibiting GSR and block TTD not by suppressing androgen secretion as it had been postulated before *(Hadziselimovic et al., 1979; Hadziselimovic et al., 1980)* but via suppression of a possible MIS effect on the gubernaculum *(Hutson, 1987; Hutson & Donahoe, 1986; Hutson & Watts, 1990)*. MIS has been further investigated as a factor mediating TTD, because in patients with persistent Mullerian duct syndrome (PMDS), which is a familial autosomal recessive disorder due to mutations in the MIS and/or MIS II receptor genes *(Imbeaud et al., 1996)*, both testes are located in the ovarian position in ~60-70% of cases *(Hutson et al., 1987)* and the gubernaculum is feminine-like i.e. thin and elongated *(Hutson et al., 1994)*. However, there is strong evidence against a significant role of MIS in GSR and TTD. Normal TD occurs in fetal rabbits immunized against bovine MIS despite partial retention of the Mullerian ducts *(Tran et al., 1986)*. In addition, semi-purified bovine MIS failed to induce in vitro DNA synthesis and cell division of cultured fibroblasts from fetal porcine gubernaculums *(Fentener van Vlissingen et al., 1988)*, while MIS appears to have a minimal proliferative activity on rat gubernacular cells in vitro due to a week expression of MIS II receptors *(Kubota et al., 2002)* (culturing rat gubernaculae in the presence of testes from MIS mutant mice do not decrease thymidine uptake compared with the effect of control or $MIS^{+/-}$ testes *(Emmen et al., 2000a))*. Furthermore, mice homozygous for targeted mutations in the MIS or MIS II receptor genes present with normally descended testes and normal GSR, while ovarian descent is not observed in transgenic femal mice overexpressing MIS *(Bartlett et al., 2002; Behringer et al., 1994; Mishina et al., 1996)*. However, it should be noted that male homozygous Tfm/MIS double mutant mice presents as pseudermaphrodite with severely impaired TTD although the Tfm and MIS mutants taken individually do not demonstrate impaired TTD *(Behringer et al., 1994)*. This fact may imply

that there is an as yet unknown mechanism via which androgen may regulate MIS activity on the gubernaculum *(Catlin et al., 1993)* or vice versa to govern TTD. A further argument against the proposed role of MIS on GSR and TTD is that, nearly all patients with inta-abdominal UT do not have persistent Mullerian ducts, while the intra-abdominal location of the testes in PMDS has been suggested to be due to the anatomic connection of the testes with the Mullerian ducts (anatomic blockade by the persistent ducts) rather than due to the absence of GSR *(Guerrier et al., 1989; Hutson, 1986; Josso et al., 1997)*.

2.3. The role of Insulin-like 3

In an in vitro study on the proliferation of fetal pig gubernaculum cells (*Fentener van Vlissingen et al., 1988)*, it was found that the extract from fetal porcine testis during the first phase of TD contains a low molecular weight (<3.5kD) fraction to which gubernaculum cells are responsive. It was suggested that this bioactive fraction probably contains the factor(s) that initiate TD. A number of polypeptide growth factors (epidermal growth factor (EGF), insulin, fibroblast growth factor, platelet-derived growth factor and transforming growth factor β) and testicular hormones tested (androgens, MIS (see above), inhibin), showed only minor stimulatory activity on gubernacular cells and were therefore excluded as putative regulators of TTD. This unknown factor received the name "descendin" and later it was proposed *(Visser & Heyns, 1995)* that it corresponds to a novel hormonal peptide of molecular weight ~26 kD secreted by the fetal testis cells that was named "gubernaculotropin".

Recently, based on gene knock-out mice studies *(Kubota et al., 2001; Nef & Parada, 1999; Zimmermann et al., 1999)*, a new hormone named insulin-like 3 (Insl3) with "descendin"/ "gubernaculotropin"-like activity has been identified and probably represents the long sought-after factor "descendin" or "gubernaculotropin". The Insl3 gene product, also known as Leydig cell insuline-like (Ley I-L) hormone or relaxin-like factor (RLF), is a member of the insulin peptide hormone superfamily *(Adham et al., 1993; Pusch et al., 1996; Zimmermann et al., 1997)* which includes at least eight members (insulin, insulin-like growth factor-I and II, relaxin, early placenta insulin-like peptide (Insl4 or EPIL) *(Chassin et al., 1995)*, insulin-5 (Insl5 or RIF-2) *(Conklin et al., 1999; Hsu, 1999)*, and insulin-6 (or RIF-1; higly expressed in the spermatocytes and round spermatids of rat and human) *(Hsu, 1999; Kasik et al., 2000; Lok et al., 2000)*) implicated in the regulation of diverse biological functions including cell growth, cell differentiation and energy balance. The mature hormone, like insulin, is composed of an A and a B chain linked by disulfide bonds *(Adham et al., 1993)*. The Insl3 gene is expressed in a differentiation and sexual dimorphic pattern *(Adham et al., 1993; Ivell & Bathgate, 2002)*. Maximal expression is observed at both fetal and mature adult-type Leydig cells (which replace fetal Leydig cells between post-natal days 5-15) whereas it is only weakly expressed in prepubertal immature Leydig cells and the ovary (with a sole exception so far known to be the ruminant ovary that exhibits a very high expression) *(Ivell et al., 1997; Zimmermann, et al., 1977)*. Insl3 transcripts are found in the developing testis of all mammalian species so far examined *(Adham et al., 2000)* but not in the gubernacular bulb or in other neighbouring tissues *(Nef & Parada, 1999)*. They are first detected at 13.5 dpc in Leydig cells (exclusive source in the testis) *(Ivell et al., 1997)*, remain constant through the 3rd postnatal week, when increases are

observed coincident with the first wave of spermatogenesis and germ cell maturation and reach their highest level in adult testis *(Adham et al., 2000)*. In contrast, in females, transcripts are first detected at post-natal day 6 in theca cells of small antral follicles (expression is correlated with the selection of the follicles to become pre-ovulatory *(Irving Rodgers et al., 2002)* and in a lesser degree in the luteal cells of the ovary *(Zimmermann et al., 1997)*.

It has been shown that homozygous ($Insl3^{-/-}$) male mice are viable but sterile, exhibiting bilateral UT located high in the abdomen due to developmental abnormalities of the gubernaculum. The gubernaculum resembles that of the wild type females and is characterized by the appearance of a flat thin bulb, elongated cord, absence of normal structural organization into outer (myoblasts) and inner (mesenchymal) layers, presence of some muscular development but absence of mesenchymal core *(Kubota et al., 2001; Nef & Parada, 1999; Zimmermann et al., 1999;)*. Apart from the gubernaculum and testicular position, the remainder of the genital tract (including regression of CSL) as well as sexual behaviour and serum testosterone levels appears normal. These results indicate that Insl3 stimulates the outgrowth and differentiation of the gubernacular primordium in an androgen-independent way in male mice, while androgen-mediated regression of the CSL occurs independently from Insl3 *(Nef & Parada, 1999; Zimmermann et al., 1999)*. Although Insl3 expression increases dramatically during puberty *(Zimmermann et al., 1997)*, which suggests an additional role for this hormone in male fertility, the inta-abdominal testes in the newborn Insl3 knock-out mouse are histologically normal. However, if left in this position until adulthood, histology deteriorates to the Sertoli-cell-only state leading to infertility in 100% of knock-out adult mice *(Nef & Parada, 1999; Zimmermann et al., 1999)*. It has been recently shown that intra-scrotal orchiopexy may rescue fertility in this model *(Nguyen et al., 2002)*. Thus infertily of $Insl3^{-/-}$ mouse model may be attributed to the icreased environmental temperature resulting from TMD *(Nef & Parada, 1999; Zimmermann et al., 1999)*. Heterozygous ($Insl3^{+/-}$) male mice are fertile and may present with partial (either uni- or bilateral) UT with a gubernaculum that appears to be differentiated but not embedded in the abdominal wall causing a delay in TD during adulthood (dosage sensitivity of Insl3 for TD) *(Nef & Parada, 1999)*. In contrast, there is a mildly altered phenotype in homozygous females, with impaired fertility due to disturbed cycle length (prolongation) and increased ovarian apoptosis, particularly in follicles and corpoa lutea *(Spanel Borowski et al., 2001)*. Based on these results Insl3 seems to play an essential role in TTD, it does not seem to be essential for spermatogenesis but its potential role(s) in male and female fertility remains to be further clarified.

In vitro studies of the effect of Insl3 and androgens upon rat gubernacular proliferation activity, suggest that both are required for the induction of gubernacular development and differentiation *(Emmen et al., 2000a, Kubota et al., 2002)*. However, there is strong evidence that in vivo Insl3-mediated activity upon the gubernaculum is androgen-independent *(Adham et al., 2002)*. This conclusion is supported by results with transgenic mice overexpressing active Insl3 in the pancreas during prenatal and postnatal development of females and males. Expression of the transgenic allele in the Insl3-deficient mice rescues TMD in male mutants. On the other hand, all transgenic females display bilateral inguinal hernias and descended ovaries over the blader

(attached to the abdominal wall via the well-developed CSL and the gubernaculums) due to Insl3-induced gubernacular development (shortenin of gubernacular cord, enlargement of gubernacular bulb and differentiation of the bulb into mesenchyme in the center and myoblasts in circumferential layers). Administration of DHT during prenatal development suppresses the formation of the CSL allowing the ovaries further descend into the processus vaginalis.

A critical aspect concerning Insl3 function and the underlying molecular mechanisms governing gubernacular differentiation awaits identification of a physiological receptor. Recently, a transgenic insertion mutant mouse strain called crsp (cryptorchidism with white spotting) that exhibits a phenotype strikingly similar to Insl3 mutants has been generated *(Overbeek et al., 2001)*. Homozygous mutants (crsp/crsp) of both sexes exhibit a decreased early postnatal viability, lower body weight and white spotting (WS) visible at post-natal days 3-5 as a variable sized white streak on the back in the midtrunk region plus a white belly patch. crsp/crsp males are completely sterile, presenting with small bilateral UT located in a high intra-abdominal position with an extended gubernaculum. However, the rest of the genital system appears normal and peripheral blood testosterone levels within normal range. In contrast, crsp/+ males as well as crsp/crsp and crsp/+ females share a wild type phenotype. Surgical scrotal descent of the homozygous mutants' testes reveals presence of sperm in the epididymis, indicating that crsp is, like Insl3, not essential for spermatogenesis. Complete arrest of spermatogenesis is attributed to high temperature environment of the intra-abdominal testes. The crsp transgenic integration is accompanied by a ~550 kb deletion in the distal part of chromosome 5 upstream of the Brca2 gene (corresponding to the previously characterized human 13q12-13 region) *(Couch et al., 1996)* affecting several genes. Direct sequencing of the affected region led to the identification of a new gene named Great (G protein-coupled receptor affecting testis descent) *(Overbeek et al., 2001)*, mutations of which have recently been proposed to be responsible for the phenotype in crsp mice *(Gorlov et al., 2002)*. Great gene is composed of 18 exons *(Overbeek et al., 2001)* and encodes a novel G protein-coupled receptor (GPCR) named mouse GREAT (mGREAT) *(Gorlov et al., 2002)*. In particular, mGREAT is an orphan leucine-rich repeat-containing GPCR (LGR) that belongs to the same subgroup of LGRs with a Drosophila melanogaster LGR (DmLGR3), a snail LGR (Lymnacea stagnalis, LsLGR), and two human LGRs (LGR7 and LGR8) *(Hsu et al., 2002)*. Expression of Great is restricted to the testis, brain, and skeletal muscles, with the highest level of expression in the gubernaculum. LGR8 has been proven to be the human ortholog of mGREAT with 82% overall identity at nucleotide and amino acid level and same exon-intron structure *(Gorlov, et al., 2002; Hsu et al., 2002)*. It has been recently shown that INSL3 specifically activates GREAT/LGR8 receptors through the adenylate cyclase pathway in the gubernaculum leading to gubernacular cell proliferation *(Hsu et al., 2002; Kumagai et al., 2002)*.

Two points of interest concerning crsp mutation are also worth-noting. First, UT has been reported in some patients with Rieger syndrome type II (hereditary glaucoma). A gene responsible for this syndrome has been mapped close to BRCA2 *(Phillips et al., 1996)*. The relevance of this gene to the crsp mutation remains to be elucidated. Second, there may be a relationship between crsp-induced WS phenotype and

inactivation of a gene responsible for UT. Melanoblasts and somatic nerves are derived from the neural crest during development, while neural crest derivatives are possibly implicated in TD (UT in human is often associated with neural tube defects) *(Hutson & Beasley, 1988)*. Furthermore, it has been suggested that proper innervation of the cremaster muscle and gubernaculum are essential for normal TD *(Hutson et al., 1997)*. Additionally, there is at least one more example of association among UT, WS and behavioural abnormalities (suggesting abnormalities of the neural system) described in foxes homozygous for a semidominant mutation in the gene encoding the STAR protein *(Belyaev et al., 1981)*. Taken all these together, it could be assumed that crsp mutation may in some way disturb proper functioning of embryonic neural crest cells resulting both in UT and WS.

The observation that in utero exposure to exogenous estrogens in rodents and humans *(Gill et al., 1979; Greene et al., 1942; Hutson et al., 1987; Raynaud et al., 1958; Shono et al., 1996)* as well as the absence of Insl3 hormone in mice *(Kubota et al., 2001; Nef & Parada, 1999; Zimmermann et al., 1999)* both induce bilateral inta-abdominal UT in newborn males, has triggered investigation for a possible relationship between these two hormones. It has been suggested that UT induced by the nonsteroidal synthetic estrogen diethylstilbestrol (DES) is due to failure of gubernacular development *(Raynaud et al., 1958; Shono et al., 1996)*, but the exact mechanism is poorly understood. It has been demonstrated that estrogens inhibit fetal Leydig cell development *(Hadziselimovic et al., 1980)* in a fetal pituitary gland- and androgen-independent way *(El Gehani et al., 1998; Grocock et al., 1988; Hutson, 1987; O Shaughnessy et al., 1998)*. Prenatal exposure to estrogens, such as 17α- and 17β-estradiol, estriol, DES, inhibits Insl3 gene transcription in embryonic Leydig cells, thus providing an explanation for the lack of GSR and UT *(Emmen et al., 2000b; Nef et al., 2000)*. Evidently, abnormally high levels of estrogenic molecules can overcome the normal fetal buffering mechanisms (serum α-fetoprotein that binds efficiously estrogen compounds *(Keel & Abney, 1984; Nunez et al., 1974)* and estrogen sulfotransferase in Leydig cells, which facilitates the catabolism of estrogens *(Hobkirk & Glasier, 1992; Song et al., 1995)*) that control the concentration of free estradiol (either produced in situ by Sertoli cells *(Dorrington & Armstrong, 1975; Pomerantz, 1980)* or concentrated by the testis *(Kelch et al., 1972)* (from exogenous sources) in Leydig cells. Estrogen inhibitory effect must be mediated via indirect estrogen-receptor (ER) transcriptional control (Insl3 proximal promoter does not reveal sequences obviously related to estrogen-response elements) *(Zimmermann et al., 1998)* of Insl3 regulatory genes possibly *(Majdic, et al., 1997)* other than SF1 *(Emmen et al., 2000b; Nef et al., 2000).* SF1 is a direct mediator of Insl3 gene transcription in Leydig cells *(Zimmermann et al., 1998)*. Intrestingly, Insl3 expression reappears between post-natal days 0-7 suggesting that Insl3 gene transcription is permanently blocked in the fetal-type Leydig cell population. Exposure of adult males to estrogens does not block Insl3 gene expression *(Nef et al., 2000)*. This indicates that Insl3 gene regulation in fetal and adult population of Leydig cells is different. In fetal Leydig cells, Insl3 expression is estrogen-sensitive and gonadotropin-independent, whereas in adult Leydig cells, the expression is estrogen-insensitive and gonadotropin-dependent *(Balvers, et al., 1998)*.

Human INSL3 gene maps to chromosome 19p13-p12, and consist of two exons: exon 1 encodes for a signal peptide (that is present in the preproprotein), the B chain and the first eight aminoacids of the C-peptide; exon 2 encodes for the rest of the C-peptide and the A chain *(Burkhardt et al., 1994)*. Insl3 appears to be necessary for the development of the mesenchymal component of the gubernaculum (the predominat muco-polysacharide structure in human, dog, or pig gubernaculum in contrast to the gubernaculum of rodents which is mainly composed of cremasteric muscle with a mesenchymal core) *(Bartlett et al., 2002; Donaldson et al., 1996)*. The $Insl3^{+/-}$ male mouse phenotype appears to resemble many cases of human TMD, where partial TD at birth often is self-corrected *(Nef & Parada, 1999)*. However, only two studies so far *(Marin et al., 2001a; Tomboc, et al., 2000)* have implicated INSL3 gene mutations (3 in total) in human TTD while the majority of studies have failed to indicate such an association *(Baker et al., 2002; Koskimies et al., 2000; Krausz et al., 2000; Lim et al., 2001; Marin et al., 2001b; Takahashi et al., 2001)*. Furthermore, a missens mutation affecting the structure of LGR8 has been recently reported among 60 affected individuals *(Gorlov et al., 2002)*. Although these results point to the notion that mutations involving the human INSL3 and/or the LGR8 genes are not common causes of UT, it is difficult to interprete their phenotypic effects and there is a great need for further functional studies in order to clarify their potential influence on TD. The exact signifficane of this hormone in the pathophysiology of TD awaits generally further testing.

3. The role of Homeobox genes

Other genes possibly implicated in TTD include homeobox (Hox) genes. Hox genes were first discovered within the homeotic complex (HomC) of Drosophila *(Akam, 1989)*. They encode transcription factors all of which share a helix-turn-helix DNA-binding motif, the homeodomain *(Kissinger et al., 1990)*. In mammalian genomes, four Hox complexes designated HoxA, B, C, D and a total of 39 genes are found, located on four different chromosomes *(Branford et al., 2000)*. These genes display remarkable conservation throughout evolution with nearly the same sequence in mice as in humans *(Duboule & Dolle, 1989)*. Thus, murine Hox a, b, c, d (chromosome 6, 11, 15, 2, respectively) correspond to human HoxA, B, C, D (chromosome 7, 17, 12, 2, respectively) *(Kolon et al., 1999)*. They have a key role in the morphogenesis of segmental structures along the primary anteroposterior body axis, such as branchial arches, vertebrae, cranial nerves and ganglia *(Botas, 1993; Krumlauf, 1994)*. They are expressed in many cell types acting as master regulatory switches or transcription factors that specify axial identity. In addition, they control the growth and differentiation of cells *(Botas, 1993*). The single Drosophila Abdominal B (AbdB) gene at the 5' end of the HomC (which specifies the identity of the Drosophila posterior segments) is represented by 5 paralogous groups within mammalian Hox clusters (16 genes in total) *(Krumlauf, 1994)*. These AbdB-related genes constitute a dinstinct subfamily of HOX genes that exhibit posterior domains of expression *(Krumlauf, 1994; Izpisua Belmonte et al., 1991)*, including the developing genitourinary system in vertebrates *(Celniker & Lewis, 1987; Dolle et al., 1991)*. Specifically the AbdB-related Hoxa-10 gene is expressed in posterior domains of the developing mouse embryo, including intermediate mesoderm, which gives rise to the gonads and genitourinary tract *(Benson et al., 1995)*.

Recent studies of murine knockout models of Hoxa-10 *(Satokata et al., 1995; Rijli et al., 1995)* have demonstrated male mutants which are fully viable, but present with uni- or bilateral UT (small testes varying from perinephric to lower abdominal space in location) as well as developmental abnormalities of the gubernaculum and GFN with resultant abnormal spermatogenesis and sterility. Anterior homeotic transformations of the male reproductive tract with the proximal ductus deferens transformed into the epididymes have also been reported *(Benson et al., 1996)*. Male homozygotes are normally virilized, in contrast to testicular feminization *(Lyon & Hawkes, 1970)* and GnRH-deficient *(Radovick et al., 1991)* models of TMD. Therefore, hormonal derangements appear unlikely to cause TMD in Hoxa10-deficient mice *(Satokata et al., 1995; Rijli et al., 1995)*. In situ hybridization at 15.5 dpc indicates that both the gubernacular cord and bulb are sites of strong Hoxa10 expression in the male mouse embryo and this expression continues postnatally *(Satokata et al., 1995)*. Furthermore, analysis of the gubernaculum in Hoxa10-deficient males reveals that shortening of the cord and outgrowth of the bulb have failed to occur *(Satokata et al., 1995)*. Cremasteric myocytes, although present, are disorganized and reduced in number *(Satokata et al., 1995)*. Furthermore, the processus vaginalis, the inguinal canal and the scrotal sac are either missing or partially formed *(Rijli et al., 1995)*. Since the above findings are combined with a morphological (anterior) transformation of vertebrae (anterior homeotic transformation of L1 vertebra to T13, causing an extra T13 vertebra) and spinal nerves (i.e. the last thoracic nerve is transformed to an intercostal nerve identity, the first lumbar nerve is transformed to a last thoracic nerve identity, and the second to fifth lumbar nerves are transformed to first to fourth lumbar nerve identity) *(Rijli et al., 1995; Satokata et al., 1995)*, it has been speculated that TMD in this model might be due to incorrect specification of GFN motoneurons and/or abnormal target tissue innervation *(Rijli et al., 1995)*. Mutations of Hoxa-11, also expressed in paramesonephric mesenchyme, affect similarily the male reproductive tract development. Direct comparison of gubernacular defects (abnormally thin, elongated with apparent failure of formation of the cord and muscular bulb) do not reveal any overt differences between Hoxa-$10^{-/-}$ and Hoxa-$11^{-/-}$ *(Branford et al., 2000)*. Similarily Hoxa-$10^{+/-}$/Hoxa-$11^{+/-}$ transheterozygotes display analogous male reproductive tract defects. However, Hoxa-$10^{-/+}$ and Hoxa-$11^{-/+}$ male mice *(Branford et al., 2000)*, present with normal TD and gubernacular development. Thus, Hoxa-10 and 11 appear to be functionally redundant in the development of the male reproductive system, such that the quantitative loss of any of the two Hoxa-10/Hoxa-11 alleles results in similarly severe abnormalities *(Branford et al., 2000)*.

HOXA10 is located at the human chromosome 7 and consists of 2 exons. An increased amount of genetic alterations (point mutations and polymorphisms) located in exon 1 of this gene have been identified in some boys with UT compared to normal controls *(Kolon et al., 1999)*. This was most evident in familial cases of UT. However, functional analysis of the mutations has not yet been performed and specific associations between mutations and TTD remain to be studied. Further examination of the paralogous genes of HoxA10 (HoxB10, C10, and D10) and its orthologous genes (HoxA9 and A11) will also help elucidate the role of these regulatory genes in TTD.

INGUINOSCROTAL PHASE OF TESTICULAR DESCENT

1. The role of abdominal pressure and gubernaculum

The second phase of TD, ISTD, is currently thought to be regulated by a combination of mechanical and hormonal factors. It is hypothesized that the abdominal pressure caused by the growth of the viscera *(Frey et al., 1983; Frey & Rajfer, 1984; Quinlan et al., 1988)*, combined with putative gubernacular morphological and compositional changes contribute to the testes push down through the inguinal canal *(Hutson et al., 1997)*. Conditions that result in UT hypothetically associated with decreased intra-abdominal pressure include prune-belly syndrome, cloacal exstrophy, omphalocele, gastroschisis, and a number of syndromes that include both UT and congenital abdominal wall muscular defects or agenesis *(Kaplan et al., 1986; Koivusalo et al., 1998)*. Abdominal pressure probably has an ancillary role in TTD, but thereafter it plays a more significant role during ISTD *(Attah & Hutson, 1993; Quinlan et al., 1988)*. Androgens may also play a role together with abdominal pressure at this stage of development to deliver the testis into the scrotum *(Frey & Rajfer, 1984; Frey et al., 1983)*. *Frey et al. (1983)* demonstrated experimentally that in the presence of androgen a silicone prosthesis descends from the abdominal cavity into the scrotum, but this occurs less frequently when androgen is removed.

The role of the gubernaculum in ISTD remains controversial. Regression of the bulky bulb, which is particularly prominent in the pig, was thought to be sufficient to allow the testis to reach the scrotum *(Wensing, 1968; Wensing, 1973a; Wensing, 1986)*. A significant active migratory phase in human *(Heyns, 1987)* and rodents *(Fallat et al., 1992)* has been demonstrated, although this is disputed by some authors in favor of involution and eversion of the gubernacular cone *(Elder et al., 1982; Hadziselimovic et al., 1979; Radhakrishnan & Donahoe, 1981)*. Recently it has been suggested that the gubernacular bulb actively proliferates after birth in rodents and possibly differentiates into new cremaster muscle cells *(Hrabovszky, et al., 2002)* while in human *(Costa et al., 2002)*, gubernacular connective tissue undergoes such an extensive remodeling during TD that finally gubernacullum becomes an essentially fibrous structure rich in collagen and elastic fibers. Such changes decrease its size and, thus contribute to other forces that cause the testis move toward the scrotum. Due to lack of smooth muscle cells, and the small amount of striated muscle cells, active contraction of the human gubernaculum is less likely to be an important factor in ISTD.

2. The inguinoscrotal phase of testicular descent is androgen-dependent

It is generally agreed that ISTD is androgen-dependent *(Hadziselimovic, 1983; Hutson & Beasley, 1992; Schneck & Bellinger, 2002)*. Migration of the gubernaculum beyond the inguinal region is absent in gonadotropin-deficient animals *(Grocock et al., 1988)* as well as in both animals with testicular feminization and humans with complete androgen resistance *(Hutson, 1986)*. Furthermore, about 50% of animals treated prenatally with the anti-androgen flutamide also have deraranged gubernacular migration and delayed regression *(Goh et al., 1994a; Husmann & McPhaul, 1991a; Husmann & McPhaul, 1992; McMahon et al., 1995; Spencer et al., 1991)* Regression of the gubernacular bulb appears androgen-dependent since in the human with

complete androgen resistance the gubernaculum remains enlarged, with failure of extra-cellular matrix resorption *(Hutson & Donahoe, 1986; Radhakrishan & Donahoe, 1981)*.

The way (direct or indirect) of androgenic action on the gubernaculum remains controversial. It seems quite possible that androgens act idirectly on the gubernaculum to mediate ISTD. AR localization studies in humans have demonstrated the presence of the receptor in the cremaster muscle but not in the gubernaculums *(Johansen & Klein, 1993)*. However, these studies were performed on postnatal tissues only, and it is not presently known whether the AR is expressed in the gubernaculum or cremaster muscle at the time of human ISTD. Genetic alterations (mutations or polymorphic CAG trinucleotide repeat expansions of AR exon 1 which are directly associated with underviriliztion) appear to be an unlikely cause of isolated UT in human *(Lim et al., 2001b; Sasagawa et al., 2000; Suzuki et al., 2001; Wiener et al., 1998)*. Specific ARs have been found in cultured porcine gubernacular fibroblasts *(Oprins et al., 1988)*, but the total AR concentration as well as both receptor binding affinity and capacity have been reported to be significantly lower than in recognized androgen target organs such as the prostate and urethra, and comparable to that of male or female striated muscle *(Heyns & Pape, 1991; Heyns et al., 1988)*. 5α-reductase activity has been reported to remain constant throughout gestation in the gubernaculum of the pig, at lower levels than those seen in prostate or urethra *(Heyns et al., 1993)*. The AR level of expression at the mesenchymal core of rat gubernaculum declines during ISTD *(Husmann & McPhaul, 1991b)*. The total amount of specific androgen bound in the neonatal rat gubernaculum has been reported to be 20% the amount measured in the urogenital sinus *(George & Peterson, 1988)*.

2.1. The genitofemoral nerve hypothesis

Quite recently a new hypothesis has been generated according to which, androgens act primarily on the GFN, rather than directly on the gubernaculum. As a result, the GFN releases a neurotransmitter (proposed to be the calcitonin gene-related peptide (CGRP)) that acts as a second messenger of androgenic stimulation on the gubernaculum to mediate ISTD (GFN hypothesis) *(Hutson & Beasley, 1987)*. The origin of the GFN hypothesis lays on the experiments of *Lewis (1948)* who transected the GFN in newborn rats and prevented by this means TD, while testing the "traction theory". At that time the cremaster muscle was being considered as a source of muscular traction to pull the testis into the scrotum. The idea was that since the cremaster muscle is supplied by the GFN, transection of the nerve would paralyze the muscle and prevent TD. Many workers were critical to this experiment because they believed that contraction of the cremaster would pull the testis up to the groin, rather down to the scrotum. Nevertheless, it was intriguing that denervation blocks a process that is under apparent androgenic control, leading to the speculation that androgens act via the nerve *(Hutson & Beasley, 1987)*. Androgens should lead to prenatal GFN modification *(Hutson & Beasley, 1987)*, as even in species with postnatal gubernacular migration, the nervous system would require earlier modification. Studies with antiandrogens confirm that ISTD, although occurring postnatally in rodents, can only be blocked by prenatal treatment *(Goh et al., 1994a; Husmann & McPhaul, 1991a; Husmann & McPhaul, 1992; Shono, et al., 1994; Spencer et al., 1991)*.

The GFN hypothesis is consistent with the results of distal transection of the gubernaculum in neonatal rats, which prevents gubernacular postnatal migration and ISTD while proximal transection which preserves gubernacular nerve supply (the GFN supplies the gubernaculum from its posterio-caudal surface *(Tayakkanonta, 1963; Larkins & Hutson, 1991)*) fails to block ISTD *(Beasley & Hutson, 1988; Bergh et al., 1978)*. Furthermore, this hypothesis predicts that neuronal anomalies affecting the GFN nucleus should be associated with UT. This is in fact the case for children with spina bifida, particularly when the myelomeningocele is in the high lumbar region (above L4; the origin of GFN) *(Hutson et al., 1988)*. A similar result can be obtained by neonatal rat spinal cord transection at the level of low thoracic-high lumbar region leading to a 75% incidence of UT *(Hutson et al., 1988)*.

There is plenty of evidence supporting the role of CGRP in stimulating the developing gubernaculum. In organ culture, neonatal rodent guberaculae show a dose-dependent response to exogenous CGRP by rhythmic contractions *(Park & Hutson, 1991; Momose et al., 1992)*. Prior transection of the GFN results in the gubernaculum becoming more sensitive to CGRP *(Shono et al., 1995)*, which is consistent with up-regulation of CGRP receptors *(Terada et al., 1995)*. CGRP receptors are most abundant during the period of gubernacular migration to the scrotum *(Yamanaka, et al., 1993)*. Prenatal flutamide treatment increases the density of CGRP receptors in the gubernaculums *(Terada et al., 1994)*. In mutant trans-scrotal rats (TS; congenitally TMD from unknown causes-natural model of UT), CGRP receptors are diminished and the gubernaculum is resistant to exogenous CGRP *(Goh et al., 1993; Terada et al. 1995)*. However, after GFN transection or after selective sensory denervation with capsaicin (which depletes CGRP from the nerves) gubernacular contractility, in response to exogenous CGRP in organ cultures, is restored due to an increase in the gubernacular receptor content *(Hrabovszky & Hutson, 1999; Terada et al. 1995)*.

A role of the GFN and its neurotransmitter CGRP in TD has been well established *(Beasley & Hutson, 1987; Beasley, & Hutson 1988; Bergh et al., 1978; Goh et al., 1993; Hrabovszky & Hutson, 1999; Lewis, 1948; Momose et al., 1992; Park & Hutson, 1991; Shono et al., 1995; Terada et al., 1994; Terada et al., 1995; Yamanaka et al., 1993)*. However, the exact mechanism by which circulating androgens act on the GFN is not yet known. Intrauterine flutamide exposure during gestational days 16-19 leads to UT in rats at a frequency of ~50% *(Husmann & McPhaul, 1991a; Spencer et al., 1991)*. The exact mechanism is uncertain, but it has been suggested that flutamide prevents circulating androgens from masculinizing the GFN during this critical phase of development *(Hutson & Beasley, 1987)*. A specific search for AR in the fetal rat lumbar spinal cord shows androgenic binding as early as 15 days of gestation *(Cain et al., 1994a)*, which is consistent with androgens stimulating differentiation of the GFN and its nucleus. Furthermore, a significant decrease in the neural diameter and the number of myelinated axons within GFN ipsilateral to UT has been reported in flutamide-treated rats *(Husmann et al., 1994)*.

Previous studies focused on the motor nucleus of the GFN (L1-L2 in the spinal cord) as the primary target organ of circulating *androgens (Barthold et al., 1994; Barthold et al., 1996; Goh et al., 1994a; Goh et al., 1994b; Larkins, 1991)*. The GFN motor nucleus is sexually dimorphic in

rodents, but antiandrogen treatement causes just minor decrease in its cell content *(Barthold et al., 1996; Goh et al., 1994b)*. The sexual dimorphism of cell number in the newborn rat is lost after flutamide treatment but due to an increase of the number of cell bodies in females and not due to a significant loss of cell bodies in males as expected *(Goh et al., 1994a)*. Furthermore, it has been claimed that androgens have no effect on the GFN mortor nucleus since both in rats *(Barthold et al., 1994)* and mice *(Larkins et al., 1991)* with testicular feminization, its size is found to be similar to that of normal animals while rat GFN motoneurons virtually lack CGRP immunoreactivity *(Barthold et al., 1994; Newton et al., 1990; Schwindt et al., 1999)*.

Dense CGRP immunoreactive varicose fibres surrounding GFN motoneuron cells in male rats which are absent in females have been described *(Newton et al., 1990)*, and recently it has been shown that the rat sensory nucleus of the GFN (L1-L2 dorsal root ganglia) is also sexually dimorphic *(Hrabovszky et al., 2000)*. In addition, it has been reported that flutamide decreases the number of cells both in male and female rat GFN sensory nucleous, but a significant decrease of the CGRP immunoreactive cells is detected only in males *(Hrabovszky et al., 2000)*. Furthermore, it has been shown that in TS rats the sensory nucleus of the GFN ipsilateral to an UT contains significantly more CGRP immunoreactive cells compared to the contralateral normally descended testis *(Hrabovszky et al., 2001)*. It has been hypothesized that TMD in this strain may be due to a primary defect of CGRP receptors and the increased number of CGRP immunoreactive cells represent an insufficient compensatory mechanism. Based on these results, the GFN hypothesis has been recently modified to include a possible role of circulating androgens acting on the sensory nucleus of GFN instead of the motor nucleus as previously thought *(Hrabovszky et al., 2000)*. Despite the extensive work, the GFN hypothesis is still questionable since CGRP has been shown to fail to induce murine TD *(Houle & Gagne, 1995)*. Additionally, CGRP is a neuromuscular neurotransmitter that has been demonstrated to act on the muscular component of the rodent's developing gubernaculum, which is primarily cremasteric muscle *(Husmann & Levy, 1995)*. Human gubernaculum consists mostly of mucopolysacharides and does not contain almost any muscular component (target of CGRP action) *(Costa et al., 2002; Heyns et al., 1990).* This fact most likely excludes CGRP from playing a significant role in human TD (*Husmann & Levy, 1995; Schneck & Bellinger, 2002)*.

Alternatively, *Levy and Husman (1995)* have proposed an important role for EGF in the mediation of androgen action. Supraphysiological doses of EGF can reduce the incidence of flutamide-induced TMD in rodents, and vice versa, decreased EGF concentrations in the serum of dams result in a significant incidence of UT and epididymal abnormalities in the offspring. EGF stimulates gonadotropin sectretion by the placenta, and it has been proposed that EGF effects may reside mainly in the regulation of placental function *(Levy & Husmann, 1995)*. However, there are no data up to now about the role of EGF in the descent of human testis.

4. EPIDEMIOLOGY OF TESTICULAR MALDESCENT

INCIDENCE OF TESTICULAR MALDESCENT

Isolated UT represents the most common congenital abnormality of the male genitalia of human and other mammalian

species (e.g. in the dog the incidence is estimated ~1.2-5% *(Boothe, 1993)*) and generally one of the most common human congenital malformations at birth, affecting more than 3.5% of male newborns *(Berkowitz et al., 1993; Ghirri et al., 2002; James & Jean, 1998; John Radcliffe Hospital Cryptorchidism Study Group, 1992; Scorer, 1964; Thong et al., 1998)*. Unilateral UT is more common than bilateral in mammals (the incidence of bilateral UT is estimated ~1.6-1.9% of cases in human *(Schneck & Bellinger, 2002)*, ~10-16% of cases in horses *(Wintzer, 1986)* and ~20.2% of cases in dogs *(Johnston, et al., 2001)*). In most cases in human, testes have descended at or below the internal inguinal ring (intra-abdominal UT comprises a small group of ~5-10%) *(Hutson et al., 1997)*. This is the case also in the dog that exhilit intra-abdominal UT in ~18% of cases *(Boothe, 1993; Johnston, et al., 2001)* (Figures 1, 2) in contrast for example to the horse *(Wintzer, 1986)* that exhibit more commonly abdominal and incomplete abdominal TD (testicle in the abdominal cavity-tail of the epidymis and vas deferens into the inguinal cannal) with a ratio abdominal/inguinal ~2:1. Racial as well as ethnic differences *(Heinonen et al., 1997; Berkowitz & Lapinski, 1996)* have been reported, although there are studies recognizing no such differencies *(Berkowitz, et al., 1993)*. In horses UT is more commonly seen in ponies *(Wintzer, 1986)*, while in the dog an increased incidence (~2.7 times greater risk) is observed in small breeds (<9.1Kg, e.g. Chihuahua, Yorkshire terrier e.t.c) *(Boothe, 1993; Johnston, et al., 2001)*. There is strong evidence that the incidence rate is much higher (up to 10 times) in premature infants (<37 GW, birth weight < 2500 g) than in full-term babies *(Berkowitz et al., 1993; Ghirri et al., 2002; James & Jean, 1998; John Radcliffe Hospital Cryptorchidism Study Group,*

Figure 1. Undescended tesis. Edwards' model applied on data from Greek population (1991-1998) indicates that the maximum incidence rate is observed during spring (peak in March) and the minimum incidence rate is observed during autumn (trough in September): x^2EDWARDS value at 2df is statistically significant ($p<0.001$)k

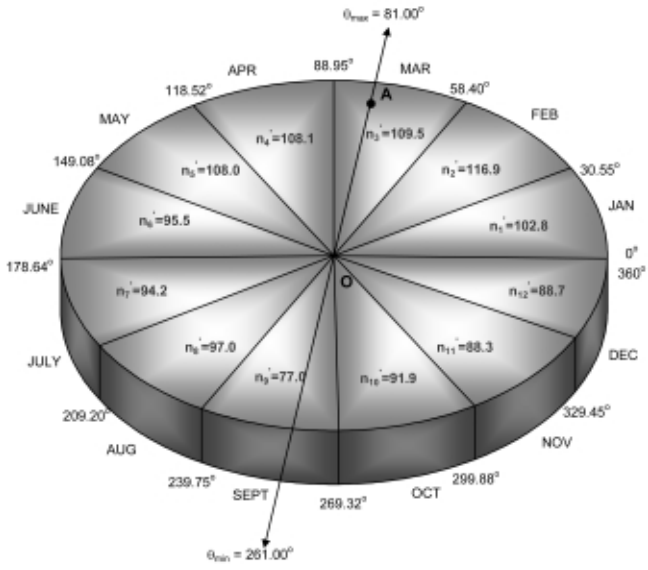
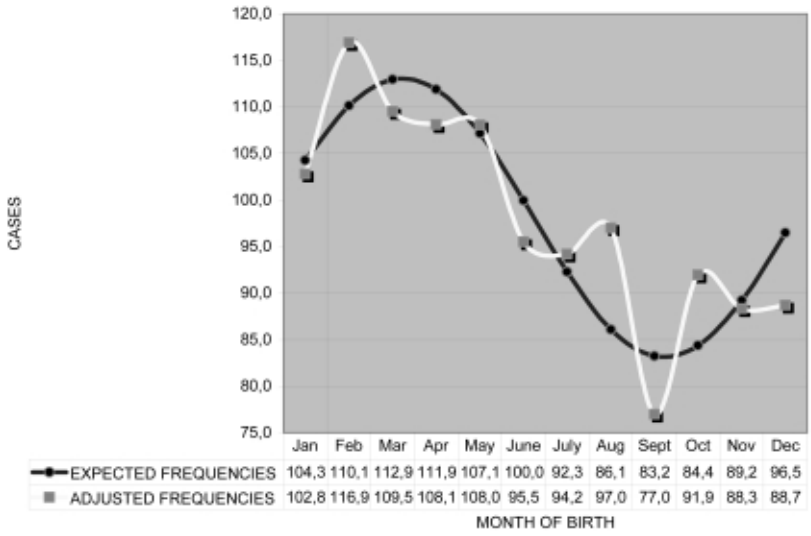


Figure 2. Undescended tesis. Adjusted frequencies fitted to the simple harmonic curve given by Edwards' test. Data are satisfactorily described by the simple harmonic curve since the two curves do not differ significantly (x^2 goodness of fit test value at 11 df is not statistically significant; p>0.2)



1992; Scorer, 1964; Thong et al., 1998). Since the testes normally descend during late pregnancy, TMD can be considered as a normal phenomenon in premature children and spontaneous descend may be expected. The completion of TD usually occurs during the first weeks after birth (approximately 70%-77% of UT will spontaneously descend early, usually by 3 months of age) so the incidence of UT by the end of infancy declines to approximately 0.7-1% and remains constant thereafter *(James & Jean, 1998; Schneck & Bellinger, 2002;)*. Factors that predict complete early postnatal TD include bilateral anomaly, normal scrotum anatomy and non-ectopic position, in contrast to the presense of a small or poorly rugated scrotum and hypospadias *(John Radcliffe Hospital Cryptorchidism Study Group, 1992)*. On the other hand factors that may indicate "late descenders" (after 1 year of life) include black or Hispanic ethnicity, family history of UT, consumption of cola-containing drinks during the pregnancy, low birthweight, and preterm delivery *(Berkowitz & Lapinski, 1996)*.

SEASONAL TRENDS IN THE INCIDENCE OF TESTICULAR MALDESCENT

Temporal analysis (investigation of secular and cyclic trends) has been widely employed as a means to elucidate the etiology of diseases. The investigation of seasonality (the most important type of cyclic variations) is especially used for the basic etiological description of congenital abnormalities. Temporal analyses suggest that there is an increasing secular trend in the incidence of UT during the last few decades in some countries *(Berkowitz et al., 1993; John Radcliffe Hospital Cryptorchidism Study Group, 1992; Scorer, 1964)*, however

there are still contrary reports *(Ghirri et al., 2002; Kaleva, et al., 2001a)*. Furthermore large regional differences among related countries have been reported (e.g. in Finland the incidence is clearly lower than in Denmark) *(Kaleva, et al., 2001a; Kaleva, et al., 2001b)*. More epidemiological studies using standardized diagnostic criteria and examination techniques are required in order to obtain a more precise estimation of the secular and regional trends in the incidence of UT, and detect possible environmental effects *(Toppari et al., 2001)*. On the other hand, there seems to be a general agreement on the seasonality of UT with spring and autumn being the seasons of significantly highest (peak in March) and lowest (trough in September) incidence respectively *(Berkowitz et al., 1995; Czeizel et al., 1981; Hjertkvis et al., 1989; Jackson & Swerdlow, 1986)*. Most researcher have attempted to attribute the above phenomenon to analogous seasonal variations of sex hormones. It has been suggested that variations in daylight length influencing the maternal pineal gland function may alter the secretion of melatonin, which in turn may influence maternal pituitary and ovarian hormonal activity and maternal hormonal balances causing alterations in fetal testicular function. The latter could be responsible for the maldescent (theory of light).

However, by strictly applying statistical tools especially designed for the investigation of seasonality in congenital malformations with respect to their recommended limitations, we have recently showed *(Mamoulakis et al., 2001; Mamoulakis et al., 2002a)* that the monthly incidence of births of children with isolated UT in Greece follows a documented cyclic pattern of simple harmonic type (peak in March/May, trough in September) similar to that observed in other studies in Northern Europe *(Czeizel et al., 1981; Hjertkvis et al., 1989; Jackson & Swerdlow, 1986)*. The detection of such a pattern in a country such as Greece with almost no significant differences in daylight length among seasons is strong evidence against the theory of light. Furthermore, we have showed that the maternal peripheral serum human chorionic gonadotropin (β-hCG) levels at the 26th GW (beginning of the ISTD) are significantly lower during winter compared to summer *(Mamoulakis et al., 2002a)*. Considering that increased incidence of UT was observed during March or May, it appears that the beginning of the ISTD in December-January or February-March period respectively, is accompanied by an increased incidence of UT. Thus low environmental temperatures at the start of ISTD may be related with an increased probability of failure of the testis to start or complete ISTD. Since ISTD is androgen-dependent, it appears that low environmental temperatures at the 26th GW are accompanied by a decrease in maternal β-hCG profiles and detrimentally affect the embryonic Leydig cell secretory function and the embryonic androgen production. It is known that human embryonic testicular androgen production in the late gestational period is influenced not only by the embryonic luteinizing hormone (LH) but also by the maternal hCG which is more potent as gonadotrophin than the LH *(Clements et al., 1976)*. The decreased embryonic Leydig cell secretory function may finally result in an increased incidence of partial/total failure of ISTD and subsequently development of UT. The above hypothesis is supported by the decreased incidence of UT in September. Our results have shown clearly that the occurrence of the 26th GW in June-July period is accompanied by a decreased incidence of UT. We have suggested that relatively high environmental temperatures at the 26th GW do not

influence detrimentally (in terms of TD) the hormonal profiles/balances of the mother or fetus with an overall result a minimal disturbance on the beginning/completion of the ISTD. Such a suggestion could explain the results on the seasonal variations of UT not only in Greece but also in Northern Europe (Hungary, Grand Britain, and Sweden) *(Czeizel et al., 1981; Hjertkvis et al., 1989; Jackson & Swerdlow, 1986)*. In contrast, the theory of light cannot explain the seasonality of births of children with UT in Greece and the similar seasonal variations in the northern and southern part of Sweden that exist despite the large differences in daylight hours between the north and south Sweden *(Hjertkvis et al., 1989)*.

In another study *(Mamoulakis et al 2002b)*, based on 1178 cases of isolated UT and 542 cases of simple hypospadias we have evaluated the epidemiological relationship, in terms of seasonality by month of birth, between these two usually associated congenital malformations *(Hjertkvis et al., 1989; John Radcliffe Hospital Cryptorchidism Study Group, 1992; Weidner, et al., 1999)* (Table 2) We have shown that the monthly incidence of hypospadiac births in Greece follows a documented cyclic pattern of simple harmonic type with autumn being the season of statistical predominance (peak in October) and spring the season of lowest incidence (trough in April). Considering that increased incidence of hypospadiac births is observed during October, it appears that the beginning (8th GW) of the crucial period of normal differentiation of human male external genitalia (8th-16th GW) takes place in late winter. Since both crucial embryonic periods for the final TD and the differentiation-development of the male urethra are considered to be androgen-dependent, the existence of an androgen stimulator that follows a cyclic pattern of variation with a trough (diminished androgen production by the fetal testis) in winter may be speculated. Under these settings it could be hypothesized that the coincidence of the 8^{th} or 26^{th} GW with the winter period could increase the risk for the phenotypical expression of hypospadias or UT, respectively. β-hCG is a possible example of such an androgen stimulator since it shows seasonal variation both in early *(Kiely et al., 1995)* and late *(Mamoulakis et al., 2002a)* pregnancy with lower levels in winter. Since no significant differences in daylight length are found among seasons in Greece, the detection of such paterns (Figures 1-4) is strong evidence against the theory of light. Seasonal alterations of weather temperature may be given as a possible example of an alternative cause contributing via the above described mechanism to the seasonal variations of UT and hypospadias observed.

Recently, based on experimental, epidemiological and clinical evidences, a new concept has been proposed according to which poor semen quality, testicular cancer, UT and hypospadias are symptoms of one underlying entity, the testicular dysgenesis syndrome (TDS), which may be increasingly common due to adverse environmental influences probably upon a susceptible genetic background *(Skakkebaek et al., 2001)*. TDS is suggested to be the result of disruption of embryonal programming and gonadal development during fetal life. We may suggest that at least some cases of TMD could be attributed to the relatively lower levels of placental β-hCG during winter months that acting upon a testis already being dysgenetic by it nature, (with a borderline Leydig cell function) possibly result in disanalogous lower levels of androgens produced. If these relatively lower placental β-hCG levels are observed early (8^{th}—9^{th}) or late in gestation (26^{th} GW) the manifes-

Table 2. Undescended testis and hypospadias: Data from Greek population (1991-1998)

		Undescended testis			Hypospadias		
Month of birth	**Total male live births**	**Observed frequencies**	**Adjusted frequencies[1]**	**Expected frequencies**	**Observed frequencies**	**Adjusted frequencies[1]**	**Expected frequencies**
	(m_i)	(n_i)	(n_i')	(E_{Ei})	(n_i)	(n_i')	(E_{Ei})
January	33346	98	102.8	104.3	46	48.5	46,4
February	31423	105	116.9	110.1	43	48.1	42,6
March	33851	106	109.5	112.9	44	45.7	39,4
April	33338	103	108.1	111.9	24	25.3	37,6
May	36261	112	108.0	107.1	33	32.0	37,9
June	35167	96	95.5	100.0	47	47.0	40,1
July	39001	105	94.2	92.3	46	41.5	43,7
August	37144	103	97.0	86.1	66	62.5	47,8
September	36346	80	77.0	83.2	50	48.4	51,1
October	36526	96	91.9	84.4	49	47.1	52,8
November	34060	86	88.3	89.2	46	47.5	52,4
December	34712	88	88.7	96.5	48	48.6	50,1
TOTAL	421175	1178	1178	1178	542	542	542

[1] Adjusted frequencies are used in order to adjust Edwards' test for a variable population at risk. They are calculated by the formula ni' = c ni ™mi / k mi where c is a scale factor such that ™ni' = ™ni and k is the number of the sectors in which the given time span (one year) is divided (i.e. k = 12). (E_{Ei}) Expected frequencies for Edwards' test are proportional to [1 + _ Cos (_i-_max)] (see Figures 1-4 and ref. 334 for details)

Figure 3. Hypospadias. Edwards' model applied on data from Greek population (1991-1998) indicates that the maximum incidence rate is observed during autumn (peak in October) and the minimum incidence rate is observed during spring (trough in April): x^2EDWARDS value at 2df is statistically significant ($p<0.01$)

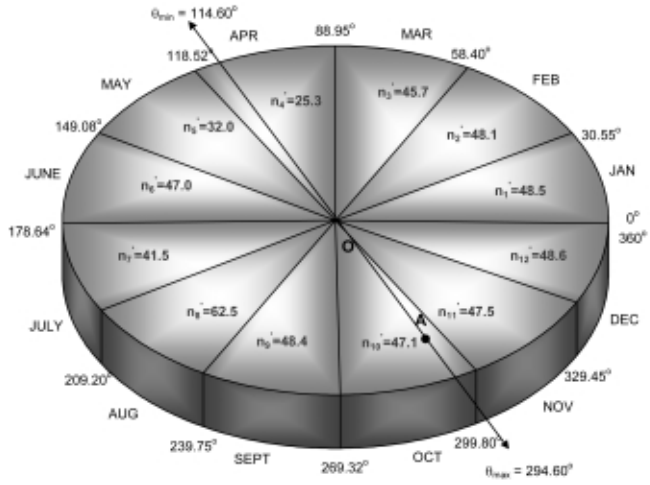
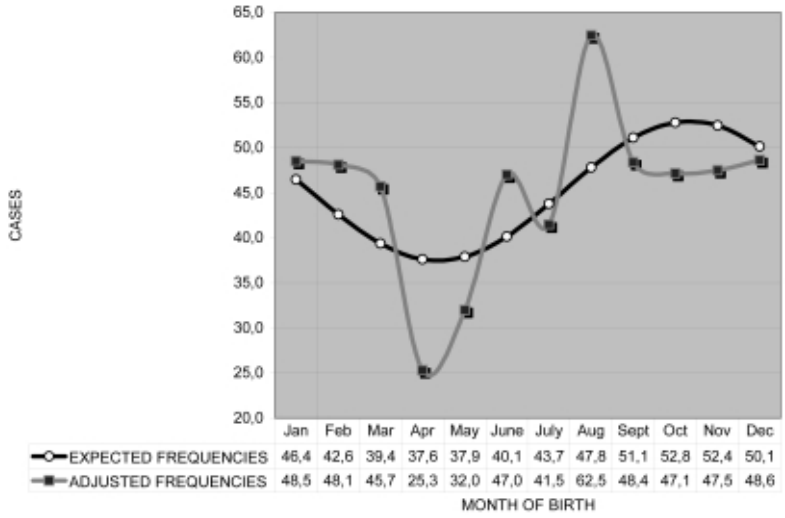


Figure 4. Hypospadias. Adjusted frequencies fitted to the simple harmonic curve given by Edwards' test. Data are satisfactorily described by the simple harmonic curve since the two curves do not differ significantly (x^2 goodness of fit test value at 11 df is not statistically significant; $p>0.2$)



tations may be hypospadias or UT respectively. The presence of TDS manifestations may vary with the severity of underlying testicular dysgenesis.

RISK FACTORS OF TESTICULAR MALDESCENT

Establishing epidemiological factors that affect the risk of UT becomes more complicated due to the complex interactions among anatomy, heredity, hormonal milieu, and socioeconomic or environmental conditions and there is not always a uniform agreement among the authors. Nevertheless, the risk factors for the presence of an UT at birth and by the 1st year of age need to be considered. As described above, UT is more common in premature and low birthweight male newborns. However, after adjusting for birthweight it has been shown that gestational age is not an independent risk factor for UT. On the other hand, the effect of birthweight is not influenced by adjusting for gestational age. Therefore, low birthweight is the principal determinant of UT at birth and at 1 year of age, independent of the length of gestation *(Hjertkvist et al., 1989; Jones et al., 1998; Mayr et al., 1999; Weidner et al., 1999)*. Other risk factors that have been reported include: congenital malformations malformations *(Biggs et al., 2002; Hjertkvist et al., 1989; John Radcliffe Hospital Cryptorchidism Study Group, 1992; Weidner et al., 1999)* (mainly hypospadias, other genitourinary and digestive complications, dislocation/subluxation of the hip), complicated pregnancy *(Biggs et al., 2002; Hjertkvist et al., 1989; Jones et al., 1998; Mayr et al., 1999)* (toxemia of pregnancy, oligohydramnio, pregnancy induced hypertension, pre-eclampsia, hyperemesis), cesarean section (*Hjertkvist et al., 1989)*, twin gestation (*Berkowitz et al., 1993)*, breech presentation (*Biggs et al., 2002; Jones et al., 1998)*, maternal smoking (*Biggs et al., 2002)*, analgetics consumption during pregnancy *(Berkowitz & Lapinski, 1996),* maternal blood group (A Rh+ and B Rh+) *(Ghirri et al 2002),* nuliparity *(Biggs et al., 2002; Hjertkvist et al., 1989; Mayr et al., 1999)*, maternal obesity *(Berkowitz & Lapinski, 1996)* and, family history of UT *(Berkowitz & Lapinski, 1996)*. It has been reported that the occurrence of UT in families is 1.5-4% among the fathers, 6.2 among the brothers, and 0.67 among first-degree male relatives of index patients with UT, supporting a multifactorial pattern of inheritance *(Czeizel et al., 1981)*.

5. CONSEQUENCES OF UNDESCENDED TESTIS: IMPAIRED FERTILITY & TESTICULAR CANCER

Although UT is occasionally considered as a minor congenital abnormality, its close association with subfertility or infertility *(Chilvers & Pike, 1989; Hadziselimovic, 1984; Leissner et al., 1999; Rozanski & Bloom)* and the fact that it represents a well-established risk factor for testicular cancer *(Chilvers et al., 1986; Depue 1983; Henderson et al., 1979; Krabbe et al., 1979; Morrison, 1976; Moss et al., 1986; Pottern et al., 1985; Schottenfeld et al., 1980)* indicate its clinical and epidemiological importance.

A. IMPAIRED FERTILITY IN PATIENTS WITH A HISTORY OF TESTICULAR MALDESCENT

1. Epidemiology of impaired fertility

Uni- or bilateral UT is a common etiologic factor of azoospermia *(David et al., 1979)*. The incidence of azoospermia is estimated around 0.4-0.5% in the general population *(Hadziselimovic & Herzog, 2001)*. However, about 89% of untreated patients with bilateral TMD develop

azoospermia (almost all men are infertile)*(Hadziselimovic, 2002; Hadziselimovic & Herzog, 2001; Weidner, 2002)*. Among men who have untreated unilateral UT, a percentage of 50-70% are azoospermic or oligozoospermic*; (Weidner, 2002)*. However, the incidence of azoospermia in unilateral UT has been reported to be around 13% regardless the patient is treated or untreated *(Hadziselimovic, 2002)*. It has been postulated that paternity would be a better index for verification of fertility than sperm count *(Lee, 1993)* since it is known that men with subnormal sperm counts can father children (*Barfield et al., 1979)*. Fertility data among men attempting paternity indicate that this index is significantly compromised in men with previous bilateral UT (61.5-65.3%) but not (as initially thought) in men with previous unilateral UT (89.7%) compared with the general population (93.2-94.6%) *(Lee et al., 1996; Lee et al., 1997; Lee & Coughlin, 2001; Lee & Coughlin, 2002)*. Furthermore, men with a single testis regardless of the origin of the loss (unilaterally maldescended testis not found at surgery or found to be atrophic at orchiopexy and was either retained or orchiectomized) have similar paternity rates compared to either men with corrected UT or controls *(Lee & Coughlin, 2002)*. There is evidence of subfertility requiring a longer exposure to regular intercourse without contraception (>12months) only in men with previous bilateral UT *(Lee et al., 1996; Lee et al., 1997)*. Neither preoperative testicular location *Lee et al., 2000)* nor small testicular size at orchidopexy *(Lee et al., 2001)* in men with previous unilateral UT appear to be a major determinant of fertility according to paternity, sperm counts or hormone levels. Higher FSH levels, lower sperm counts, and parenchymal suture placement at orchidopexy correlate inversely, both in men with previous unilateral UT compared with controls *(Coughlin et al., 1998; Lee et al., 1998)*.

2. Pathogenesis of impaired fertiity

The pathogenesis of reduced fertility seen in patients with UT has not been fully clarified and the exact cellular and molecular mechanisms involved remain still elusive. The following alterations have been discussed aetiologically: epididymal malformations, decreased number of tubules containing spermatogonia, decreased number of spermatogonia per tubule, mild concomitant hypogonadal situation, generation of sperm antibodies, contralateral testicular damage, increased apoptotic rate in germ cells, microdeletions in the euchromatic region of the Y chromosome long arm (Yq11).

2.1. Epididymal malformations

Epididymal anomalies are associated with TMD in 36-43% of cases *(Gill et al., 1989; Koff & Scaletscky, 1990; Mollaeian et al., 1994)*. They can be classified into two groups: anomalies of ductal fusion and anomalies of ductal suspension. Anomalies of ductal fusion usually accompany intraabdominal and higher inguinal location of the testes *(Gill et al., 1989; Mollaeian et al., 1994)*. They are consisted of loss of continuity between testis and the epididymis or an absent or attenuated segment of the epididymis or vas deferens. The most common anomaly of ductal fusion is a flimsy attachment of the head of the epididymis to the testis (50%), whereas complete separation or atresia of the epididymis and atresia of the vas deference occurs in 5-21%. Anomalies of ductal suspension occure more frequently in the more distal testes *(Gill et al., 1989; Mollaeian et al., 1994)*. They are classified as an angulated epididymis (separated from the testis) by a wide mesentery (80%), a partially separated epididymis with an elongated tail associ-

ated with a long loop vas deferens (12%) and a normally attached epididymis with an elongated tail associated with a long loop vas deference (8%). Such anomalies may coexist with an excellent testicular histology and may possibly explain the decreased fertility of some patients with TMD.

2.2. Impaired testicular histology

Impaired fertility has been linked to the reduced number of testicular germ cells because patients with the lowest total germ cell counts have the poorest spermiograms in adulthood *(Hadziselimovic et al., 1987a)*. The balance between germ cell proliferation, differentiation and apoptosis (see below) is critical to the maintenance of normal spermatogenesis. Disruption of the fine regulation of either of these processes may lead to infertility. Normally, testicular germ cells mature from the primitive gonocytes (fetal stem cell pool) that appear at 8th GW *(Holstein et al., 1971)* through a series of steps, including the appearance of fetal spermatogonia at 15th GW *(Holstein et al., 1971)* and adult dark spermatogonia (Ad; adult stem cell pool *(Clermont, 1966)*) at 3 months of age *(Seguchi & Hadziselimovic, 1974)* to primary spermatocytes (the first meiotic form) by 3-4 years *(Seguchi & Hadziselimovic, 1974)*. Therefore, during the prepubertal period, two major maturational steps occur within the testis: a) at 2-3 months of age the adult stem cell pool is established and replaces the fetal stem cell pool; disappearance of gonocytes, appearance of Ad spermatogonia, and dramatic reduction in the total number of germ cells per tubule *(Hadziselimovic et al., 1986; Huff et al., 1989; Huff et al., 1991; Huff et al., 1993)* and b) at 4-5 years of age an increase in germ cell maturation, and proliferation as well as the onset of meiosis is observed; transient appearance of primary spermatocytes (transformation of Ad spermatogonia into primary spermatocytes), prophase of the first meiotic division is accompanied by increases in the total number of both germ cells and Ad spermatogonia (*Hedinger, 1982; Huff et al., 1989; Huff et al., 1993; Nistal & Paniagua, 1984; Schindler et al., 1987;Scorer & Farrington, 1971)*.

The histologic hallmarks associated with UT are evident between 1st and 2nd year of life and include interstitial (peritubular) fibrosis *(Elder, 1988)*, hypoplasia of Leydig cells (the earliest postnatal histologic abnormality; evident from the first month of life) *(Huff et al., 1991),* reduced numbers of Leydig cells *(Hadziselimovic et al., 1986; Huff et al., 1989; Huff et al., 1993)*, age-independent/testicular location-age dependent Sertoli cell special degeneration pattern (dilated elements of rough endoplasmic reticulum, vacuolization of the cytoplasm, mitochondria with poorly preserved cristae, increase in electron density of the matrix, elongation of the nuclei, irregularities of the nuclear membrane) *(Rune et al., 1992)*, delayed disappearance of gonocytes, delayed apearance of Ad spermatogonia, failure of primary spermatocytes to develop and reduced total germ cell counts *(Huff et al., 1989; Huff et al., 1991; Huff et al., 1993; Huff et al., 2001)*. Reduced total germ cell counts have been associated with defects in the two normal spermatic maturational steps during the prepubertal period *(Huff et al., 1989; Huff et al., 1991; Huff et al., 1993; Huff et al., 2001)*, as well as to the enhanced Sertoli cell special degeneration pattern *(Rune et al., 1992)*. Males with UT are born with germ cells, although the number of these cells may be reduced *(Cortes, 1999; Hadziselimovic, 1977; Hadziselimovic et al., 1987; Hedinger, 1994)*. No reduction in the mean number of germ cells is detected during the first 7 months of age *(Huff et al., 1991)*. The

abnormal persistence of the untransformed gonocytes to Ad spermatogonia results in a total germ cell count that is similar to normal controls until about the 7th month, when secondary degeneration of untransformed gonocytes leads to a decrease in the total germ cell count. The persistence of high total germ cell counts in the first year of life is the basis for the traditional concept that UT is histologically normal until the second year of life *(Huff et al., 2001)*. However, a reduction in the number of spermatogonia has been reported to start as early as 6 months of age in intra-abdominal testes *(Hadziselimovic et al., 1987b)*. It has also been shown (based on the evaluation of biopsies using the spermatic index) that both intra-abdominal and intra-canalicular testes appear to have the same fertility potential *(Hadziselimovic et al., 1987a; McAleer et al., 1995; Wilkerson et al., 2001)* exhibiting a sharp decline in the spermatic index during the first 1.8 years, which reaches a critically low value as early as 8-9 months of age *(Wilkerson et al., 2001)*.

2.2.1. Hypothalamic-pituitary axis function

Several observations favor the hormonal (testosterone)-dependent explanation for germ cell differentiation and maturation during testicular development. Patients with CAIS present with defects in the two postnatal steps of spermatogenesis (Sertoli cell-only syndrome and absence of Ad spermatogonia by the 1st year of life, absence of primary spermatocytes and Leydig cell hyperplasia) *(Hadziselimovic, et al., 1999)* indicating that both the transformation of gonocytes into Ad spermatogonia and the development of primary spermatocytes are androgen-dependent steps. Furthermore, in normal male, the first maturational step is accompanied by a transient surge in serum follicle stimulating hormone (FSH), LH, testosterone levels, and a mini-peak of testicular weight and volume (*Cassorla et al., 1981; Forest et al., 1974; Siebert, et al., 1982)*. These findings suggest that a surge in luteinizing hormone releasing hormone (LHRH) causes LH release. The latter stimulates Leydig cell production of testosterone which triggers the maturation of germ cells. The cause of normal onset of meiosis at 4-5 years of age is the subject of continued study. An increase in the levels of LH in urine *(Hadziselimovic et al., 1986)* and increased prominence of juvenile Leydig cells *(Hadziselimovic, 1977)* simultaneous with the appearance of primary spermatocytes in normal boys suggest that the onset of meiosis may also be stimulated by a minor increase in hypothalamic-pituitary activity.

In UT, Leydig cell hypoplasia is one of the prominent early in life findings indicating a possible defective gonadotropin secretion *(Hadziselimovic et al., 1986; Hadziselimovic & Herzog, 1976; Huff et al., 1991)*. Furthermore, it has been reported that the normal surge in LH and testosterone at 2-3 months is blunted in boys with UT *(Gendrel et al., 1980)*. These findings suggest that in infants with UT Leydig cells are understimulated by a defective hypothalamic-pituitary axis and, therefore cannot produce a surge in testosterone of sufficient magnitude to trigger normal germ cell maturation and the formation of an adequate adult stem cell pool. The cause of the defective onset of meiosis in patients with UT is also a subject of debate. Low levels of urinary LH and FSH *(Hadziselimovic, 1987)*, and impaired LH and FSH responses to stimulation with gonadotropin releasing hormone (GnRH) *(Canlorbe et al., 1974; Forest, 1979)*. However, it should be noted that there are also contrary reports suggesting a normal gonadotropin secretion in infants with UT *(Bartolini et al., 1979; Cacciari et al., 1976;*

DeMunick Keizer-Schrama & Hazebrock, 1986). The fact that similar (but less severe, of later onset, and less progressive) changes are also seen in the contralateral descended testis (CDT) in cases of unilateral TMD, possibly supports the theory of hypogonadotropic hypogonadism as a cause of the increased incidence of infertility seen in males with unilateral UT *(Clermont, 1966; Hadziselimovic et al., 1986; Huff et al., 1991; Huff et al., 1993; Huff et al., 2001, McAleer et al., 1995)*. However, as we have recently shown *(Ono & Sofikitis, 1997)*, a thermal injury on the CDT in cases of unilateral UT could also explain the impaired function of the scrotal testis and the impaired fertility observed in such cases (see below). Nevertheless, it should be noted that the development of the CDT in unilateral UT remains controversial *(Leissner et al., 1999)* since there are also reports suggesting a primary (intrinsic) abnormality in both testes even when only one is descended *(Skakkebaek et al., 2001)*, while others claim that the CDT is not affected in unilateral TMD *(Kirby et al., 1985; Mancini et al., 1965)*.

2.2.2. The role of apoptosis

It has been recently suggested that the degenerative process of testicular germ cells in UT is associated with increased apoptotic DNA fragmentation of these cells *(Guo et al., 2001; Heiskanen et al., 1996; Henriksen et al., 1995a; Jara et al., 2002; Nandi et al., 1999; Ogi et al., 1998; Ohta et al., 1996; Shikone et al., 1994; Socher et al., 1997; Tomomasa et al., 2002; Wang et al., 1998; Watts et al., 2000; Yin et al., 1997; Yin et al., 1998)*. Sertoli and Leydig cells do not show apoptosis in UT *(Ohta et al., 1996)*. The normal cycle of a cell includes growth, differentiation and death. Death may occur by two different mechanisms: necrosis and apoptosis *(Buja et al., 1993)*. Apoptosis is a distinctive form of cell death that is defined by characteristic morphological and biochemical events that result in the efficient elimination of cells from a tissue without eliciting an inflammatory response *(Kerr et al., 1972; Wyllie et al., 1980)*. The hallmark of apoptosis is the rapid internucleosomal fragmentation of cell DNA by nuclear endonuclease into multimers of 180-200bp *(Schwartz & Osborne, 1993; Schwartzman & Cidlowski, 1993)*. Observations on the cellular morphological changes include condensation and margination of nuclear chromatin, cytoplasmic condensation, budding of the cell membrane, cellular shrinkage and fragmentation into membrane bound apoptotic bodies which are rapidly phagocytosed by macrophages or other neighbouring cells *(Kerr et al., 1972; Majno & Joris, 1995)*. Apoptosis may be caused by noxious agents but, unlike necrosis, it may also occur spontaneously or after a physiologic stimulus such as a surge or withdrawal of a hormone *(Tapanainen et al., 1993)*. Deletion of a subset of testicular germ cells by apoptosis occurs normally in the adult mammalian testis *(Allan et al., 1992; Bartke, 1995; Billig et al., 1995)* as well as in the prepubertal human testis *(Heiskanen et al., 1996)*. It has been recently recognized that apoptosis serves as an important physiologic mechanism to limit the germ cell population to numbers which the Sertoli cells can support *(Allan et al., 1992; Bartke, 1995; Billig et al., 1995; Sinha Hikim et al., 1995)*. In addition to physiologic germ cell apoptosis, massive increases in germ cell apoptosis are observed after various testicular injuries, including toxicant exposure *(Blanchard et al., 1996; Brinkworth et al., 1995; Lee et al., 1997; Lee et al., 1999; Richburg & Boekelheide, 1996)*, radiation (*Hasegawa et al., 1998; Meistrich, 1993)*, treatment with chemotherapeutic com-

pounds *(Meistrich, 1993)*, alterations of hormonal support *(Billig et al., 1995; Henriksen et al., 1995b; Sinha Hikim et al., 1995; Tapanainen et al., 1993; Troiano et al., 1994; Woolveridge et al., 1999)* and raised testicular temperature *(Lue et al., 1999; Yin et al., 1997; Yin et al., 1998)*, indicating that a specific pathway is activated when testicular environment cannot support spermatogenesis.

The reason for the increased incidence of apoptosis in testicular germ cells in terms of UT remains unclear. Two putative testicular injuring factors have been suggested to trigger apoptosis *(Wang et al., 1998)*, acting either concurrently or independently, via molecular mechanisms which still have not been fully clarified; defective hormonal support due to impaired hypothalamic-pituitary-gonadal axis and hyperthermia due to abnormal testicular location (see above). There is a controversy regarding the level of apoptosis in the (CDT) in cases of unilateral TMD. It has been reported that the CDT exhibits similarly enhanced level of apoptosis *(Wang et al., 1998)*, but there are also contrary reports suggesting either no apoptotic elevation in the CDT *(Ohta et al., 1996; Watts et al., 2000; Yin et al., 2002)* or even a significantly increased labeling of apoptotic cells per tubule in the CDT compared with its maldescended partner *(Heiskanen et al., 1996)*. Since a direct thermal injury on the CDT is not obvious, it has been claimed that increased apoptosis in the CDT might be mediated by an immune reaction (autoantibody response against the development of testicular components) induced indirectly by the thermal injuring effect on the maldescended testis *(Rapaport et al., 1969)*. High titers of antisperm surface antibodies have been recently reported in a number of children with TMD independent of testis location or orchiopexy. It has been suggested that TMD may elicit an autoimmune response against sperm antigens before and during puberty possibly due to impairement of the blood-testis barrier by the elevated temperature *(Sinisi et al., 1998)*. However, in a longitudinal case control study including 61 pubertal boys with UT corrected surgically at a prepubertal age, we have failed to detected an autoimmune response against sperm surface antigens (Mamoulakis et al., preliminary experiments), while antisperm surface antibodies have also not been detected in prepubertal boys with unilateral UT (*Mirilas & De Almeida, 1999)*. The similar effect on both testes has been taken as an evidence to exclude thermal injury as the sole cause of defective germ cell proliferation and favor the theory of the deficient hypothalamic-pituitary-gonadal axis *(Hadziselimovic, 1977)* (see above). We have recently reported an increase in contralateral testicular temperature associated with increased blood flow in CDT *(Ono & Sofikitis, 1977)*. This novel mechanism could explain the detrimental effects of UT to its descended partner *(Ono & Sofikitis, 1977)*.

Briefly, three distinct systems have been so far implicated in the regulation of the apoptotic signal pathway in the testis in order to secure normal spermatogenesis. These include the p53 protein, the Fas-system and the Bcl-2 family of proteins *(Richburg, 2000)*. Abnormal deviation of these systems due to abnormal hormonal state and/or elevated testicular temperature may play a role in the increased testicular apoptosis identified in the gonads of patients with TMD leading to their impaired fertility.

The tumor suppressor phosphoprotein p53 is a widely described regulator of both cell proliferation *(Tsukuda et al., 1993)* and intra-cellular apoptotic-signaling pathway

(Skeikh & Fornace, 2000). It has been implicated to both spontaneous and injury-induced apoptosis in mitotically active spermatogonia and possibly in meiotic and postmeiotic testicular germ cells as well *(Beumer et al., 1998; Hasegawa et al., 1998; Yin et al., 1998)*. It has been proposed that p53 is the main inducer of heat-enhanced UT-related testicular germ cell apoptotis in mice *(Socher et al., 1997)*. However, the p53-dependent pathway is responsible only for the initial (first) phase (androgen-independent) *(Ohta et al., 1996)* of apoptosis enhanced by heat and is Fas/FasL-independent *(Yin et al., 2002)*, while subsequent apoptosis involves a p53-independent pathway (*Yin et al., 1998)* mediated via the Fas/FasL system *(Yin et al., 2002)*. (see below). Early reports have suggested that p53-mediated apoptosis could be a a protective mechanism in the human and other species for the avoidance of propagation of unrepairable DNA damage induced by high temperatures (*Yin et al., 1998)*. Another point of interest is that p53 does not seem to play a role in the apoptosis observed in the epididymis of the cryptoepididymis mouse model *(Jara et al., 2002)*. The exact molecular mechanism by which p53 mediates high temperature-induced germ cell apoptosis in UT is not clear. It has been shown that the testicular orphan receptor-2 (TR2) expressed in pachytene primary spermatocytes *(Lee et al., 1996)* is repressed via a p53-dependent signaling pathway in the Rhesus monkey *(Mu et al., 2000)*. The TR2 is a master regulator that controls many signaling pathways, and it is likely that it plays very important role in the process of spermatogenesis *(Mu et al., 2000)*. It has been hypothesized that higher testicular temperatures may increase p53 with a subsequent repress of TR2 which in turn negatively influences spermatogenesis on the level of primary spermatocytes securing the avoidance of damaged DNA replication transmission *(Mu et al., 2000)*.

The Fas-signaling pathway is composed of the interacting proteins Fas (CD95/APO-1) and Fas ligand (FasL, CD95L/APO-1L) *(Nagata & Goldstein, et al., 1995)*. Fas is a type I transmembrane protein belonging to the tumor necrosis factor/nerve growth factor superfamily, that contains a “death domain” and is capable of initiating apoptosis when stimulated by receptor cross-linking or binding to its ligand FasL (a tumor necrosis factor-related type II transmembrane protein) *(Nagata & Goldstein, et al., 1995)*. The FasL-Fas interaction triggers the death of cells expressing Fas *(Nagata & Goldstein, et al., 1995)*. The Fas expression is universal but particularly is seen in thymus, spleen and nonlymphoid tissues, while FasL expression is generally more restricted to lymphoid organs and interestingly, it is abundant in the testis, with a suggested localization to Sertoli cells *(French et al., 1996)*. Besides the role of Fas-system in maintaining the immune privilege status of the testis *(Bellgrau et al., 1995)*, the identification of both FasL (constituitively expressed in Sertoli cells) and Fas (in select types of germ cells) in rodent and human testis implicates this system in the regulation of spontaneous germ cell apoptosis and implies a key role of the Sertoli cell as a paracrine regulator of spermatogenesis *(Lee et al., 1997; Pentikainen et al., 1999)*. The Fas-signaling system is also implicated in the apoptosis induced after testicular injury (exposure to toxicants, radiation, hyperthermia) *(Lee et al., 1997; Lee et al., 1999)*. Especially after testicular hyperthermia (in contrast to toxicant exposure), there is a selective increased expression of Fas, while FasL is not upregulated *(Lee et al., 1999)*. This implies that hyperthermia selectively targets the germ cells;

the supporting capacity of Sertoli cells is not altered (Sertoli cells are not obviously affected/FasL is not upregulated) but the affected germ cells are eliminated by the up-regulation of Fas *(Lee et al., 1999)*. Recently it has been shown that the Fas pathway is responsible for the second (p53-independent) phase of apoptosis in TMD *(Yin et al., 2002)*. There is a controversy regarding the role of androgens on Fas-signaling pathway, since both up-regulation *(Nandi et al., 1999)* and down-regulation *(Woolveridge et al., 1999)* of testicular Fas gene expression in correlation to germ cells apoptosis after testosterone withdrawal has been reported. Apart from the two apparently interactive but independent apoptotic pathways responsive to heat stress in UT (p-53- and Fas-signaling pathways), there appears to be additional yet unknown sequential apoptotic patways *(Yin et al., 2002)*.

The Bcl-2 family of proteins is a widely recognized group of apoptotic regulators *(Adams & Cory, 1998; Korsmeyer, 1999)*. This family consists of both antiapoptotic (e.g. Bcl-2, Bcl-xL, Bcl-w) and proapoptotic (e.g. Bax) members *(Gross et al., 1999)*. The ratio of the above molecules provides information on the susceptibility of cells to a stimulus inducing death *(Oltvai et al., 1993)*. There is evidence that Bcl-2 family members and their regulated expression are critical for functional spermatogenesis. Bax-deficient male mice exhibit an inappropriate accumulation of premeiotic germ cells as well as accelerated apoptosis of mature germ cells that enter meiosis leading to complete cessation of sperm production and infertility *(Knudson et al., 1995)*. Bcl-w-deficient mice are also infertile *(Print et al., 1998)*. Transgenic mice misexpressing Bcl-2 in spermatogonia display an accumulation of spermatogonia prior to puberty but during adulthood exhibit loss of germ cells in the majority of the tubules leading to infertility *(Furuchi et al., 1996)*. Transgenic mice overexpressing Bcl-2 or Bcl-xL are also infertile and display a disorganization of the seminiferous epithelium *(Rodriguez et al. 1997)*. Intrestingly, both Bax and Bcl-2 have been involved in the testicular germ cells apoptosis induced by hyperthermia *(Yamamoto et al., 2000)* or androgen withdrawal *(Woolveridge et al. 1999).* In addition, it has been reported that these proteins may play an important role in the germ cell apoptosis observed in experimentally-induced unilateral UT *(Xu et al., 2000)* and cryptoepididymis mouse models *(Jara et al., 2002)*.

2.2.3. Microdeletions of the Y chromosome and testicular maldescent

The role of the Y chromosome in sex determination is well established (see above). However it has been shown that the control of spermatogenesis is another Y chromosome-closely related function *(Tiepolo & Zuffardi, 1976)*. *Tiepolo and Zuffardi (1976)* were the first to hypothesize a correlation between Y chromosome deletions and male infertility *(Tiepolo & Zuffardi, 1976)*. These authors examined the karyotype of 1170 men and observed large deletions including the entire heterochromatic region of the Y chromosome long arm (Yq12) and an undefined amount of the adjacent Yq11 part in six azoospermic males. They suggested that the deletions were the cause of the azoospermia and it was postulated that a genetic factor located in Yq11 was important for male germ cell development. This gene cluster was defined as “azoospermia factor” (AZF). During the subsequent years, many genes have been mapped on the Y chromosome, while the development of sequence tagged sites (STS)- and yeast artificial chromosome clones (YAC)-based mapping revealed the

genetic complexity of the AZF locus. These analyses permited the detection of interstitial submicroscopic deletions not visible at cytogenetic level (microdeletions). They are detectable only by STS-polymerase chain reaction (PCR) or Southern hybridization. Three nonoverlapping regions (spermatogenesis loci) may be deleted in infertile men (AZFα, AZFb and AZFc) *(Vogt et al. 1996)* containing genes that are expressed in the testis and are therefore considered candidate genes for the AZF phenotype (AZFα: USP9Y, DBY; AZFb: RBMY; and AZFc: DAZ) *(Foresta et al., 2001a)*. It is currently clear that Yq11 microdeletions represent the most frequent genetic etiology of severe testiculopathy with an overall incidence ~8% in infertile patients *(Foresta et al., 2001b)*.

The association of Yq11 microdeletions in TMD has only recently started to be evaluated. Up to now the results are inconclusive. Yq11 microdeletions have been sporadically reported in patients with a history of UT *(Dada et al., 2002)*, while two studies have failed to detect such defects in a total of 48 patients (either infertile or not) (*Cortes et al., 1998; Fagerli et al., 1999)*. Only in one study has been reported a high incidence (11/40;27.5%) of Yq11 microdeletions in patients with a history of unilateral orchidopexy presenting with clinical, hormonal, seminal and testicular cytological features of severe bilateral testicular damage *(Foresta et al., 1999)*. Based on these findings it has been suggested that Yq11 microdeletions are responsible for the severe bilateral testicular damage that can be phenotypically expressed by unilateral UT and infertility probably because of altered testicular responses to mechanisms regulating TD. In order to evaluate the probability that specific genetic loci of the Y chromosome associated with the regulation of spermatogenesis (AZFα, AZFb, AZFc) play a role in the production of factors stimulating/impeding TD, we conducted a screening of more than 180 children with all possible phenotypical expressions of uni- or bilateral TMD (anorchia/total atrophia, intra-abdominal location, inguinal/upper scrotal location, retractile testis and ectopia). Genomic DNA was extracted from peripheral blood lymphocytes using a salting out technique and amplified by routine PCR. The set of STS primers and the conditions of amplification were chosen according to current laboratory guidelines for molecular diagnosis of Yq11 microdeletions *(Simoni et al., 1999)*. PCR products were separated and visualized under UV light on 2% agarose gels using ethidium bromide. In none of these patients did we detect Yq11 microdeletions (Mamoulakis et al., preliminary experiments). Our results provide strong evidence against a direct causative role of Yq11 microdeletions in the development of TMD.

B. TESTICULAR CANCER IN PATIENTS WITH A HISTORY OF TESTICULAR MALDESCENT

1. Epidemiology of testicular tumor formation

Although many risk factors have been proposed for the etiology of testicular cancer in human, only a history of UT is a well established one *(UK Testicular Cancer Study Group, 1994a; UK Testicular Cancer Study Group, 1994b)*. UT is associated with testicular neoplasia not only in humans but also in other mammalian species such as horses and dogs *(Boothe, 1993; Johnston et al., 2001; Wintzer, 1986)*. The magnitute of the relative risk is a point of considerable disagreement varying among several reports between 5-50 times greater and 9.2-13.6 times greater in humans and dogs, respectively, compared to the general popu-

lation *(Cortes et al., 2001; Farrer, 1985; Johnston et al., 2001; UK Testicular Cancer Study Group, 1994b; Whitaker, 1988; Woodhouse, 1991)*. No increase in risk seems to exist in cases of men with a history of spontaneous TD *(Moller et al., 1996)*. The relative frequency of a history of UT in men with testicular tumors (TT) is 15-fold for unilateral and 33-fold for bilateral UT *(Stone et al., 1991)*, respectively. Location of the UT also affects the relative risk of developing a TT; the higher the position the testis, the greater the risk of developing a malignancy *(Martin, 1982; Martin & Menck, 1975)*. Inta-abdominal testes have a greater risk of malignancy, compared with testes palpable in the groin *(Stone et al., 1991) (Campbell, 1942)*. The most common TT that develops from an UT is seminoma in human *(Abratt et al., 1992; Batata et al., 1980; Martin, 1979; Martin, 1982; Stone et al., 1991)*, while the incidence of seminoma and Sertoli cell tumors is almost equal in dogs with TMD *(Johnston et al., 2001)*. Other rarer histological types encountered in men with a history of TMD include embryonal carcinoma, teratocarcinoma, and choriocarcinoma (*Batata et al., 1980)*. TT develop usually during or after puberty although there are sporadic case reports of development before 10 years of age (for review see *Doi et al., 2002)*. In childhood, seminoma is not as common compared to adulthood *(Miller et al., 1999)*. Although rare in the general population, the most common TT associated usually with intra-abdominal UT in childhood is mature teratoma *(Doi et al., 2002)*.

2. Pathogenesis of testicular tumor formation

The cause of the increased risk for malignant testicular degeneration of the UT is unclear. It has been speculated that TT may arise due to an intrinsic testicular abnormality responsible also for the initial TMD, rather than due to secondary dysplasia caused by abnormal temperature *(Giwercman et al., 1988)*. Although a secondary effect cannot be ruled out, the theory of an intrinsic pathologic process affecting both testes seems more plausible. It is supported by the evidence of a ~3.5-fold increased risk of the CDT in cases of unilateral UT (~15-20% of all TT occur in the CDT) *(Batata et al., 1980; Hutson & Beasley, 1992; Johnson et al., 1968; Martin, 1982; Prener et al., 1996).* Furthermore, among men with bilateral UT and unilateral TT, 15% of the contralateral to the primary tumor testes develop malignancy *(Schneck & Bellinger, 2002)*.

In adults, carcinoma in-situ (CIS) germ cells are currently considered the precursors for all germ cell tumors, except spermatocytic seminoma *(Skakkebaek et al., 1987)*. These cells are presumed to be malignant gonocytes derived from primordial germ cells that escaped normal differentiation in utero.[501] The incidence of CIS is estimated ~1.7% in adults with a history of TMD *(Giwercman et al., 1989)*. CIS is more commonly detected in intra-abdominal testes *(Ford et al., 1985)*. Germ cell tumors have been noted to develop in 40-50% of adults during a 5-year period from the diagnosis of CIS *(von der Maase et al., 1986)*. The interval between the diagnosis of CIS and the development of seminoma in an UT may last more than 10 years, while spontaneous regression of CIS has not been recorded *(Sigg & Hedinger, 1983)*. On the other hand, CIS seems to be an extremely rare finding before adulthood *(Cortes et al., 2001)*. In a large series of testicular biopsies taken at surgery (before adulthood) from 1335 patients with UT, CIS was detected only in 6 patients (2 with intra-abdominal testes, 3 with abnormal external genitalia besides

TMD and 1 with abnormal karyotype [45,X/46,XY]). Based on these results, the authors recommended examination for testicular neoplasia by testicular biopsy at orchidopexy only in cases accompanied by these characteristics. However, the biologic behaviour of CIS in the prepubertal testis has not yet been clarified. The predictive value of the identification of placental-like alkaline phosphatase positive germ cells (a reliable immunohistochemical marker for confirmation of the CIS histological diagnosis) *(Giwercman et al., 1991)* in prepubertal testicular biopsy speciments for future malignancy has been questioned *(Engeler et al., 2000)*. Therefore, the value of routine testicular biopsy at orchidopexy during childhood as a possible aid in the identification of patients at risk for future testicular neoplasia is questionable. It is controversial whether orchiopexy affects the natural history of development of TT, although there is emerging evidence to support that prepubertal orchiopexy may lessen the risk *(Moller et al., 1996; Prener et al., 1996; Raina et al., 1995; UK Testicular Cancer Study Group, 1994b)*. It should be emphasized that orchiopexy will allow a more thorough examination of the testis for earlier detection of futural malignant degeneration.

6. MANAGEMENT OF TESTICULAR MALDESCENT: HORMONAL TREATMENT & SURGERY

Early management of boys referred for an empty scrotum is mandatory in order to maintain testicular function. Objective of any therapeutic intervention is the positioning of the gonad in the scrotum before 1 year of age. Spontaneous descent occurs in the majority of cryptorchid children by 3 months of age and it is very rare thereafter. Consideration for earlier intervention should be made to prevent complications of UT that may be manifested before the first year of life.

CONSERVATIVE TREATMENT

There are two types of conservative treatment for the UT, administration of hCG and administration of GnRH-a or LHRH. The administration of androgen was abandoned because of the side effects of precocious puberty. The mechanism of action of hCG and GnRH, is an increase in testosterone production by stimulation at different levels of the hypothalamic-pituitary axis. This treatment is based on experimental observations that the inguinoscrotal phase of testicular descent is androgen mediated, involving testicular synthesis of the active metabolite in high local concentrations *(Rajfer & Walsh, 1977)*. hCC stimulates Leydig cells directly to produce testosterone. GnRH stimulates the pituitary to release LH promoting the testicular production of testosterone. Serum testosterone levels during therapy for UT in prepubertal boys are much higher with hCG treatment than with GnRH treatment *(Rajfer et al., 1986)*. Re- examination of boys treated with hormonal therapy must be performed in a 6-month base, because reascent has been reported in up to 25% of patients. Hormonal treatment is not indicated in patients with previously operated testes.

Success rates of hCG treatment have varied considerably, from 14% to 59% (*Adamsen et al., 1989; Ehrlich et al., 1969; Job et al., 1982;)*. Treatment regimens have also varied, and the most effective treatment was demonstrated to be a total dose of at least 10,000 IU to achieve maximal stimulation of the Leydig cells and avoid the complications of doses exceeding 15000 IU *(Job et al., 1982)*. A typical treatment schedule is 1500 IU injected intramuscularly twice per week for 4 weeks. FSH appears to affect the spontaneous descent of the

testis and to induce the production of LH receptors; however, studies evaluating the efficacy of a combined treatment with hCG and FSH have shown inconsistent results *(Giannopoulos et al., 2001; Hoorweg Nijman et al., 1994; Saggese et al., 1989)*.

Side effects of hormonal treatment include increased rugation and pigmentation of the scrotum. Rarely, there is an increase in the size of the penis and development of pubic hair. These side effects are regressing after cessation of therapy. Significant increase in weight increment velocity was seen in 7- to 9-year-olds boys treated with a dose of 10,000 IU *(Adamsen et al., 1989)*. Furthermore, the administration of hCG in immunosuppressed patients should also be avoided because of a transient decrease in the absolute number of total peripheral blood lymphocytes, total T cells, T helper- inducer cells, and of CD8+ subsets during therapy. In addition, the percentage of CD8+ cells and lymphocyte response to the mitogen concanavalin A decreased significantly with hCG treatment and returned to normal after hCG withdrawal *(Maghnie et al., 1991)*.

Results of GnRH administration trials may have been overestimated because of the inclusion of patients with retractile testes; the reported success rates ranged from 32 to 65% *(Hadziselimovic, 1982; Witjes et al., 1990)*. *Rajfer et al. (1986)* found that TD into the scrotum occurred in 19% of patients treated with GnRH and in 6% of those treated with hCG. One multicenter study found a therapeutic gain in 8.1% of children with bilateral UT and no effect in children with unilateral UT *(Olsen et al., 1992)*. A success rate of 9% for LHRH nasal spray and 8% for placebo was reported in 252 prepubertal boys with 301 UT *(De Muinck Keizer-Schrama et al., 1987)*. These results confirmed similar findings reported by *Pyolala et al. (1995)*, who performed a metaanalysis of 33 studies from 1958 to 1990 and assessed the results of LHRH and hCG in the treatment of 3282 boys with UT.

SURGICAL TREATMENT

The overall efficacy of medical treatment is less than 20% for cryptorchid testes and is dependent on testicular pretreatment location. Therefore, surgery remains unequivocally the treatment of choice in the management of the UT.

The analysis of surgical therapy for UT by *Docimo (1995)* revealed an orchiopexy success rate equal to 92% for testes below the external ring, 89% for inguinal testes, 84% for microvascular orchiopexies, 81% for standard abdominal orchiopexies, 77% for staged Fowler-Stephens orchiopexies, and 67% for standard Fowler-Stephens orchiopexies *(Docimo et al., 1995)*.

The age at which orchiopexy is best performed is considered to be about 12 months, and certainly before the third year of life, prior to morphological damage to the UT. There is some preliminary evidence to suggest that orchopexy should be performed earlier than 12 months *(Yamanaka et al., 1991)*.

If a clinically evident hernia appears in an infant who has an ipsilateral UT, an orchiopexy should be performed at the same time as the herniotomy, irrespective of the age of the infant. Failure to do so would make subsequent orchiopexy extremely difficult, because of the production of fibrous adhesions in the region of the internal ring.

A. Standard orchiopexy

The key steps of standard orchiopexy are: a) complete mobilization of the testis and spermatic cord, b) repair of the patent processus vaginalis by high ligation of the hernia sac, c) skeletonization of the sper-

matic cord without sacrificing vascular integrity in order to achieve tension-free placement of the testis within the dependent position of the scrotum, and d) creation of a superficial pouch within the hemiscrotum to receive the testis.

Experimental evidence has demonstrated that transparenchymal suture fixation causes testicular damage by inducing an inflammatory reaction regardless of suture size and material. In comparison, dartos-fixed testes demonstrated complete circumferential adherence with only 5% or the animals in the dartos pouch group demonstrating an inflammatory response; normal spermatogenesis was found in 94% of the animals *(Bellinger et al., 1989; Dixon et al., 1993)*. Based on these studies, transparenchymal suture should be avoided. There are only two exceptions: for tethered testis when its position is tenuous after complete mobilization of the spermatic cord and short-term external fixation to a nylon button is required, and for testicular fixation of the ipsilateral and contralateral testes due to clinical torsion of the spermatic cord. A significant variation of the technique, however, is to completely avoid deep transparenchymal placement of the sutures by superficial placement of the sutures just under the tunica albuginea. The sutures used in these cases are fine, permanent monofilarnent suture, such as 5-0 or 6-0 Prolene, on a noncutting needle. It is important that the surgeon has to be familiar with the intragonadal vascular anatomy in such cases, especially when ligation of the spermatic vessels is contemplated. The collateral arterial circulation through the deferential artery, which communicates with the internal spermatic arterial system both in the spermatic cord and at the lower pole of the testis, was found to be compromised in all testes with placement of a traction transparenchymal suture *(Jarow, 1991)*.

B. Techniques for the highly undescended testis

Occasionally, greater mobilization of the proximal spermatic cord structures does not provide adequate length to allow tension-free placement of the testis within the scrotum. Greater cord length can be obtained by mobilizing the spermatic vessels medially. The spermatic vessels are usual the limiting factor in these circumstances. The Prentiss manoeuvre was described in 1960 and occasionally is helpful in adding length to the spermatic vessels by positioning the spermatic vessels medially and thereby choosing the hypotenuse of the triangle, or most direct course to the scrotum, created by the natural course of the vessels laterally through the internal ring.

C. Management of intra-abdominal testis

I. Laparoscopy

Laparoscopy is a safe and effective method for localization a nonpalpable testis. The accuracy of transperitoneal laparoscopy to locate a nonpalpable testis approaches 100% and subsequently defines the management options. Once the presence or absence of the testis is verified by diagnostic laparoscopy, an orchiopexy or orchiectomy may be performed with laparoscopic assistance. The initial purpose of diagnostic laparoscopy is to plan the subsequent surgical approach or to avoid an open exploration. The three most frequent findings at diagnostic laparoscopy for a nonpalpable testis are: a) blind-ending vessels above the internal ring, b) cord structures entering the internal ring, and c) an intra-abdominal testis. If blind-ending spermacic vessels are found, no further surgical

investigation is necessary. The testis is considered "vanished" as the result of a prenatal vascular accident. When spermatic vessels, even hypoplastic ones, are visualized entering the internal ring, inguinal exploration is mandatory. The intra-abdominal testis can be managed by either an "open" surgical or laparoscopic orchiopexy procedure or by orchiectomy. The orchiopexy can either be staged or performed with complete mobilization of the testis into the scrotum in one procedure. The choice of repair technique depends on the viability of the testis, the anatomy of the paratesticular structures, the distance of the testis from the scrotum, the status of the contralateral testis, and, most important the surgeon's experience and ability. A micro-surgical autotransplantation of the intra-abdominal testis is an extreme measure with few indications.

II. Fowler-Stephens orchiopexy

The management of the high inguinal or intra-abdominal testis occasionally demands ligation of the testicular vessels. The testicular artery and veins often limit the distal mobility of these testes. It was not until 1959 that Fowler and Stephens studied the vascular anatomy of the testis and devised a means to repair the highly undescended testis preserving its blood supply via collateral circulation. The testis has three arteries for blood supply: the testicular artery (primary), the deferential artery of the vas deferens, and the cremasteric arteries. When the spermatic vessels are divided, blood supply to the testis is dependent on collateral circulation from the deferential artery, a branch of the inferior vesical artery, and the cremasteric system, a branch of the inferior epigastric artery.

The technique described by Fowler and Stephens was originally a one-stage procedure, but it may also be performed in two stages. If an one-stage repair is decided to be performed, it is critical early in the dissection that a wide pedicle of peritoneum be preserved with the vas deferens in order to maintain collateral blood flow. Careful inspection is required to identify the primary and collateral vessels to the testis, as well as the course of the vas deferens. It is very common than anatomic variations of the vascular supply to the testis are present in associated cases of a long-looping vas. This (a long-looping vas) most important requirement for successful Fowler-Stephens orchiopexy is present in less than one third of boys with true intra-abdominal testes. Children with a long-looping vas that extends down to the inguinal canal are the ideal candidates for an one-stage repair. True intra-abdorninal testes lie at least 1 cm above the internal ring and have shortened spermatic vessels. If a long-looping vas is present, it is carefully mobilized. Once it has been determined that the testis cannot be placed in its scrotal position, the spermatic vessels are divided. The Fowler-Stephens test is a method to evaluate the collateral blood supply to the testis. It is performed by temporarily occlusion of the testicular artery with a vascular clamp for 5 minutes and inspection of the testis for color as well as after a small incision in the tunica albuginea of the testis to document arterial bleeding. A carefully inspection is needed to distinguish the tunical blood supply which may be preserved despite testicular arterial occlusion from the blood supply to the testicular parenchyma. If the blood supply is at this point considered tenuous the performance of a two-stage repair allow the development of the collateral circulation.

The anticipated advantages of a two-stage orchiopexy with spermatic vessel ligation are: a) the development of the collateral blood supply to compensate for divi-

sion of the main blood supply to the testis, and b) a greater mobilization of the testis in order to place it within the scrotum *(Elder, 1989; Elder, 1992)*. After 6 months or longer re-operation must be performed to complete the vasal peritoneal pedicle mobilization using the standard Fowler Stephens technique.

III. Microvascular autotransplantation

Silber & Kelly (1976) first reported the performance of a microvascular anastomosis to bring extra blood supply to the testicle after mobilization of a high inta-abdominal testicle in a child with prune-belly syndrome. Initial series were limited to older boys whose internal spermatic artery was large enough to be anastomosed to the inferior epigastric artery, but the technique was demonstrated to be successful in boys younger than 2 years of age *(Harrison et al., 1990)*. Overall success rates are in excess of 80% *(Bianchi, 1984; Bukowski et al., 1995a; Bukowski et al., 1995b; Harrison et al., 1990; Wacksman et al., 1982)*. Bukowski and associates reported a 96% success rate for testicular autotransplantation over a 17-year period *(Bukowski et al., 1995a)*.

D. Complications of orchiopexy

Complications of orchiopexy include testicular retraction, hematoma formation, ilioinguinal nerve injury, postoperative torsion (either iatrogenic or spontaneous), damage to the vas deferens, and testicular atrophy. Atrophy of the testis is the most devastating complication, but it is a rare finding after the performance of standard orchiopexies. Devascularization with atrophy of the testis can result from skeletonization of the cord, from overzealous electrocautery, from inadvertent torsion of the spermatic vessels during passage of the testis into the scrotum or as a result of ligation and division of the spermatic vessels during a Fowler-Stephens orchiopexy. Devascularization may also be caused by excessive axial tension on the spermatic vessels, particularly if the collaterals are poor. Some degree of post-haematoma is common. In contrast, the development of a large haematoma is an uncommon finding after the performance of standard orchiopexies. Large haematoma increases the risk of infection and abscess formation. Although retraction is usually a result of inadequate retroperitoneal dissection, insufficient cord length precludes successful orchiopexy no matter how much dissection is performed. If the patent processus vaginalis is ligated within the canal and not at the level of the internal ring, the peritoneum remains adherent to the spermatic vessels, and complete retroperitoneal mobilization of vessels is not feasible.

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MALE ACCESSORY GLAND INFECTIONS AND INFERTILITY

Enzo Vicari, Sandro La Vignera, Alessandro Arancio, Aldo Eugenio Calogero

Section of Endocrinology, Andrology and Internal Medicine, Department of Biomedical Sciences, University of Catania, Catania

Corrispondenza:
Prof. Enzo Vicari, Catt. Endocrinologia, Sez. Endocrinologia, Andrologia e Medicina Interna, Dip. Scienze Biomediche, Università di Catania, Osp. Garibaldi, P.zza S.M. Gesù, 95123 Catania

MALE ACCESSORY GLAND INFECTIONS : BACKGROUND

Male accessory gland infections (MAGI) are of worldwide distribution in both industrialized and developing countries. Except the uncommonly acute, symptomatic conditions, MAGI have an underhand beginning, and a chronic, symptomless or rather paucisymptomatic course. Clinically, MAGI include: a) the "uncomplicated MAGI", known as prostatitis or prostatitis syndromes, when the inflammation is limited to the urethro-prostatic regions; b) the "complicated MAGI", when the inflammatory process involves more accessory glands and, thus, it is better defined as prostato-vesiculitis (PV), prostato-vesiculo-epididymitis (PVE) or epididymo-orchitis (EO).

Prostatitis

Prostatitis is the most common prostate disease, resulting in more physician visits than either benign prostatic hyperplasia or prostate cancer (NIH, 1990). Prostatitis is also the most common urologic diagnosis in men under 50 years of age, and the third most common in older men (Collins et al., 1999).

In men living in northern Finland, the occurence of prostatitis is higher (14.2%) (Mehik et al., 2000) than that reported in other parts of Europe (5-9%) (Weidner et al., 1999) or in the world.

Physicians, urologists and pathologists have reported a broad range of epidemiological prevalence of prostatitis. Particularly, a physician's diagnosis of prostatitis ranges from 1% to 11%, using data from the Olmsted County Study of Urinary Symptoms and Health Status among men (Roberts et al., 1998). In this community-based study, only 4% of the nearly 2 million visits for prostatitis per year were recorded as "acute prostatitis", suggesting that chronic prostatitis is quite common. On the other hand, 8% (Collins et al., 1998), 11.5% (Nickel et al., 2001) or 16% (Collins et al., 2002) of men have symptoms that urologists have diagnosed as prostatitis over a year. Furthermore, the histopathologic prevalence of prostatitis ranged from 6% to 44% in patients undergoing prostate biopsy, according to Roberts et al. (1998).

Prostatitis is today more frequently referred to as "prostatitis syndrome". According to the duration of the symptoms, this condition is described as either acute or chronic, when they are present for at least 3 months. The predominant symptoms include pain at various localizations (prostate/perineum 46%; scrotum and/or

testes 39%; penis 6%; urinary bladder 6%; lower back (2%) (*Zermann et al., 1999*) and lower urinary tract syndromes (LUTS) (pollakiuria, difficulty urinating, pain/increased pain on urination).

Prostatitis is diagnosed and classified by symptoms, microbiological and cytological testing of expressed prostatic secret and/or segmented urine samples (*Meares & Stamey, 1968*).

Prostatitis actually may be classified according to the NIDDK Workshop, US National Institutes of Health (*Krieger et al., 1999*) in four categories:

Category I Acute bacterial prostatitis

Category II Chronic bacterial prostatitis

Category III Chronic abacterial prostatitis /chronic pelvic pain syndrome:

- **IIIA.** Inflammatory chronic pelvic pain syndrome (significant leukocytes in semen; expressed prostatic secretion; third voided urine specimen);
- **IIIB.** Non-Inflammatory chronic pelvic pain syndrome (non-significant leukocytes in semen; expressed prostatic secretion; third voided urine specimen);

Category IV Asyntomatic inflammation of prostate (histological prostatitis)

Since bacterial prostatitis (acute or chronic) accounts for a low percentage (5-10%), the most significant proportion of the urologic population studied is affected by chronic nonbacterial prostatitis (60-65%) or prostatodynia (30%) (*Weidner et al., 1991).* Recent data suggest that chronic nonbacterial prostatitis may actually have a cryptic, nonculturable (biofilm bacteria) microrganism-based etiology: the microbiological workup of these specimens are further complicated by the presence of inhibitory substances present in the prostatic secretion, and of previous courses of antibiotics.

In patients complaining of prostatitis, the quality of health is similar to that of patients with unstable angina, recent myocardial infarction, or active Crohn's disease (*Schaeffer et al., 2002*). Therefore, the National Institutes of Health Chronic Prostatitis Symptom Index (NIH-CPSI), available online at http://www.QLMed.org/nih-cpsi, accurately measures three major domains of the chronic prostatitis syndrome: pain, voiding dysfunction, and impact/quality of life. The utility of this symptom index has been confirmed by three years of clinical and research use (*Nickel et al., 2001*).

PROSTATO-VESICULITIS AND PROSTATO-VESICULO-EPIDIDYMITIS

Chronic prostato-vesiculitis (PV) and prostato-vesiculo-epididymitis (PVE) may be regarded as complicated MAGI, due to an ascending spread of urethral pathogens via the ejaculatory duct into the vas deferens up to the epididymes. PV or PVE epidemiological data are scanty. These conditions have been defined as "occult pathology" (*Doble & Carter, 1989*) o "enigmatic syndromes" (*Nickel et al., 2002*) because of the multiple clinical challenges.

In patients with PV, there is no clear relationship between the extention of the inflammation changes visualised by ultrasound and the severity of the symptoms or the presence of leukocytospermia. However, patients with PV represent an important clinical MAGI subgroup for several reasons:

a) they complain of symptoms even in unilateral forms (*Christiansen & Purvis, 1991*) with a frequency higher than that observed in patients with prostatitis;
b) some symptoms overlap with those of patients with prostatitis (spermatorrhea,

haematospermia, lower abdominal discomfort or perineal/pelvic discomfort), others with those of patients with PVE (funicolar pain);

c) since PV represents mainly a complication of prostatitis, its presence is associated with post-flogistic organic and/or functional sequelae of the lower reproductive genital tract, up to severe dyspermia in almost 40% of the patients affected (*Purvis & Christiansen, 1993*);

d) a correct diagnosis of PV prevents a possible further extension of the inflammatory process to the epididymus (PVE) with a more severe sperm impairment. Indeed, a careless clinical and ultrasound approach to patients with MAGI results in an understimed incidence of PV or PVE. In the last 20 years, the number of items recorded in the PubMed are 3368 for prostatitis and only 59 for PV and 1724 for PVE (considered secondary to PV). The greater number of citation for PVE compared to the scanty citations of PV may likely reflects the onset of the most complicated form of MAGI due to a lack of a correct precocious diagnosis of PV.

Epididymitis

The incidence of epididymitis is approximately 600,000 cases per year. The highest prevalence is in young men (19-35 years of age). The disorder is a major cause of hospital admission among soldiers (causing approximately 20% of the admissions). The condition, described as discomfort or pain in the scrotum, testicle or epididymis (one or both sides) of at least 3 month duration, is usually associated with a past or concurrent history of sexually transmitted urethritis (N. Gonorrhoeae or C. trachomatis) in young men. In older men, it is more often associated with bacterial prostatitis or PV (infections with typical uropathogens: i.e. enterobacteriaceae, gram positives aerobes, C. Trachomatis or Ureaplasma urealyticum) (*Christiansen & Purvis, 1991; Krishnan & Heal, 1991*).

PREVALENCE OF MAGI AND INFERTILITY

It is known that MAGI is identified by WHO among diagnostic categories affecting male reproductive function and fertility (*WHO, 1993*). Chronic, mainly symptomless MAGI may contribute to infertility to a various extent, depending on the site of inflammation (*Weidner et al., 1999; Ochsendorf, 1999; Vicari, 1999; Agarwal et al., 2003*) and/or on the inflammatory response in terms of leukocytospermia and its products: reactive oxygen species (*Vicari, 1999; Vicari, 2000; Vicari & Calogero, 2001; Vicari et al., 2002*) and/or cytokines (*Depuydt et al., 1996; Diemer et al., 2003*).

The presence of MAGI is diagnosed in patients with oligo-, astheno- or teratozoospermia who fulfil the WHO conventional criteria (*WHO,1993*) (Fig. 1).

MAGI has been reported to account for a wide percentage (1.6 - 15%) of male infertility in various infertility clinic settings (*Comhaire et al., 1986; Andreeßen et al., 1993; Vicari, 2000; Diemer et al., 2003*). The broad range of prevalence reported in the literature reflects various factors:

1. difference in the consulting physician speciality. This has a different impact on the work-up of the infertile patient with MAGI;
2. incomplete and not well defined diagnosis (“sperm infection” is an improperly used laboratory word and it should be correctly replaced by the male diagnostic category defined conventionally as MAGI);
3. lack of clinical characterization in patients initially fulfilling the conventional WHO criteria (combined: history and

physical signs; prostatic fluid and ejaculate signs) in diagnostic MAGI categories (i.e. prostatitis; PV, PVE, EO);

4. the greatest prevalence may be found in studies which include patients who require specific andrological clinical counselling for persistent infection (even after antimicrobials) and/or previous IVF programme failure.

An open debate with pros and cons on the role of MAGI in male inefertility is going on. It has been recently evaluated by an European Association of Urology (EAU) working group (*Weidner et al., 2002*). Table 1 summarizes the account (balance between values vs. limits) about MAGI and infertility, generated by scientific communities (WHO; NIH consensus) and by ultrasonographic-based evidence in this research area.

Whereas WHO included MAGI among the diagnostic categories of infertile patients and recommended some combined conventional criteria for its identifica-

Figure 1. MAGI: Conventional WHO criteria (WHO, 1993)

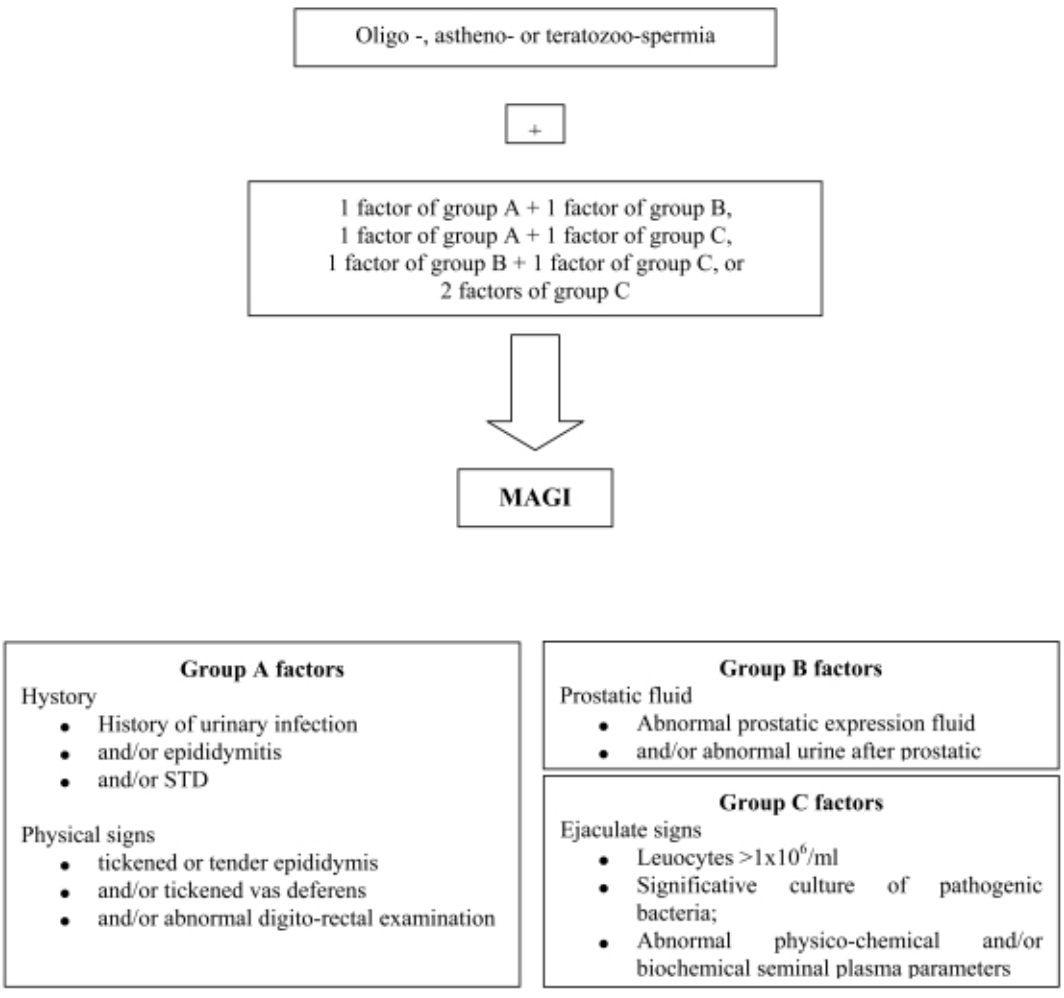


Table 1. Account (values and limits) about MAGI and male infertility

	VALUES	LIMITS
WHO criteria	Recognize and establish a combination of factors (clinical and seminological)	• Neglet the significance to define the site of inflammation (of one or more sexual glands); • Seminal leukocyte assessment can be understimated by the routinary staining; • Have inspired studies based mainly on seminal data, and poorly on the clinical expression of inflammed glands.
NIH Consensus	Gives a new classification of prostatitis, including the prostatitis syndromes.	• Neglets complicated MAGI (PV; PVE): this results in a lower number of items in PubMed (59 and 1724 citations for PV and PVE, respectively, compared to 3368 for prostatitis); • The 3 major domains of symptom index (NIH-CPSI) pain, voiding dysfunction, and impact/quality of life are not much applicable to an andrological, infertile population.
Reports about scrotal and rectal ultrasound testings	Various ultrasound criteria have been considered indicative of chronic inflammation and can differentiate prostatitis, PV, PVE.	• Neglets to perform additional seminal data about host inflammatory response (leukocyte assessment through immunocytochemistry; ROS hyperproduction) in different infertile MAGI categories (prostatitis, PV, PVE).

tion, the lack of "site-diagnosis", based on the clinical characterization of the adnex glands, has determined contrasting and uncertain conclusions, this has also originated several negative influences, such as:

a) the enrollment of non-homogenous cohorts of infected patients, which in turn has provoked contrasting and not comparable effects on the main sperm parameters (*Weidner et al., 1999*);
b) the disproportionate number of items in PubMed among putative clinical MAGI categories: 3368, 59 and 1724 items about prostatitis, PV and PVE, respectively;
c) understatement of ultrasound features in patients with chronic MAGI;
d) lack of synergy and application between the recent knowledge of basic research of inflammatory response (leukocy-

tospermia and ROS hyperproduction) and a clinical ultrasound-based approach. This is enough to lead us to think that for many years the above parameters or measurements of inflammatory response have been preferentially evaluated in patients with idiopathic infertility rather than in patients with proven MAGI;

e) Finally, a careless diagnosis of MAGI has not allowed to make an early diagnosis and managemnent of PV, which may be regarded as an intermediate condition of MAGI, before it becomes PVE.

HOW TO MAKE A CORRECT DIAGNOSIS

The algorithm for the assessment and treatment of MAGI is based, in our approach, into three sequential levels (Figure 2). Each level includes relative "process" broken down into specific "actions" and "outcomes".

a) To suspect/identify an aspecific MAGI "first-line testing" is enough (i.e. history, physical uro-genital examination, sperm analyses including seminal leukocytes);

b) When an initial diagnosis of MAGI is suspected, "second-line testing" (i.e. microbiological tests and ultrasound scans - didymo-epididymal and rectal prostato-vesicular ultrasonography) allows to confirm and assess MAGI, both from the ethiological and localization point of view, respectively;

c) In patients with complicated (microbial or amicrobial) MAGI (PV or PVE), "third-line testing", i.e. leukocytospermia (accurately assessed by immunocytochemical staining), reactive oxygen species production (by chemioluminescence analysis) aids to explore the host inflammatory response and to direct the therapeutical course in a more rational manner.

The promoted "actions" for each level are based from the following evidences:

- In the first level, the "actions" are identified by conventional WHO criteria for MAGI (Figure 1) (*WHO, 1993*). In particular, the presence of oligo (sperm concentration 5-19 millions/ml), astheno (forward progressive motility a+b, after 1 h = <25%) and/or terato-zoospermia (normal oval forms: 14-29%) in combination with other factors (see Figure 1), allows to establish a suspected (ethiological) diagnosis of MAGI (microbial or amicrobial);
- In the second level, the localization of the inflammatory process is based on the presence of a significant number of ultrasound abnormalities for each infected gland (i.e. >2 in the prostatitis; >4 in the PV; >6 in the PVE) (Table 2). These abnormalities have been found stronger associated with elevated (>10^5 CFU/ml) bacteriospermia and ROS hyperproduction in recent studies on selected infertile infected patients (*Vicari, 1999; Vicari, 2000*);
- In the third levels, in patients with complicated MAGI, the extension of infectious /inflammaion process to more sexual glands potentially enhances the host inflammtory response, in terms of leukocytospermia and production of metabolic spermiotoxyc leukocyte-related products (ROS, cytokine) (*Ochsendorf, 1999; Vicari, 1999; Vicari, 2000; Agarwal et al., 2003*). Since the above sperm oxidative stress seems to persist even following antimicrobials (*Omu et al., 1998; Vicari*, *2000*), the presence of an alteration of the above parameters suggests a subsequent rational pharmacological treatment (Figure 3) (*Vicari & Calogero, 2001; Vicari et al., 2002*).

Figure 2. Diagnostic algorithm to make a clinical decision in the assessment of patients with MAGI.

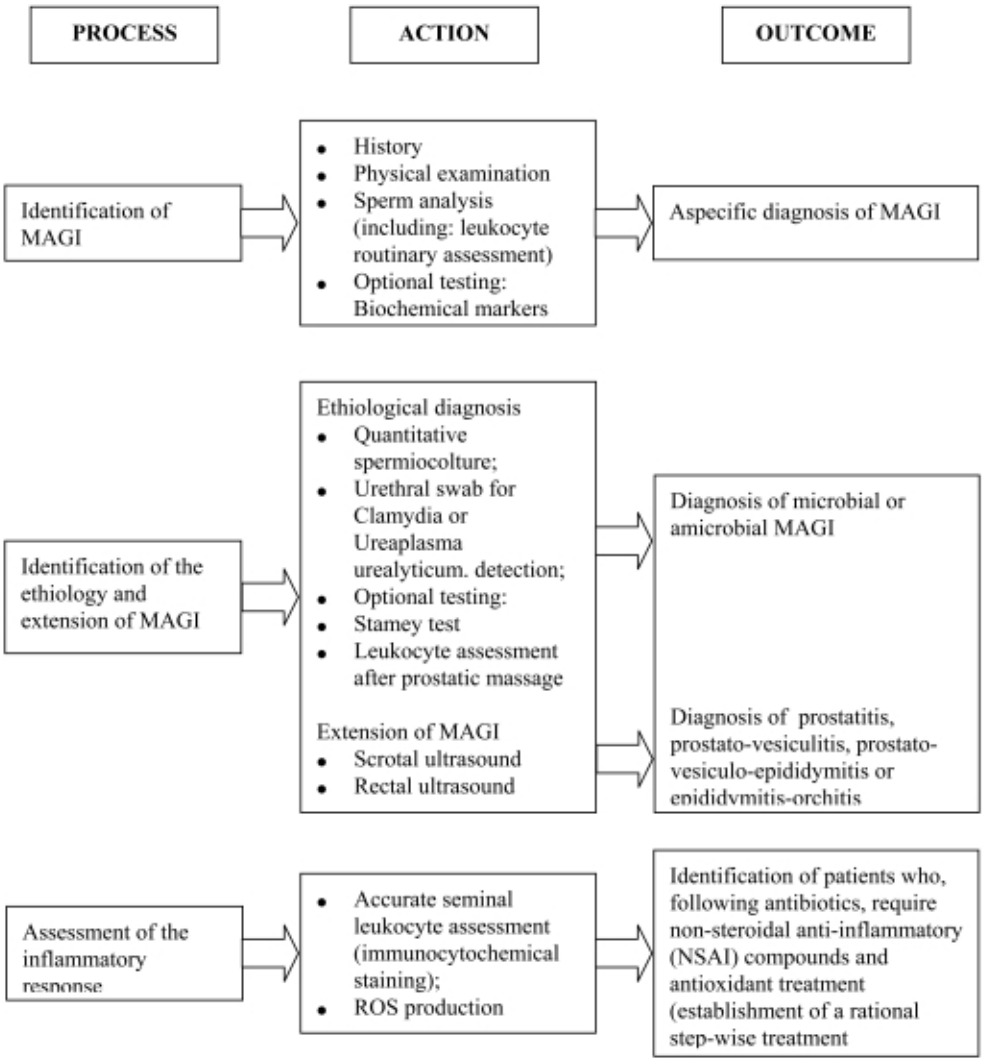


Table 2. Ultrasound (US) findings considered indicative of MAGI (from Vicari, 1999)

1. PROSTATITIS alone It was suspected in presence of 2 or more of the following US signs: a.1) glandular asymmetry; b.1) hypoechogenicity associated with oedema; c.1) hyperechogenicity associated with areas of calcification; d.1) dilation of the periprostatic venous plexus.
2. PROSTATO-VESICULITIS (PV) In additing, to the above mentioned US inflammatory iuxta-prostate changes, fulfilled 2 or more of the following US signs: a.2) enlargment and asymmetry; b.2) tickening and calcification of the glandular epithelium; c.2) policiclic areas separated by hyperechogenic septs
3. PROSTATO-VESICULO-EPIDIDYMITIS (PVE) The PV-group specific US signs associated with 2 or more of the following US signs: a.3) an increased size of the head (diameter cranio-caudal >12 mm) and/or tail (diameter cranio-caudale >6 mm) of the epididymis; b.3) presence of multiple microcyst lesions confined to the epididymal head and/or tail; c.3) oedematous hyperechoic epididymis; d.3) a large hydrocele

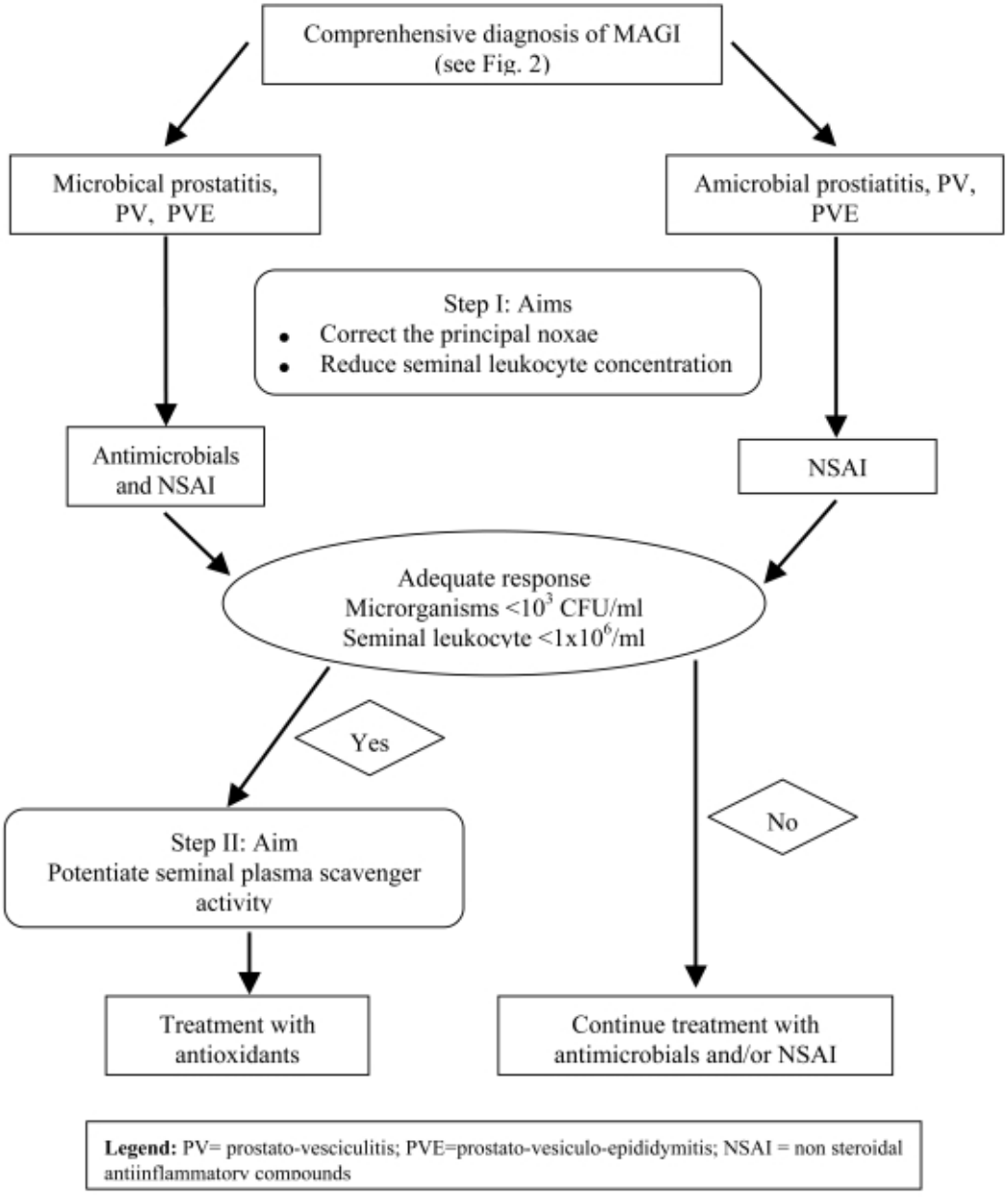
WHEN AND HOW TO TREAT INFERTILE PATIENTS WITH MAGI

Only patients with a clear clinical evidence of MAGI require a full scale treatment. Symptomatic or commonly asymptomatic MAGI should be treated with antibiotics when a morphological and/or the functional sperm alteration is present, or when there is a sub-obstruction of the seminal tract or a proven mono or multiple glandular infection. On the other hand, the presence of bacteriospermia or leukocytospermia alone does not necessarily mean that there is a glandular infection, since temporary inflammatory episodes are likely present in the majority of sexually active men (*Purvis & Christiansen, 1993*). Furthermore, bacteriospermia may represent contamination, colonization or infection. The treatment algorithm of patient with MAGI and problems of couple's fertility is summarized in the Figure 3.

A prolonged exposition/interaction between the inflammatory noxae on one hand, and sperm and sexual glands on the other hand, enhances an inflammatory response through the release of seminal reactive oxygen species (and cytokines), which may persisted even following antibiotic treatment in patients with complicated MAGI (PV and PVE) (*Vicari, 2000*). This justify a multi-step treatment of patients with PVE, who have a significantly higher production of ROS compared to patients with prostatitis or PV, using as first-line therapy, antibiotics (fluoroquinolones, macrolydes, or doxycicline are potentially effective); as second-line therapy, non-steroidal antiflammatory (NSAI) compounds and finally as third-line therapy, antioxidants (combined carnitine

plus acetilcarnitine) (*Vicari & Calogero, 2001*; *Vicari et al., 2002*). When this sequential pharmacological management is adopted, a significant increase of sperm (forward motility and viability; WBC and ROS production) and reproductive (pregnancy rate) parameters has been recently observed in patients with PVE (*Vicari & Calogero, 2001; Vicari et al., 2002).*

Figure 3. Treatement of the infetile patient with MAGI



IMPACT OF INFECTED SPERM ON ASSISTED REPRODUCTIVE TECHNIQUES (ART)

The influence of seminal bacteria and/or leukocyte on the outcome of IVF or ICSI (*Michelmann, 1998*) is influenced by three factors which have little in common with in-vivo conditions:

1. sperm process (swim-up, Percoll) with antibiotic buffered media;
2. the small amount of inseminated spermatozoa (100,000) in IVF;
3. the short cultivation time.

In contrast to viruses (i.e HIV, HBV, HCV), the presence of bacteria in the ejculate does not influence ART program. The reason is that ejaculate preparation techniques, performed immediately after semen liquefaction, not only separate motile spermatozoa but also wash them to get rid of bacterial impurities through various mechanisms: a) antibiotics (penicillin and streptomycin) as an additive to the medium; b) washing procedure with centrifugation and swim-up; c) high dilution factor.

Thus, if at the beginning of IVF the 29% of all ejaculates is contaminated, after insemination medium exposure as well as in the culture media no bacteria were detectable (*Forman et al., 1987*). The same result was found by *Cottell et al. (1996*) who did not find bacteria in the culture medium of embryos on the day of embryo transfer. Furthermore, in this study, fertilization, cleavage, and pregnancy rates were independent of the microbial presence.

Short-term antibiotic treatment of asymptomatic patients before ART should be done with caution, since the pregnancy rate after in-vitro fertilization and embryo transfer does not improve (*De Geyter et al., 1994*), and it may also be detrimental to IVF outcome in some cases (*Liversedge et al., 1996*).

CONCLUSIONS

Chronic symptomatic, or more commonly asymptomatic MAGI may lead to male infertility when the noxae, acting for long periods of time, produce sperm and extended (to more accessory glands) glandular phlogosis/infection -related effects, enhancing a host inflammatory response, in terms of semen ROS and pro-inflammatory cytokine (IL-1β; IL-6; IL-8; TNFα) hyperproduction. These bioactive substances may persist even following antimicrobials, since the initial antioxidant capacity (mainly based on epididymal biological micronutrients of the seminal plasma) is progressively exhausted, thereby impairing sperm function by inducing DNA damage and/or apoptosis (*Agarwal et al., 2003; Sanocka et al., 2003*).

The approach to the infertile patients with MAGI requires a comprenhensive clinical investigation, including the characterization of the different MAGI infertile subgroups by ultrasound scans, microbiological investigation, immunocytological leukocyte determination, and biochemical measurement of ROS production (*Vicari, 1999*). Only when infertile men show a clear clinical evidence of MAGI, a sequential step-wise pharmacological treatment (antibiotics → NSAI → antioxidants) may be established.

Microbiological investigations and suppletive cytological (immunocitochemistry leukocyte assessment; ROS analysis) are not necessary if the suspected pathogens (microbiotes, leukocytes) are not able to induce severe and extented glandular infections, proven through the lack of a significative number of didymo-epididymal and prostato-vesicular post-phlogistic abnormalities at the ultrasound scans. These conditions may be defined as transitory phlogistic processes, due to noxae with low glan-

dular reactive impact and have a negligible impairment on fertility.

Taken in these terms, infertile patients with proven clinical MAGI represent a group of patients who may be treated rationally. Furthermore, a carefull approach including ultrasound (didymo-epididymal and rectal prostato-vesicular) investigation always helps to prevent a further extension of the inflammatory process, which in turn may lead to a worst reproductive prognosis.

A difference must be made when the influence of bacteria is evaluated in-vitro conditions. During ART, sperm processing (wash and swim-up) in an antibiotic rich culture medium effectively eliminates organisms and seems to be devoid of effects in IVF or ICSI outcome. However, the addition of antioxidant compounds to culture media or during sperm preparation (*Aitken et al., 1991; Guerin et al., 2001*) could be useful to reduce oxidative stress during sperm centrifugation, even in the specimens obtained from patients with well-diagnosed PV or PVE, in whom ART is indicated following in vivo unsuccessfull treatment.

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