Plenary Lectures

(PL 01 to PL 06)

The history of the testis and testosterone treatment

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The biological effects of the testes and testosterone (T) are known since antiquity. Aristotle (384-322 BC) knew the effects of castration and his hypothesis on fertilization is one of the first scientific encounters in reproductive biology. Over centuries castration has been performed as punishment, but also to preserve the soprano voices of prepubertal boys. The Chinese imperial (and other oriental) courts employed castrates as overseers of harems; the last of the Chinese eunuchs died in 1996.

The era of testis transplantation and organotherapy was initiated by John Hunter in London who transplanted testes into capons in 1786. The intention of his experiments was to prove the "vital principle" as the basis for modern transplantation medicine, but Hunter did not consider endocrine aspects. Adolph Berthold postulated internal secretion from his testicular transplantation experiments in 1849 in Göttingen and is thus considered the father of endocrinology. Following his observations, testicular preparations were used for therapy, popularised by self-experiments by Charles-Édouard Brown-Séquard in Paris (1889), which can at best have had placebo effects. Nevertheless, testis preparations were consumed until quite recently for the enhancement of virility. In the 1920s Sergio Voronoff transplanted testes from animals to men, but their effectiveness was disproved by the Royal Society of Medicine in 1927. Experimentation with testicular transplantation continued to be popular in Russia. Today testicular transplantation is being refined by stem cell research and germ cell transplantation.

Modern androgen therapy started in 1935 when Ernest Lacquer isolated T from bull testes in Amsterdam. In the same year T was then chemically synthesized independently by Adolf Butenandt in Göttingen and Leopold Ruzicka in Basel. Since T was ineffective orally it was either compressed into subcutaneous pellets or was used orally as 17_-methyl T, now obsolete because of liver toxicity. In the 1950s longer-acting injectable T enanthate became the preferred therapeutic modality. In the 1950s and 1960s research concentrated on the chemical modification of androgens in order to emphasise their anabolic effects. Although anabolic steroids have largely disappeared from clinical medicine, they continue to live an illegal life for doping in athletics. In the 1970s the orally effective T undecanoate was added to the spectrum of preparations. In 1992 WHO, NIH and FDA postulated preparations of natural T mimicking physiological serum levels, a demand first met by a transdermal scrotal film. Non-scrotal skin patches followed and finally in 2000 transdermal T gels became available. The most recent additions to T substitution therapy, the short-acting buccal T and the long-acting injectable T undecanoate, also fulfil the demand for physiological serum levels.

Reproductive epigenetics and transgenerational toxicity

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The term 'epigenetics' refers to heritable non-sequence based mechanisms that regulate gene activity. Three main

types of mechanisms, including DNA methylation, RNA-associated silencing and histone modifications have been associated with the epigenetic silencing of genes. To date, the most well studied

DNA modification associated with the modulation of gene activity is methylation of cytosine residues within CpG dinucleotides occurring at about 25 million sites throughout the genome. DNA methylation plays a role in regulating genes during development and has been implicated in gene regulation, genomic imprinting (variation in the expression of a gene according to its maternal or paternal origin), and X inactivation. Abnormalities in DNA methylation have been linked to cancer as well as growth and behavioral defects. DNA methylation is catalyzed by DNA (cytosine-5)-methyltransferases (DNMTs), is initiated in the germ line and then further modified during early embryo development. In the field of male-mediated developmental toxicity, adverse effects on the offspring in animal studies often occur at levels too high to be accounted for by mutagenesis, leading to the suggestion that alternative mechanisms, including epigenetic processes, may be affected.

Recent studies have reported that male-mediated effects on the progeny can be passed across generations and have implicated epigenetic mechanisms. In addition, a number of recent studies have linked the use of assisted reproductive technologies with growth and genomic imprinting disorders in children; epigenetic processes appear to be involved as the imprinting disorders found were associated with DNA methylation abnormalities. However, in the latter studies it was unclear whether the birth defects were related to the underlying infertility or the treatments (i.e. ICSI, superovulation, culture conditions) being used.

Since DNA methylation events and enzymes are well conserved across mammals, the rat and mouse have served as excellent models relevant to human studies. Using the rodent model, our results and those of others indicate that DNA methylation is highly regulated in the male germ line and implicate alterations in the enzymes involved in DNA methylation, the DNMTs, and DNA methylation, with abnormalities in germ cell as well as embryo development.

The presentation will describe recent studies on the timing and mechanisms underlying the acquisition and mainteneance of DNA methylation patterns in gametes and early embryos as well as the consequences of altering these patterns. Three models relevant to male-mediated effects will be reviewed, DNMT deficiency, effects of cytosine analogues and defects in folate (methyl donor) pathways.

Health of children conceived through intracytoplasmic sperm injection, in vitro fertilization, and natural conception

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In vitro fertilisation has been carried out for nearly 30 years and in developed countries 1% or more of births are from assisted reproductive therapies (ART). These children now represent a significant proportion of the population but until recently little has been known about their health.

Some of the morbidity associated with assisted reproductive therapies does not result from the techniques as such but result from the underlying health risks of being sub fertile.

Much of the increased risk associated with ART is related to higher birth order. However, there are apparent specific increased risks of intrauterine and subsequent perinatal problems, and urogenital malformations in boys, even in singleton ART infants. There is no apparent increase in problems within families.

Long term follow-up of ART children to reproductive age and beyond is necessary.

Keywords : childhood, assisted reproduction, outcome

Gene regulation in spermatogenesis and spermiogenesis - insights and implications for human infertility

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No abstract provided.

Stem cells in spermatogenesis

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The highly efficient process of sperm production is dependent on proper hormone balance, local cellular interactions and, more importantly, on the biological activity of spermatogonial stem cells (SSCs), the stem cells of germline. Because of the ability to transmit genes from one generation to the next, SSCs represent the only replicating, potentially totipotent, population of cells in the adult body. A major breakthrough in understanding SSC biology was represented by the successful demonstration of spermatogonial transplantation and subsequent proliferation and differentiation of the transplanted cells in the recipient animal (1). Germ cell transplantation represents, to date, the only bioassy for SSCs and appears to have relevant potential applications. Manipulations of these cells in vitro, before transfer to a recipient subject, represents an opportunity to modify germline for generating transgenic experimental animals and introduction of new genes directly into the male germline could be of great interest for agricultural, zoological and clinical purposes. By FACS analysis and transplantation technology, surface antigenic profile of SSCs has been partially defined, however none of the markers identified is specific for SSCs, since they are expressed also by other germ cells and/or by somatic cells in the testis (2). Using Hoechst staining of isolated seminiferous tubule cells and FACS analysis, we were able to identify a testis "side population" (T-SP) population that, by transplantation technology, we demonstrated to be enriched in stem cell activity, evaluated 2 months after transplantation (3). Further characterization of T-SP SSCs by double vital staining with Hoechst and rhodamine, allowed us to understand that T-SP SSCs represent only a subset of SSCs and that a variety a SSC subpopulations, possibly endowed with different functional properties, could exist (4). It has been demonstrated that a combination of growth factors, such as GDNF, bFGF and EGF, induces the proliferation of spermatogonia with stem cell potential in vitro, thus providing the possibility to manipulate SSC genome. Transgenic mice and rats have been produced by retroviral infection of SSC (5) and, more recently, homologous recombination has been demonstrated to be feasible in mouse SSC (6). On this direction, we analyzed the feasibility of the in vitro Cre delivery to germ cells in order to obtain gene inactivation in stem cell germline (7).

Conditional mutagenesis represents a powerful approach to analyze gene functions in mammalian cells. Site-specific recombinases such as the bacteriophage P1 recombinase Cre have been used to induce gene modification in a spatial and/or temporal manner (8). However, the use of site-specific recombination in genetic studies is limited by difficulties to express the recombinase in cells at the desired time and place. Recently a new tool for the in vitro deletion of loxP flanked genes has been described. In this approach enzymatically active cell-permeable Cre recombinase is delivered to cultured target cells (9). The HTNC (HIS-TAT-NLS-CRE) recombinase has been shown to induce deletion of lox-flanked gene in about 90% of ES cells or primary splenocytes (9). Genetic analysis in spermatogonial stem cells (SSC) is highly warranted but, presently, no Cre transgenic mice are available to perform conditional gene inactivation in postnatal SSC.

To test if HTNC induces conditional gene inactivation in germ cells, we thought to treat germ cells isolated from ROSA26R mice and to detect recombination events by means of germ cell transplantation. Genetic analysis has demonstrated that after transplantation, donor-derived spermatogenic colonies originate from a single SSC thus germ cell progeny inherit the founder SSC genotype (1). We anticipated that recombination events occurring in HTNC-transduced SSC will generate colonies of donor-derived spermatogenesis where germ cell progeny bear the same recombination events. Recombinant colonies would therefore be easily recognizable as blue colonies in the recipient testis after X-gal staining owing to the activation of the ROSA 26R locus.

Germ cells were isolated from adult ROSA26R mice and enriched in SSC by Percoll purification (10). Germ cell aliquots were treated in vitro with HTNC and transplanted in the testis of busulfan-treated recipient mice. Two months after transplantation, animals were sacrificed and testis analyzed to detect donor-derived blue colonies generated from modified SSC. Donor-derived blue colonies were found in 6 out of 11 tranplanted testis. We have demonstrated, for the first time, the feasability of in vitro conditional gene inactivation in stem cell germline. This experimental strategy may be used to perform conditional gene inactivation in order to analyze gene functions in germ cells during spermatogenesis. The possibility to perform postnatal gene inactivation may be relevant for the study of lox-flanked genes whose inactivation is either embryonically lethal or interferes with prenatal germ cell development. This strategy may also be used to introduce stable genome modification in mice germline.

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Pathophysiology of erectile dysfunction

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In unicellular organism genetic information to the offspring is just duplicated, and therefore, subsequent generations are identical to the preceding ones (parthogenetic reproduction). It is, overall, a monotonic, asexual reproduction. The individual diversity of the more complex organisms derives from the development of sexual reproduction, which requires two distinct partners (the couple) having distinct phenotypic and behavioural futures (maleness and femaleness).

Couple reproduction allows billiards of possibility to exchange genetic material and is of great evolutionary advantage and very favourable for species evolution. However, sexual reproduction between heterogeneous individuals carries patent risks. Although the couple reproduction geometrically increased genetic benefits, threats and problems are similarly amplified. In fact, due to partner interdependence, sexual reproduction can be problematic either for reproductive or for sexual reasons, resulting in one hand in couple infertility and in the other one in couple sexual dysfunction. In fact, couple sexual function should be viewed as the net result of the sexual fitness of both partners.

Only few decades ago it was generally thought that the majority of male sexual dysfunctions (including erectile dysfunction) were mostly related to psychological problems, and to anxiety in particular. Hence, at that time, the only recognized treatment was psychotherapeutic, i.e psychoanalysis and behavioral therapy. Nowadays, it is well recognized that this picture is limitative, as biological and relational domains, beside psychological components, have a relevant impact on male sexual response. Indeed, male sexual response can be seen as an integrated feed-forward interaction among an intra-psychic system (the individual's sexual identity and sense of well-being), a biological system (cardiovascular, hormonal, neuronal), and a relational system (the context for a sexual relationship).

Understanding the relative contribution of each of these components is essential for a correct diagnostic and/or therapeutic approach to male sexual dysfunction and, in particular, to erectile dysfunction (ED). From a biological point of view, male sexual activity is essentially characterized by a T-driven synchronization of sexual desire, arising in the brain, and its transmission to the periphery, allowing penile erection. The most important pathway underlying the penile erection is the nonadrenergic/noncholinergic signalling, which through the release of nitric oxide (NO), leads to an intracellular increase of cyclic GMP (cGMP), the main secondary messenger mediating tumescence in the penis. Interestingly, both cGMP formation and degradation are affected by testosterone (T). Because T positively controls both the initiation (NOS) and the end (PDE5) of the erectile process, its net effect on erection might result even null. Hence, erections are still possible in hypogonadal conditions where a decreased cGMP formation, due to impaired NO production, is counterbalanced by a reduced cGMP hydrolysis.