PREFACE

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Estrogens were traditionally recognized as exclusively female sex hormones. Since their discovery in the early 40-ties of the 20th century until last decade it was thought that these hormones either caused suppression of gonadal function or did not exert any major influence in the male. Nevertheless, estrogens are produced in men and their main sources are the testes, adipose tissue, and the adrenals. Moreover daily production and blood levels of estradiol (E_2) in men are higher than those formed in postmenopausal women.

In 1988, investigations from the Medical University of Lodz, in Poland, revealed that E_2 might be a hormonal signal that participated in initiation of spermatogenesis via stimulatiozy action on the first spermatogonia. This finding was supported by further and animal experimens and clinical observations. In 2000, Ebling and co-workers, investigators from the United Kingdom and Australia, induced experimentally complete spermatogenesis by treating hypogonadal animals with extending in that way the concept that E_2 may have a physiological role in male gametogenesis. These observations are being reviewed by our team in the first article of this special issue of the ANIR.

The importance of E_2 in male physiology was strengthened by the identification of the estrogen receptor (ER) in the males. In the middle 90-ties transgenic mice with lack of ER (ER knock out) or the enzyme aromatase catalyzing the conversion of testosterone into E_2, were produced. An important contribution to the research on the localization of ER and the source of estrogen production within the male reproductive system was made by the group of Barbara Bilinska, from the Jagiellonian University of Kraków. She was kind to accept our invitation to delineate here the current view on this topic.

Doc. Dr. med. Jolanta Slowikowska-Hilczer form the Medical University of Lódz reviews the hot topic on "Xenoestrogens and Male Reproduction". She addresses the question on another environmental xenobiotics (especially xenoestrogens) may be related to the increasing incidence of the male reproductive system anomalies. These endocrine disrupting chemicals may in some aspects mimic the action of endogenous E_2 and bind to ER, despite their different chemical structure. Beside this, xenoestrogens change hormonal metabolism and influence genomic imprinting. The main conclusion is that although the physiologic action of estrogen in the male reproductive system is of vital importance, it is necessary to realize that most of xenoestrogens, as endocrine disruptors, may exert both estrogen-like and anti–androgenic activity and in this way may be harmful to the male reproductive system.

The reproductive part of this issue is completed by a review from Drs E. Koukkou, G. Mitios and D.A. Adamopoulos, our hosts form Athens. Besides, the comprehensive review of the biochemistry and pharmacokinetics of antiestrogens and androgens, they describe a novel application of the combined administration of an antiestrogen (tamoxifen) together with a weak androgen (testosterone undecanoate) to improve sperm parameters in men with idiopathic oligozoospermia. The rationale of this presently most successful medical therapy of male subfertility, is that antiestrogen action leads to an increase in gonadotropin and sex steroids secretion and this effect is expected to have a beneficial effect on sperm number. The simultaneously applied weak androgen may directly stimulate male reproductive tract elements,that improving the quality of spermatozoa. Thank you Dimitris (and your colleagues) for your contribution.

The most unexpected and fascinating clinical data outside male reproductive tract originate from the Department of Endocrinology of Modena University, Italy, headed by Prof.

Cesare Carani. The review by Dr. Lucia Zirilli, Dr. Vincenzo Rochira and Prof. Dr. Cesare Carani is focussed on observations in men with inherited inactivating mutations of the gene encoding aromatase (congenital estrogen deficiency) and men with inherited inactivating mutations of the gene encoding ER (congenital estrogen resistance). Data from both situations have considerably extended our knowledge about the role of E_2 in the formation of bone stroma and in the inhibition of bone linear growth after puberty in men. Before their work as well as studies by other investigators, these effects were attributed to testosterone action. Congenital estrogen deficiency in men results, among others, in osteoporosis, continuing linear growth into adulthood and unfused epiphyses. The therapeutically applied transdermal preparations of E_2 to these men produced epiphyseal closure, increased bone density and growth inhibition. The review describes the tremendous progress and expertise of this therapy in prepubertal boys with the disease, which is a very new issue. Thank you Cesare (and your Colleagues) for agreing to prepare a review on this very novel, hot topic.

New data indicate also the role of estrogens in the function of the cardio-vascular system. In men with estrogen resistance a precocious arteriosclerosis state was noted whereas in men with congenital estrogen deficiency an unfavourable lipid profile was reported . The link of estrogens to the development of arteriosclerosis seems, however, to be bipolar. In this context, it should be stated that some results indicate a possible role of E_2 in promoting the development an atherogenic lipid milieu in men. The review of this controversial topic originates from the cooperative research team of Lodz Medical University (Departments of Cardiology, Cardiosurgery and Andrology) and makes an important contribution to the content of this issue.

I do believe that the novelties of the reviews presented have established estrogen action in men as a dynamic new area that broadens in scope with growing number of organs related to estrogens for their physiological function. Nonetheless, estrogenic disruptor action is in males need special attention. It seems that the recent advances in both fields may be translated into approaches for the proper management of relevant reproductive dysfunction in the male but also opening new therapeutic potentialities.

Regulatory Role of Estradiol in the Induction of Spermatogenesis: Clinical Correlations and Animal Experimentations

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I. INTRODUCTION

Sexual maturation is initiated by the hypothalamic secretion of gonadotropin releasing hormone (GnRH) followed by secretion of pituitary gonadotropins FSH and LH, stimulating the downstream secretion of sex steroids by the gonads. In some studies independent actions have been ascribed to FSH, directly stimulating of the function of the seminal tubules function, and to LH, stimulating sex steroid biosynthesis by Leydig cells [Chemes et al., 1976; Bartlett et al.,1988]. In other studies synergistic effects of LH and FSH have been reported to account for the response of the seminal tubules [Lostroh, 1969; Russel and Clermont, 1977; Sharpe, 1984].

The main male sex hormone is testosterone (T), necessary for male sexual differentiation, puberty and the maintenance of male sexual characteristics. Testosterone participates in the induction and maintenance of spermatogenesis, acting through Sertoli cell's androgen receptor [Bremner et al., 1994]; however, its relative role is still under debate.

The testis secretes also some other hormones, that may take part in the regulation of spermatogenesis, but their roles are not clearly understood; one of them is estradiol (E_2). Since its discovery in the 40'ies and until the 90'ies in the 20th century, E_2 was recognized as "female" sex hormone, harmful for testicular function. However, estrogen receptor (ER) is widely distributed in testicular cells, suggesting a role of estrogens in the regulation of testicular function [for review see Bilinska et al, in this issue of ANIR].

In human testis ERb is likely to be the main receptor that mediates the effect of estrogens. ERβ is localized in the nuclei of spermatogonia, spermatocytes and early developing spermatids of adult men [Makinen et al., 2001]. Furthermore, the demonstration of abundant ER's in human spermatozoa [Aquila, 2004], supports the concept for the possible involvement of estrogens in the male reproductive function. However, these molecular studies did not address the question about a specific role of estrogen in the kinetics of spermatogenesis, especially in the induction of this process during pubertal development.

II. ROLE OF ESTROGEN IN THE INDUCTION OF SPERMATOGENESIS IN MAN

An outlook on human spermatogenesis

Spermatogenesis can be divided into three phases. The first, premeiotic, phase concerns the youngest cells of the germ cell line - the spermatogonia. Most of these cells proliferate to give rise to spermatocytes, while the remainder maintain their own number by renewing themselves. The second phase involves the primary and secondary spermatocytes that go through a process of meiotic divisions, leading to the formation of haploid cells, the spermatids. The third phase concerns the spermatids, each of which goes through a complex metamorphosis, leading to the production of a highly differentiated motile cell, the spermatozoon.

In men there are two kinds of spermatogonia, types A and B. The nuclei of A spermatogonia does not show heterochromatin. Cells with nuclei which stain less heavily with hematoxylin are named A-pale spermatogonia, while others which stain more heavily are called A-dark [Clermont, 1966]. A-pale spermatogonia divide mitotically and give rise

to B spermatogonia (one mitosis) and following another mitosis become meiotic spermatocytes. B spermatogonia divide and transform initially into preleptotene, and then leptotene, zygotene and pachytene primary spermatocytes. Diakinesis of secondary spermatocytes completes meiotic division and initiates spermiogenesis.

A-dark spermatogonia do not divide and are quiescent. However, when the number of A-pale spermatogonia is diminished, for example after irradiation, A-dark spermatogonia become active and transform into A-pale, and thereafter start to proliferate. So, A-dark spermatogonia act as reserve of stem cells. They are repopulated (renewed) by re-differentiation of A-pale spermatogonia when it is needed [for review De Rooij and Russel, 2000] *(Fig.1)*.
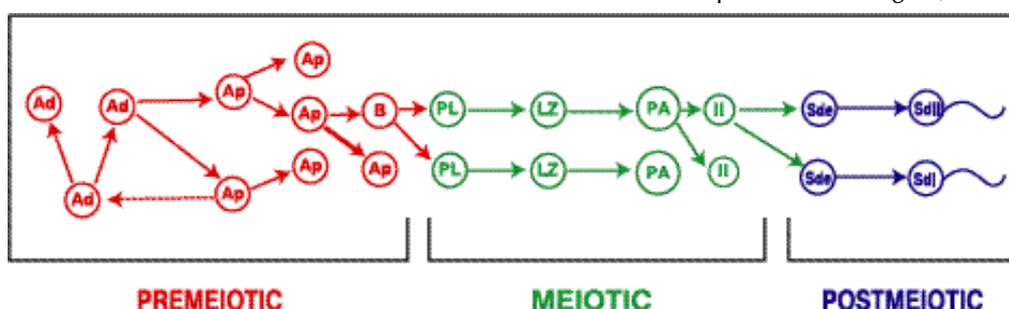

Figure 1: Human spermatogenesis. A-dark spermatogonia (Ad) represent stem cells; A-pale spermatogonia (Ap) continuously proliferate and differentiate into B spermatogonia (B) and renew themselves (thick arrow). Ap can also redifferentiate into Ad (dotted arrow). B divide and simultaneously differentiate into preleptotene meiotic spermatocytes (PL). Meiotic division is represented by: LZ – leptotene/zygotene phase, PA – pachytene phase (pachytene spermatocytes) and II – secondary spermatocytes. Postmeiotic cells are early spermatids (Sde), the haploid cells that differentiate into late spermatids (Sdl) (include mature spermatozoa). *[Based on de Rooij and Russell, 2001]*

After birth, most of germ cells in the seminal tubules are fetal germ cells, called gonocytes. Subsequently they transform into spermatogonia and it is suggested that after the first year of life gonocytes should not be present in the seminal tubules *[Jörgensen et al., 1993]*. The total number of premeiotic germ cells increases from 13 to 83 millions during 0-10 year of age *[Müller and Skakkebaek, 1983]*. Single spermatocytes are seen before puberty but generally meiosis is initiated at the beginning of puberty, indicating the importance of prepubertal period in life for the creation of germ cell number and specifically the number of premeiotic germ cells. The onset of the release of first spermatozoa (spermarche) occurs at median age of 13.4 years (range 11.7-15.3) and takes place when median testicular size is about 11 mL *[Nielsen et al., 1986b]*. Adult levels of T secretion follows these changes and are not observed until 2 years after spermarche *[Nielsen et al., 1986a]*.

Human testis as a source of estrogen

It is generally assumed, that in men the main source of E_2 is the aromatization of testosterone in peripheral tissues.

However, it was demonstrated that in boys with bilateral cryptorchidism an increase FSH concentration in response to a single intravenous injection of GnRH appeared in parallel to the rapid (within 60 min.) rise in the E_2 levels *[Kula and Chilarski, 1987]*. This suggested a stimulatory role of FSH to generate E_2 secretion from the intratesticular pool. Furthermore, in normal adult men a single intramuscular injection of human chorionic gonadotrotropin (hCG), a natural ligand of LH receptor within the testis, is followed by the increased secretion of E_2 (maximal after 24 hours), followed by increased testosterone secretion (maximal after 72 hours) *[Oszukowska et al., 2003]*. In an adult patient with Leydig cell tumor the secretion of E_2 in response to hCG was up to 10 times higher, while secretion of testosterone was lower than in healthy men. Interestingly, hypersecretion of E_2 in this man did not disappear immediately after resection of the tumor-bearing-testis, indicating that impaired testicular steroidogenesis, favoring E_2 over testosterone secretion, might be a lesion not exclusively located within Leydig tumor's cells.

These results indicate that as in prepubertal boys with cryptorchidism, in normal adult men and in a patient with Leydig cell tumor, the testes were more eager to secrete E_2 than testosterone. This indicates the importance of testicular estrogens both in physiology and pathology. Recent data indicate that the mean testicular contribution to peripheral blood E_2 levels in man is 57% *(de Ronde et al., 2005)*.

Leydig cell hyperplasia as a natural model of sex steroids influence on the initiation of spermatogenesis

Premature activation of the hypothalamo-pituitary-gonadal axis leads to precocious puberty. In turn, autonomous premature activation of gonadal or adrenal sources of sex steroids secretion, associated with lack of gonadotropins secretion by the pituitary, leads to pseudo-precocious puberty. This condition in males may result from a Leydig cell tumor or from uniformly distributed Leyding cell hyperplasia called testotoxicosis. Both pathologies permit to the observation of a direct influence of sex steroids on the neighboring seminal tubules. Hence, this clinical situation may serve as an experiment of nature, important for an

assessment of the hormonal regulation for the initiation of spermatogenesis in man.

In 1973 Steinberger et al. observed that overproduction of testosterone by a Leydig cell tumor resulted in the stimulation of spermatogenesis up to early spermatids in the tumor bearing testis of a 6-year-old boy with pseudo-precocious puberty, while in the biopsy of contralateral testis an absence of spermatogenesis was seen. In our study quantitative analysis of the seminiferous epithelium was performed in seminal tubules located in different distances from a Leydig cell tumor in the testis of a 7-year-old boy with pseudo-precocious puberty [Kula et al., 1980]. The increased number of spermatocytes found correlated well with the progression of Sertoli cells'maturation, whereas the increased number of spermatogonia was dependent on the vicinity of the seminal tubules to the Leydig cell tumor. Spermatogenesis was precociously complete. Although no hormonal data were available in this patient, the analysis of spermatogenesis suggested that secretory products of Leydig cells (androgens and/or estrogens) diffused to the basal compartment of the seminiferous epithelium and stimulated spermatogonia, while postmeiotic steps were more dependent on the Sertoli cells function.

In a following study [Kula et al., 1996], the precociously complete spermatogenesis was observed in a 4.5-year-old boy with pseudo-precocious puberty because of Leydig cell hyperplasia (testotoxicosis). This was associated with an excessive secretion of testosterone that was in the normal adult men range. Moreover, on hypersecretion of E_2 was found, that was even more highly expressed, being 5 times higher (117 pg/ml and 174 pg/ml, mean 145.5 pg/ml) than in adult men (27.6 ± 18.9 pg/ml). FSH and LH concentration was markedly.

Quantitative data on spermatogenesis in testicular biopsy of this boy and of two other boys with pseudo-precocious puberty due to Leydig cell hyperplasia, both aged 8 years, were compared to the data obtained from testicular biopsies of 5 year old age-matched boys, with glandular hypospadias and from testicular biopsies of 9 adult men with obstructive azoospermia [Slowikowska-Hilczer et al., 1995; Kula et al., 1996] (Fig. 2).

Boys with Leydig cell hyperplasia had complete spermatogenesis (up to late spermatids, including spermatozoa), while in the age-matched boys

Initials (age-years)	FSH (IU/L)	LH (IU/L)	Testosterone (ng/ml)	Estradiol (pg/ml)
D.J. (4.5y.)	0.8-1.0	2.4-2.9	3.5-5.0	117.0-174.0
D.B.(8y.)	0.3-0.5	10.5-11.7*	4.6-10.0	13.0-16.7-
W.T. (8y.)	-	-	5.0	120.1
Age-matched boys (n=5; 5-6 y.)	1.4 ± 1.0	0.9 ± 0.5	0.95 ± 0.15	≤1.0
Adult men (n=9; 25-35 y.)	5.6 ± 2.9	7.2 ± 2.9	4.0 ± 1.6	27.6 ± 18.9

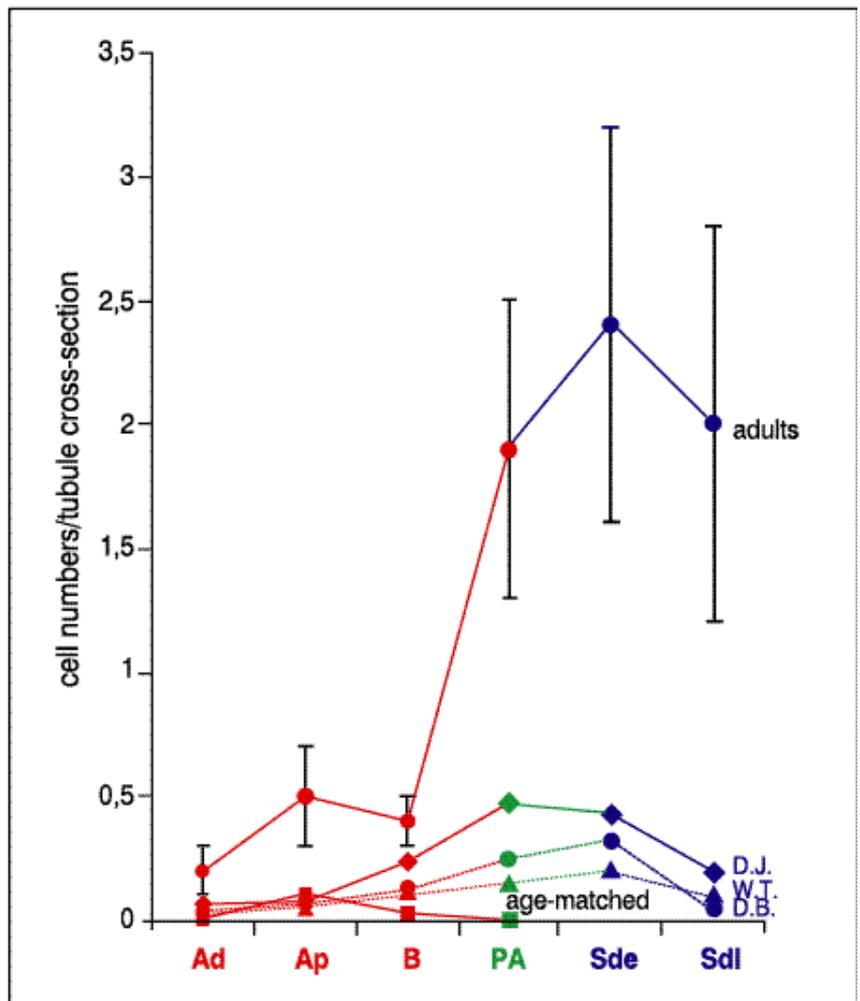*Luteinizing hormone (LH) secreting adrenal tumor [Romer et al., 1998]

Figure 2: Blood levels of hormones and the mean ($\pm$S.D.) numbers of germinal cells representing successive steps of spermatogenesis (from the left to the right) in the testis of boys with pseudo-precocious puberty (D.J.; D.B., T.W.), age-matched boys (n=5), and in adult men (n=9). Ad – A-dark spermatogonia, Ap – A-pale spermatogonia, B – B spermatogonia, PA – pachytene spermatocytes, Sde – early spermatids, Sdl – late spermatids. Note qualitatively complete spermatogenesis in pseudo-precociously pubertal boys (up to Sdl), while spermatogenesis in age-matched boys reaches only PA. *[From Slowikowska-Hilczer et al., 1995; Kula et al., 1996; modified]*

spermatogenesis reached the level of meiosis. In all 3 cases spermatogenesis suffered in quantitative terms.

In boys with precocious puberty hypersecretion of E_2 (13-100--fold increase vs. age-matched boys) and testosterone (3.5-12–fold increase vs. age-matched boys and equal to or 2-fold increase vs. adult men) were found (*Fig.2*). It appeared, therefore, that at the reduced secretion of FSH the increased levels of E_2 and testosterone were sufficient to initiate and complete the first spermatogenesis in the human. However, the quantitative aspects of spermatogenesis might require a more complex hormonal milieu, presumably a proportionally balanced FSH availability. *Figure 3* shows that in boys with pseudo-precocious puberty premeiotic steps of spermatogenesis were evidently hyperactivated in comparison to the age-matched controls.
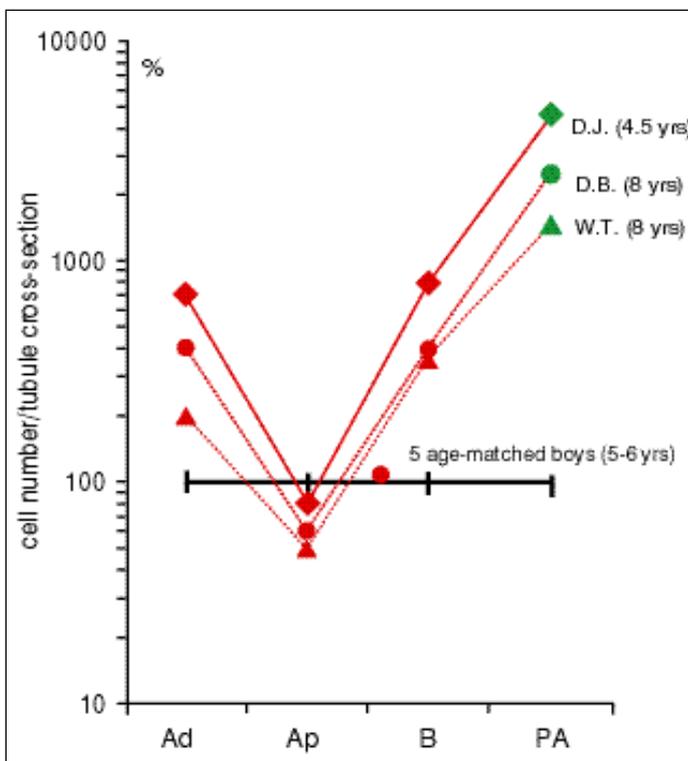

Figure 3: Mean numbers of cells representing premeiotic and meiotic steps of spermatogenesis (from the left to the right) in boys with Leydig cell hyperplasia (D.J.; D.B., T.W.) with respect to age-matched boys (n=5). Numbers are expressed as percent changes vs. age-matched boys (100%) in the logarithmic scale. Germ cells numbers per tubule cross-section include A-dark spermatogonia (Ad), A-pale spermatogonia (Ap), B spermatogonia (B) and pachytene spermatocytes (PA). Note the hyperactivation of spermatogonia differentiation (increased number of B and PA) and increased number of Ad (germinal stem cells) in boys with Leydig cell hyperplasia. *[Deducted from Kula et al., 1996]*

The hypothesis that estrogens may participate in the testicular maturation may be supported by other studies, demonstrating that increased blood level of E_2 is associated with testicular growth in normal pubertal boys *[Maruyama et al., 1987; Biro et al., 1990].*

Mutations of genes coding estrogen receptors and aromatase

In the following years, thanks to the cloning of the aromatase and ER genes in men, congenital estrogen deficiency as the result of naturally occurring inactivating mutations of the aromatase gene and estrogen resistance due to inactivating mutations of the ERa gene could be diagnosed. To date, 6 subjects with estrogen deficiency have been described (5 adult men and 1 male infant), and only one case of estrogen resistance is known. Many clinical aspects are shared by both the estrogen-resistant man and the 5 adult men with estrogen deficiency, but the possible occurrence of infertility has not been reported in all of them.

The only man known to be estrogen resistant had reduced sperm motility, but normal sperm number *[Smith et al., 1994].* A severely reduced sperm concentration and an impairment of sperm viability with germ cell arrest at the level of primary spermatocytes were found in one patient with estrogen deficiency. In a second subject, a complete germ cell arrest was found *[Maffei et al., 2004],* whereas. A third patient had slightly reduced sperm count and sperm viability *[Herrmann et. al., 2002].* Although these data may not indicate a strong link between estrogen non-availability and reduced fertility in men, only in one of them spermatogenesis was overlooked in testicular biopsy.

The effects of estrogen replacement treatment on sperm number in 3 of 5 patients with estrogen deficiency did not improve either the sperm count or the motility *[Carani et al., 1997; Faustini-Fustini et al., 1999; Herrmann et al., 2002].*

III. ANIMAL EXPERIMENTATIONS

An outlook on spermatogenesis in the rat

In rodents, as in humans, spermatogenesis includes in three phases: premeiotic, meiotic and postmeiotic. The premeiotic step compromises multiplication, self-renewal and differentiation of spermatogonia. As in men, the premeiotic step of spermatogenesis is of great importance because firstly, spermatogenesis is initiated via spermatogonia and secondly, the population of germ cells is increased via the mitotic activity of spermatogonia. One spermatogonium goes through 8 to 9 divisions before differentiating into a meiotic spermatocyte. Spermatocytes undergo only 2 meiotic divisions. Ultimately, 1 spermatogonium is capable of rendering anything from 2048 to 4096 spermatozoa, depending on the number of divisions. Thirdly, determination

of germ cell numbers is accomplished at the spermatogonia level - the appropriate ratio of germ to Sertoli cells is provided [for review De Rooij and Russel, 2000].

There are three main categories of rat's spermatogonia. The first, type A, does not display heterochromatin in the nucleus, while the second, type B, does. The third type has intermediate amount of heterochromatin and is termed intermediate (In) spermatogonium. Type A spermatogonia are the most primitive, followed by In spermatogonia and finally by B spermatogonia. Among A spermatogonia stem cells are recognized, called A-single spermatogonia, corresponding to A-dark spermatogonia in man.

A single spermatogonium have the capacity of self-renewal and differentiation to A-paired and A-alignant spermatogonia, called also primitive or undifferentiated type (UnA). A-alignant types differentiate giving 6 generations of differentiating spermatogonia, which are subsequently composed of A1, A2, A3, A4, In and B spermatogonia. Differentiating A spermatogonia in the rat are homologous with A-pale spermatogonia in man.

In the testis of a newborn rat, the fetal germ cells – gonocytes start to differentiate into first spermatogonia on about the 3rd postnatal day [Vitale et al., 1973; Roosen-Runge and Leik, 1968]. Gonocytes give rise to A-single spermatogonia but may also go immediately to A2, making first spermatogenesis shorter in duration than that seen in adult animals [for review De Rooij and Russel, 2000].

Rat represents a unique species where the neonatal period of life overlaps with sexual maturation. Gonocytes complete differentiation into the first spermatogonium at about the 5th day of life, first spermatogonia become first meiotic spermatocytes between the 10th and 19th day and the first spermatozoa are seen at about the 45th day after birth [Clermont and Perey, 1957; Kula, 1977; Russell et al., 1987]. So, the first 15 days after birth is the period during which the first appearance of A spermatogonia, their multiplication and differentiation into In and B spermatogonia, their progression into premeiotic germ cells - the preleptotene spermatocytes and the transition between preleptotene and pachytene spermatocytes take place and represents the prophase of the first meiotic division. Clermont and Perey [1957] demonstrated that in rats the seminiferous epithelium cycle is already established from day 15th of the animal's age.

Detrimental effects of estrogen administration on testicular function in adult and immature animals

The detrimental effects of estrogen administration on testicular function was known since many years [Wolf and Ginglinger, 1935]. Supra-physiological (500 mg) single dose of E_2 during the critical period of neonatal life in male rodents

resulted in numerous reproductive defects as: atrophy of the testes and sexual accessory glands in adulthood [Naslund and Coffey, 1986; Aguilar et al., 1987], impairment of germ, Sertoli and Leydig cells maturation [Gaytan and Aquilar, 1987; Gaytan et al., 1986; Pinilla et al., 1992] and decreased LH secretion [Aguilar et al., 1984; Pinilla et al., 1995].

An inhibitory effect of E_2 on testosterone biosynthesis in Leydig cells was possible and it might have been indirect, involving the negative feedback inhibition of LH secretion [Freeman, 1985; Tsai-Morris, 1986; Amador, 1989]. The pathway, via which estrogens reduce testosterone production directly, may involve inhibition of the expression of steroidogenic factor-1 [Majdic et al.,1997] and 17a-hydroxylase-C17-20-lyase [Majdic et al., 1996]. However, a short-term effect of estrogen administration focused on the onset of first the spermatogenesis was not evaluated in these studies.

The administration of pharmacologically available estradiol - estradiol benzoate (EB), in a dose of 50 mg to adult male rats for 5, 10 or 15 days caused gonadotropins suppression and induced germ cell apoptosis [Blanco-Rodriguez and Martinez-Garcia, 1996; 1997]. A possible involvement of E_2 in the modulation of germ cell death in the adult testis was suggested. This was based on the finding that the apoptosis pattern of the seminiferous epithelium cells elicited by E_2 treatment in adult rats differed from that reported after gonadotropin or testosterone withdrawal. This suggests a direct influence of estradiol on germ cells to induce apoptosis.

Revealing a stimulatory effect of estrogen administration on the initiation of spermatogenesis

Estradiol is detectable in the blood of rats as early as the 1st day of life [Pang et al. 1979], and its level increases until about the 10th day [Döhler and Wuttke, 1975, 1976]. It has been shown that aromatase activity, an enzyme necessary for a bioconversion of androgens into estrogens, is located in newborn rats exclusively within seminal tubules and undergoes sharp dislocation into Leydig cells on the 15th day after birth [Tsai-Morris et al., 1985]. Maximal capacity of FSH for stimulating estrogen synthesis within rat testis is also limited to the initial first 12 days of life [Pomerantz, 1980]. Thus, a functional role of estrogens in the initiation of spermatogenesis could be expected.

Quantitative analysis of seminiferous epithelium performed in newborn/pubertal rats after daily injections of estradiol benzoate (EB), 15 µg daily, testosterone propionate (TP), 2.5 mg daily, human menopausal gonadotropin (hMG), a preparation containing FSH activity, 7.5 IU daily and TP+hMG. Experiments were conducted between the 5th and 11th day of life followed by a 4 days pause and the rats were

autopsied on day 15th *[Kula, 1988]*.

Administration of TP did not influence significantly germ cell numbers. TP reduced Sertoli cell nuclei volume indicating inhibition of Sertoli cell function by testosterone. Administration of hMG, significantly stimulated testes weight, seminal tubule diameter, differentiation of all premeiotic germ cells into spermatocytes and produced precociously an adult-like premeiotic germ cells ratio. This indicated an important role of FSH in the initiation of spermatogenesis and in the stimulation of testicular growth. Unexpectedly, all these stimulatory effects of hMG were counteracted by TP, when TP was given together with hMG.

The results of the quantitative analysis of the seminiferous epithelium are summarized in *Figure 4*. The mean numbers of UnA and differentiating A spermatogonia were significantly increased after EB and not after other treatments. Differentiation of spermatogonia was significantly reduced and the formation of preleptotene spermatocytes from spermatogonia after EB was not changed (data not shown). The first unexpected observation was, however, that exogenous estrogen did not induce degenerative changes in germ cells or did not decrease the growth of testes as was reported before *[Gaytan and Aquilar, 1986; Gaytan et al., 1986]*. By the large our data revealed for the first time a stimulatory effect of the "female hormone" E_2 on the testes, with its influence on the level of first spermatogonia, whereas testosterone was not effective. The latter hormone seemed to be even inhibitory as it counteracted the stimulatory effect of hMG/FSH when was co-administered with it.

Indeed, it has been subsequently shown that ERb is present in germ cells such as spermatogonia, spermatocytes, round and late spermatids (in rats, mouse, non-human primates and in humans) [Saunders et al. 1998, Van Pelt et al. 1999; O'Donell et al., 2001]. The evidence of complete aromatase gene expression in rat and mice germ cells [Nitta et al., 1993; Carreau and Levallet, 1997, Bilinska et al.,2003] and the presence of ERs on different testicular cells during the development [Fisher et al., 1997; Saunders et al., 1998; Van Pelt et al., 1999; Jefferson et al., 2000; Nielsen et al., 2000] further supported the concept of the role of estrogen in the induction of spermatogenesis.

Estradiol may influence spermatogonia directly. Namely, Miura et al. [1999] have shown that 17β-estradiol stimulates and antiestrogens (tamoxifen) inhibit the self-renewal of spermatogonia both in vitro and in vivo in the Japanese eel (Anguilla japonica). The study using a non-mammalian vertebrate model, the lizard Padarcis s. sicula, demonstrated that 17β-estradiol treatment induced spermatogonial proliferation, possibly via activation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), and that this effect was neutralized by the antiestrogen ICI 182-780 [Chieffi et al., 2002]. The recent study of Vicini et al. [2006]
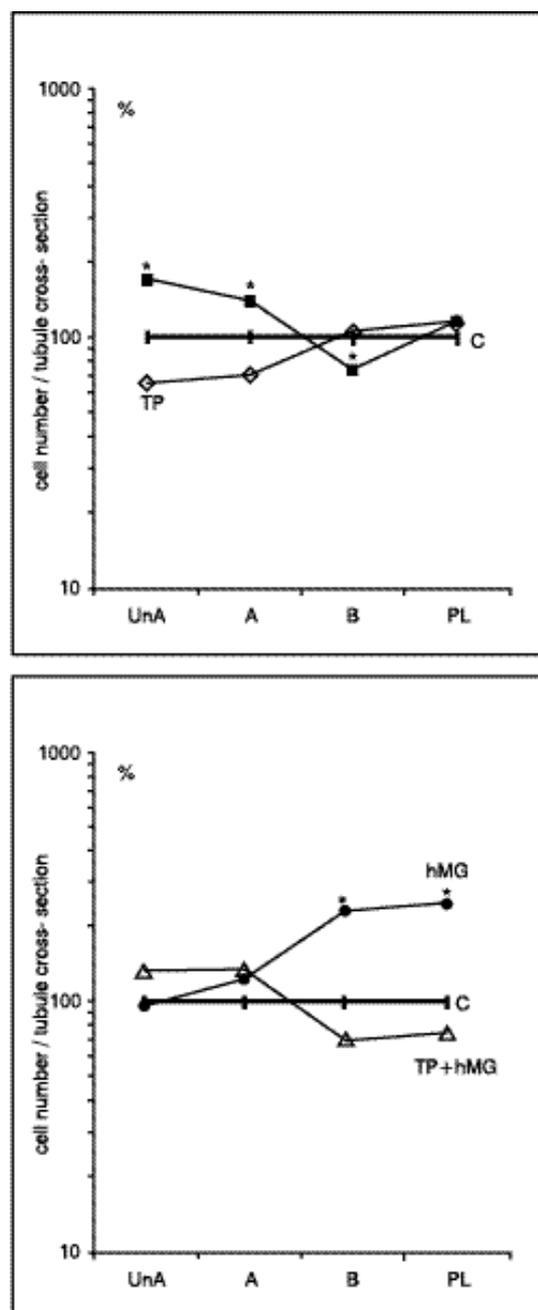

Figure 4: The influence of estradiol benzoate (EB), testosterone propionate (TP) (upper panel), human menopausal gonadortopin (hMG) and TP+hMG (lower panel) on the subsequent (from the left to the right) steps of the first premeiotic spermatogenesis. The numbers (per tubule cross-sections) of undifferentiated A spermatogonia (UnA), differentiated A spermatogonia (A), B spermatogonia (B) and preleptotene spermatocytes (PL) are expressed as a percent changes vs. control group (C) (100%). Note the logarithmic scale. Statistical analysis was performed on the absolute values for experimental vs. C group. *statistically significant vs. C (Students t-test) *[Deduced from Kula, 1988]*

demonstrated that the mouse spermatogonia derived GC-1 cell line responded to estrogens by transiently activating a signal transduction pathway that acts on the mitogen-activated protein kinases ERK1 and ERK2. A similar dose-dependent transient activation of ERKs was also observed in primary mouse spermatocytes in vitro.

In 2000, Ebling et al. reported induction of qualitatively complete spermatogenesis from pachytene spermatocytes onward by 17b-estradiol administration in mice congenitally lacking gonadotropins and thus sex steroid production. These data extended and complemented the concept that the "female" hormone estradiol may have a physiological role in male gametogenesis. Spermatogenesis proceeded in the absence of measurable androgens concentration, but circulating FSH concentration was slightly (but significantly) elevated. Although it may indicate that estradiol induces spermatogenesis via increase in FSH secretion, we did not observe the increase in FSH secretion in response to the administration of estradiol in newborn/pubertal rats *[Kula et al., 2001]*.

Estradiol enhances the stimulatory effect of FSH on testicular maturation

A hypothesis for an involvement of FSH in the stimulation of proliferation and differentiation of first spermatogonia in puberty arises from the study of Chemes et al. *[1979]*. They showed that in immature rats the administration of antibodies against rat's FSH resulted in a 25% decrease of the number of first preleptotene spermatocytes. Subsequently, Almiron and Chemes *[1988]* revealed that in the rat up to 10 days of life, the purified FSH stimulated DNA biosynthesis and mitotic activity of gonocytes and A spermatogonia. The possibility of a direct, not mediated by Sertoli cells, effect of FSH on spermatogonia may arise from the study of Baccetti et al. *[1998]*, who showed an active binding of FSH to spermatogonia and the presence of FSH receptors in spermatogonia, spermatocytes and spermatids in mice and men.

Initiation of spermatogenesis in rats was investigated under the influence of daily injections of EB in a dose of 12.5 µg/day, purified human FSH (hFSH) in a dose of 7.5 IU and EB+hFSH. All substances were administered continuously beginning from the 5th until 15th day of life. Rats were autopsied on day 16th *[Walczak-Jedrzejowska and Kula, 1999; Kula et al., 2001]*.

These results are summarized in *figure 5*. As in the previous study using hMG *[Kula, 1988]*, hFSH given alone accelerated testicular growth, increased the number and differentiation of spermatogonia, and this resulted in a 5-fold increase in the mean number of spermatocytes.
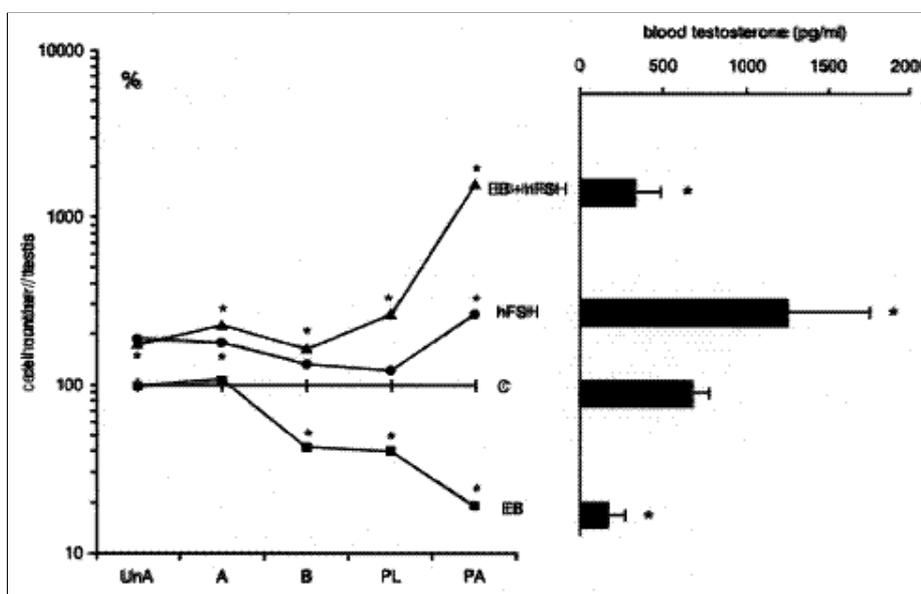
As in our previous study, EB given alone reduced differentiation of A to B spermatogonia *(Fig. 4, 5)*, together with a significant 4-fold decrease in blood concentration of testosterone but did not influence FSH or LH levels. Unexpectedly, when EB was given together with hFSH the treatment not only overcame the inhibition of spermatogonia differentiation, but multiplied hFSH's stimulatory effect on the premeiotic step of first spermatogenesis, that resulted in the 30-fold increase in the number of pachytene spermatocytes versus control values. After EB+hFSH an adult type of premeiotic germ cells ratio was precociously created.

The enhancement of hFSH stimulatory effect on the initiation of spermatogenesis by EB was associated with a significant increase in seminal tubules' diameter and the formation of tubular lumen, indicating that Sertoli cells intermediated this stimulatory effect.

Figure 5: The influence of estradiol benzoate (EB), human purified FSH (hFSH), and EB+hFSH on the subsequent (from the left to the right) steps of the first premeiotic spermatogenesis (left panel) and the mean (±S.D.) blood level of testosterone (right panel). The absolute numbers (per testis) of undifferentiated A spermatogonia (UnA), differentiated A spermatogonia (A), B spermatogonia (B), preleptotene spermatocytes (PL) and pachytene spermatocytes (PA) are expressed as a percent changes vs. control group (C) (100%). Note the logarithmic scale. Statistical analysis was performed on the absolute values for experimental vs. C group. *statistically significant vs. C (Students t-test) *[From Kula et al., 2001; modified]*.

After EB+hFSH the secretion of testosterone remained significantly reduced to 50% of control values, and this indicated that seminal tubules of maturing rats became extremely sensitive to FSH at the excess of estradiol and decreased levels of testosterone. In this last aspect, animal data resemble clinical observations, showing that in adult men hyperactivation of the premeiotic steps of spermatogenesis was associated with increased secretion of FSH together with decreased blood levels of testosterone [Kula, 1991].

The synergistic effect of estradiol and FSH observed by our group appears to be mediated by Sertoli cells. In 1997, McCalman et al. demonstrated that estradiol enhanced the stimulatory effect of FSH on the production of mRNA transcript for N-kadherin biosynthesis in Sertoli cells of 17-day-old mice. This protein is necessary for intercellular adhesions within seminiferous epithelium. In 1993, Dorrington et al. observed an interaction between FSH and 17b-estradiol or TGF b in Sertoli cells of immature rats promoting the stimulation of mitotic activity of these cells.

Unlike in previous findings [Kula, 1988] the number of A spermatogonia was not affected in our subsequent study [Kula et al., 2001]. This difference may have arise from the difference in experimental design and procedure. Firstly, in the more recent experiment daily administration of EB was continued until the 15th day of life and autopsy was performed on day 16th, while in the first study administration of EB was discontinued on day 11th followed by a 4-day pause before autopsy (on the day 15th post partum). Secondly, the dose of EB applied was lower (12.5 µg daily) in the recent as ????? to the higher dose (15 µg) used previously.

Estradiol but not testosterone stimulates repopulation of seminal tubules with germ cells disrupted by irradiation

The effective suppression of gonadotropins and intratesticular testosterone with GnRH analogs paradoxically restores repopulation of seminal tubules with germ cells and stimulates progression of spermatogenesis disrupted by irradiation of rats (Meistrich and Kangasniemi, 1997). It has been shown that testosterone is responsible for the inhibition of surviving A spermatogonia differentiation in irradiated rats (Shetty et al.,2000). However, administration of E_2, which suppresses serum LH, FSH and testosterone, produced a dramatic recovery of spermatogenesis in these animals. Combined administration of testosterone with estradiol partially counteracted the stimulatory effect of estradiol on germ cell repopulation in irradiated rats (Shetty et al.,2004). This further supports the concept that during initiation / re-initiation of spermatogenesis, estradiol may play a crucial stimulatory role, while testosterone may have a negative effect.

IV. CONCLUSIONS

The data reviewed here in suggest that estrogens may play a regulatory role for the initiation of spermatogenesis in three forms: 1) excitory, via direct influence on spermatogonia multiplication and their self-renewal, 2) stimulatory, exerted through an enhancement of FSH effects on the initiation of spermatogenesis and 3) inhibitory, exerted both via direct stimulation of germ cell apoptosis and inhibition of gonadotropin secretion.

It is of interest that administration of testosterone may counteract the stimulatory influence of FSH on the induction of spermatogenesis and may counteract the stimulatory effect of E_2 on germ cell repopulation in irradiated animals.

V. POST SCRIPTUM

Clinical, well controlled studies, are needed before use of estrogens would be envisaged for possible treatment of impaired spermatogenic function in infertile men. However, already in 1998 Sah treated 14 men, aged 23-31 years, with oligozoospermia of unknown etiology with one tablet, containing 0.0044 mg of ethinyl estradiol and 3.6 mg of methyl testosterone, daily. After 4 months of the treatment 9 men (64 %) he observed a definite improvement in the semen index (summarizing the number, the percentage of motile spermatozoa and their normal morphology). The wives of 3 patients (21 %) became pregnant within 6 months of the initiation of therapy. Subsequently, Sah [2002] published a case report where the same treatment resulted in the spectacular increase in sperm number from $0,025 \times 10^6$ or $0,075 \times 10^6$/mL (40% motility and 15% normal morphology) in pre-treatment examinations to 35.0×10^6/mL (25% motility and 25% normal morphology) after 70 days of the initiation of therapy. Semen analysis was repeated just after therapy and revealed similar results up to 70 days in the post-treatment follow-up (sperm number was 20.0×10^6/mL with 60% motility and 70% normal morphology of spermatozoa). The patient's wife conceived within 5 months after completion of therapy and delivered a healthy child in term. Unfortunately, these studies were open and uncontrolled by a proper placebo group.

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ESTROGEN SOURCE AND ESTROGEN RECEPTORS IN THE MALE REPRODUCTIVE SYSTEM

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INTRODUCTION

The testis is not only the site of androgen biosynthesis but also is a target of androgen and estrogen action itself. It is generally accepted that spermatogenesis, the main testicular function, is an androgen-dependent process. Androgen metabolism may proceed to amplify the action of testosterone through its conversion to dihydrotestosterone or its modification by aromatization to estradiol. On the other hand, excess estrogens in the male may lead to a suppression of the expression of insulin-like 3 that is responsible for testicular descent and the excess estrogens may be a cause of a feedback inhibition of the hypothalamus-pituitary-testis axis, leading to hypoandrogenemia [Ivell and Hartung, 2003].

The absence of the androgen receptors from germ cells has led to the conclusion that testosterone either influences sperm cells indirectly, via effects on Sertoli cells in which androgen receptors are expressed, or some effects of testosterone may occur directly on the germinal cells after its aromatization to estrogens and subsequent estrogen interaction with estrogen receptors (ERs).

Based on recent literature and data of our studies dealing with the action of estrogens on the male genital tract, an attempt was undertaken to summarize the role of aromatase and estrogen receptors in mediating the action of estrogens within the male reproductive system, especially in seasonally breeding rodents, bank voles.

AROMATIZATION OF ANDROGENS INTO ESTROGENS

The enzyme aromatase (also known as estrogen synthase, P450arom, and EC 1.14.14.1) is best known for its ability to catalyze the transformation of androgens such as testosterone and androstendione into the estrogens: 17b-estradiol and estrone, respectively. Aromatase is a member of the P450 cytochrome family encoded by cyp19 gene. This gene was first cloned in humans [Simpson et al., 1994]. Later on, complementary DNA encoding cytochrome P450arom has been isolated and characterized from a broad range of vertebrates. The identities of the derived amino acids sequences of P450arom from rat, mouse, chicken, trout and bovine compared to the human sequence are 77%, 81%, 73%, 52% and 86%, respectively. The tissue-specific regulation of aromatase transcription is largely explained by the existence of tissue-specific promoters. The coding region begins with exon II in human P450arom. Upstream of exon II there is a number of alternative exon I sequences. Splicing of these untranslated exons to form the mature transcript occurs at a common 3'-splice junction that is upstream of the translational start site, producing the same protein in various tissues [Simpson et al., 2002].

The aromatization of testosterone to estradiol in the testis was first postulated based on studies investigating the precise site of aromatase within the male gonad [for review, see Carreau et al., 1999; O'Donnell et al., 2001]. Subsequent studies described the distribution of immunoreactive aromatase at a cellular level which frequently matches the presence of functionally active enzyme within the cells. Interestingly, age-related aromatase expression has been described in several species; in adult animals, the principal source of estrogens are Leydig cells, whereas Sertoli cells are implicated in testicular estrogen production in juvenile animals [Carreau et al., 1999]. During last decade, several studies have shown the presence of P450 aromatase in testicular germ cells of the rat [Janulis et al., 1996; 1998; Levallet et al., 1998] corroborating previous studies on mouse [Nitta et al., 1993], brown bear [Tsubota et al., 1993], and

rooster [Kwon et al., 1995]. On the other hand, in testes of horse, pig, and ram [Almadhidi et al., 1995; Conley et al., 1996; Bilinska et al., 1997] aromatase was localized only in Leydig cells. In humans, expression of aromatase has been shown in ovaries, testes, brain, liver, adipose tissue and skin, prostate and endometrial cancers as well as in myeloid leukemia cells [for review, see Kuiper et al., 1998].

ESTROGEN RECEPTORS AND THEIR ROLE IN THE MALE REPRODUCTIVE TISSUES

Maturation of the male reproductive system at the time of puberty results in an increase in gonadal steroid hormone secretion, spermatogenesis, and expression of male sexual behavior. The increased sensitivity to the actions of androgen and estrogen is due to the appearance of functional steroid-hormone receptors.

For almost 10 years it was thought that only a single form of the estrogen receptor (ER) existed. However, in 1996, several laboratories independently announced the discovery of a second type of ER in the rat, mouse, and human [Kuiper et al., 1996; Mosselman et al., 1996].

This newly discovered receptor was termed ERβ, resulting in the renaming of the classical, known since 1986, as ERα. The two receptors are not isoforms of each other but they are the proteins encoded by separate genes located on different chromosomes. ERβ is localized on human chromosome 14, in contrast to ERα, which is present on chromosome 6 [Enmark et al., 1997].

The ERs are classified as members of the superfamily of nuclear hormone receptors, defined as a ligand-inducible transcription factors. The ER proteins are each composed of six functional domains labeled A-F. The N-terminal A/B domain is the least conserved among all members (only 17% homology between the two human ERs). This domain contains the activation function 1 (AF-1) region. The C-

the E domain, being ligand binding domain, is modestly conserved (60% homology between the two human ERs). The D small domain, the hinge region, contains signals for nuclear localization of ERs (30% homology between the two human ERs). The E domain contains the major dimerization surface of the receptors, and the second transactivation function, the activation function 2 (AF-2). The F domain (C-terminal domain) is unique to ERs among nuclear receptors. It contributes to the transactivation capacity of the receptor, and demonstrates only 18% identity between the two human ERs (Fig. 1) [Kuiper et al., 1998; O'Donnell et al., 2001].

The inactive ER exists in a complex form consisting of several heat-shock proteins. In the presence of a ligand the proteins disassociate resulting in activation of the ER by its phosphorylation. Then, receptor dimerization occurs, nuclear translocation, DNA binding and interaction with several cofactors (coactivators or corepressors). Finally, modulation of transcription starts. The ER is predominantly nuclear protein regardless of whether or not it is complexed with the hormone.

The biological effects of estrogens (genomic or nongenomic) are mediated through at least four ER pathways (for review, see Luconi et al., 2002). First, the classical ligand-dependent activation of ERs. Second, estrogen response element (ERE)-independent. Third, the ligand-independent, and fourth, cell-surface-ligand-dependent signaling. Although the molecular mechanisms involved in ligand-independent activation of ERs have been characterized to some extent, the significance of these processes for biological actions of estrogens in not yet clear.

In cells expressing only ERα and ERβ, homodimers interact with cognate DNA response elements in target gene promotors. In cell expressing both subtypes, heterodimers can be formed in addition to the respective homodimers. It is possible, that DNA response elements could exist that interact preferentially with the heterodimers. Therefore, three
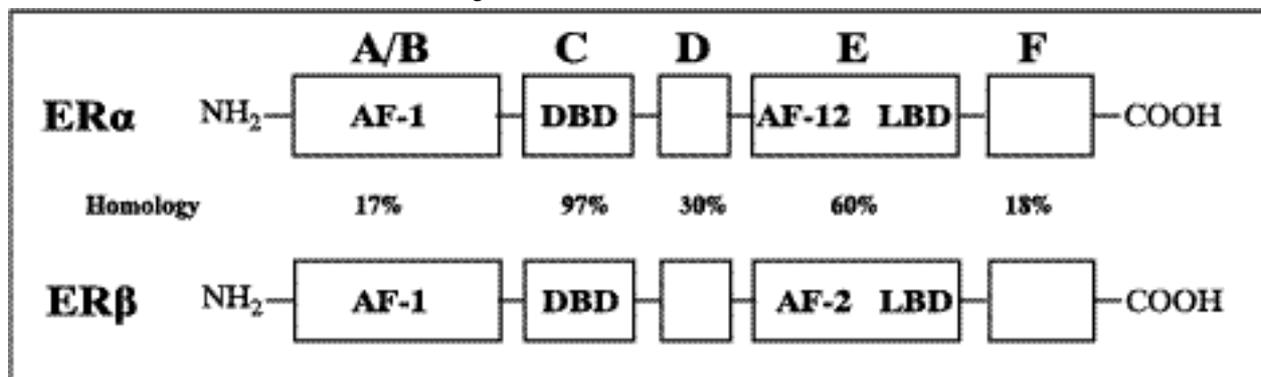
Figure 1: Comparison of the human estrogen receptor α and β proteins.

domain is the most conserved region (97%), being the DNA binding domain that contains the zinc-finger motifs, whereas

possible pathways of ER-mediated estrogen signaling are postulated. Many of the effects of estrogens on the

vasculature are mediated by classic nuclear receptors; for example, ERβ is expressed in smooth muscle cells *(Saunders et al., 1998)* but also very rapid effects on vasodilatory changes by estrogens are observed indicating that these latter effects can be mediated by membrane receptors *[Ruehlmann et al., 1998].*

There is a considerable tissue-specificity in the expression of ERα and ERβ. The ERα is mainly expressed in hypothalamus and pituitary, adipose tissue, mammary gland, uterus, cervix, vagina, kidneys, adrenals and skeletal muscles, whereas ERβ is the major receptor type present in the central nervous system, the cardiovascular system, the immune system, the urogenital tract and gastrointestinal tract, and the lungs, spleen and thymus *[Enmark et al., 1997; Kuiper et al., 1998].* The different distribution of ERα and ERβ has motivated the search for improved tissue-selective estrogen receptor modulators (SERMs). Such ligands could exert strong estrogen antagonist effects on certain tissue and estrogen agonist activity on others. In the clinical setting, these pharmaceuticals would be used for the prevention or treatment of menopausal symptoms, osteoporosis, cardiovascular disease, and breast cancer in women, or other estrogen-related conditions affecting either men or women *[for review, see Kuiper et al., 1998].*

Additionally, ERβ mRNA isoforms have been observed in humans and in a variety of mammalian species *[Scobie et al., 2002].* This may reflect either a possibility for remaining transcripts to be cloned, or between-species isoform specificity. The expression patterns of human ERβ isoforms have been determined mostly by RT-PCR and by the use of antibodies against the N- or C-terminus *[Saunders et al., 2002].* They reported that the pattern of expression of the two isoforms of ERβ in the human testis is distinct but overlapping. The wild-type ERβ1 and its variant ERβ2 are present in Sertoli cells and spermatogonia, although immunoexpression of ERβ2 appears to be more intense than that of ERβ1 in both cell types. On the contrary, in pachytene spermatocytes the expression of ERβ1 is more intense than that of ERβ2. In round spermatids only ERβ1 expression is observed, however from stage IV onwards the ERβ1 expression of these cells is lost. Saunders and her group demonstrated that the expression of ERβ2 in some cells may affect their ability to respond to endogenous or exogenous estrogens. In addition, they postulated that the expression of ERβ1 in pachytene spermatocytes and round spermatids points to estrogens having a direct impact on the survival and maturation of germ cells, being consistent with evidence from rodent studies. On the other hand, some of the isoforms share common elements between closely related species *[Lewandowski et al., 2002].* For instance, transcripts similar to human ERβcx are found in both, macaque and marmoset. In both, rat and mice, the ERβ2 isoforms have the same feature

of a novel insert in ligand binding domain between exons 5 and 6. In rodents, the binding of the ligand appears to be mostly affected in the isoforms. Therefore, it is possible that multiple ERβ mRNA isoforms, and in consequence, multitude of differentially built proteins may be considered as another level of complexity of estrogen signaling.

THE SITE OF AROMATASE ACTION AND THE DISTRIBUTION OF ESTROGEN RECEPTORS IN THE REPRODUCTIVE TRACT

Recent data on the distribution of the estrogen receptors and aromatase help in explaining the ability of testicular cells to mediate estrogen actions locally within the gonad, while studies on the distribution of aromatase in the testis have led to a new perspective on the function of this enzyme.

A growing body of evidence indicates that germ cells are responsible for estrogen formation by aromatase *[for review see Hess et al., 1997a; Carreau et al., 1999; O'Donnell et al., 2001; Carreau, 2003].* ERs and aromatase are found at all stages of testicular development in rodents. Based on immunohistochemical studies, the ERα is present in the mouse undifferentiated gonad, suggesting that estrogen may have a role in the differentiation process *[Fisher et al., 1997].* In fetal testis, Leydig cells contain ERα until birth. It should be stressed however, that during very early development androgen receptors are not yet expressed, suggesting a role for estrogen at this stage. ERα is also present in the developing efferent ductules and epididymis, whereas ERβ is expressed in the gonocytes, Sertoli and Leydig cells. However, the level of expression is much higher in the gonocytes when compared to other testicular cell types. It is possible, therefore that estrogens play a direct role in the gonocyte maturation. The fetal rat testis also possesses activity of aromatase which is firstly expressed by day 19. All together the existing data indicate that rodent fetal testis is able to synthesize estrogens and is also a target for these hormones. Moreover, early development of the male reproductive tract as well as the maturation of gonocytes are estrogen-dependent processes.

In the immature testis both ERs and aromatase are expressed. According to most authors, ERβ is expressed in the seminiferous tubules, in both, Sertoli cells and developing germ cells, whereas ERα is shown only in the interstitial area shortly after birth. During neonatal period, in Leydig cells of the rat and mice but not in the bank vole *[Bilinska et al., 2000]* ERα is expressed along with ERβ. The ERα and ERβ are also found in efferent ductules and the epididymis. In immature testis the expression of aromatase is shown in Sertoli and Leydig cels as well as in spermatogonia and primary spermatocytes In our earlier study Carpino et al. *[2001]*

indicated that Sertoli and germ cells from juvenile rat testes express immunoreactivity for aromatase, suggesting that these cells are an important source of estrogens. Further studies show that the pattern of aromatase expression is age-dependent.

In adult testis, the presence of aromatase has been reported by many researchers. It is worth mentioning that aromatase activity in the testis is higher in the adult than in any other age. The source of estrogens in rat testicular cells and, in particular, aromatase expression in purified adult rat germ cells was examined by the group of Carreau *[Levallet et al., 1998]* employing on several complimentary techniques, such as Western blot analysis, measurement of enzyme activity, immunolocalization of aromatase, and determination of P450arom mRNA content using a highly specific quantitative RT-PCR method *[for review Carreau et al., 1999; 2001]*. In testicular sections, a high degree of immunoreactivity is observed in Leydig cells and germ cells in the adluminal compartment of the seminiferous tubules, mainly in elongated spermatids, shortly before their release into the testicular lumen. With a higher magnification, a strong immunostaining for aromatase is localized in more mature germ cells within the lumen. Frequently, the cytoplasm of less mature spermatids is also immunopositive, thus providing strong evidence for the presence of P450 aromatase in the adluminal compartment of the rat testis, as previously reported in mouse, bear, and rooster. Moreover, the presence of a 55 kDa protein in mixed germ cell preparation was shown. The activity of functional P450arom was confirmed by the measurement of tritiated water released after incubation of purified germ cells with labeled androstendione, while using RT-PCR method, P450arom mRNA in Sertoli, Leydig, and germ cells was identified, which is consistent with the report of Janulis et al. *[1998]*. All the above data were described in detail and property illustrated *[Levallet et al., 1998]*. According to an earlier study by Janulis et al. *[1996]* the immunoreactivity for aromatase in rat spermatozoa, decreases along the epididymal tract from the caput to the cauda epididymis.

In sexually mature rodents ERα is expressed in Leydig cells, while in humans and primates the localization of ERα is controversial *[Saunders et al., 1998]*. There are some papers showing ERα expression in Leydig cells, while others could not detect any positive staining for the ERα *[Makinen et al., 2001; Pelletier and El-Alfy, 2000]*. The ERβ is mainly expressed in both, Sertoli and germ cells. The latter cells are also able to produce estrogens having a high activity of aromatase. According to Hess and his group *[1997b; 2001]* the highest expression of ERα is observed in the efferent ductules. Then, decreases throughout the caput, corpus to the cauda epididymis. The ERβ expression is also present in the ductus epididymis, however the pattern of expression is different. Immunoreactivity for ERβ increases from the caput to the cauda epididymis *[for review Luconi et al., 2002]*.

The ERβ seems to be a most important factor in the mechanism of estrogen action; it has biological roles that are distinct from those of ERα. Recently, deficient in ERβ (βERKO) knockout mice have been produced (see, next paragraph) and they differ from those with ERα deficiency (αERKO). Predominant expression of ERβ is detected in the urogenital tract. Both, the transitional epithelium in the bladder and the urethra, the seminal vesicles, the prostate and kidney pelvis express significant quantities of ERβ mRNA *[Hess et al., 1997b]*. The expression of ERα is much less pronounced. The high concentration of estradiol in the fluid of rat rete testis *(Hess et al., 1997b)* and the presence of estrogen receptors in the epididymis are likely related to an important role for estrogens in the male genital tract. The presence of the ERs was confirmed in human and rat epididymides *[Kolasa et al., 2003; Carpino et al., 2004a]*, while the abundance of ERβ was shown in porcine epididymis *[Carpino et al., 2004b]*.

Differential distribution pattern of the ERs suggests that estrogens may play the role in the regulation of spermatogenesis as well as in the modulation of Leydig cell function.

Apart from our study on rodent testicular cells, expression of androgen receptors, aromatase, and distribution of ERs has recently been reported in testicular,
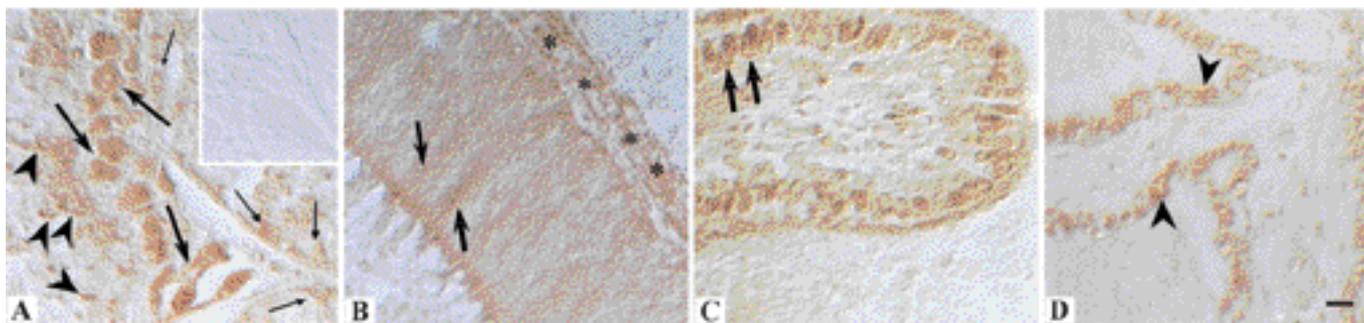

Figure 2: ERβ expression in the testis (A), corpus and cauda epididymis (B and C), and prostate (D) of the stallion. Insert represents negative control. Leydig cells – large arrows, Sertoli cells – small arrows, germ cells – arrowheads, principal epididymal cells - bold arrows, connective tissue cells – asterisks, epithelial cells of the prostate – long arrowheads. Bar represents 10 µm

epididimal, and prostatic cells of the stallion *[Bilinska et al., 2004; 2005; Hejmej et al., 2005] (Fig. 2)*. This indicates that besides androgens, the estrogens are necessary for sperm maturation, and, possibly for their storage. It should be stressed additionally that in equine testes the staining for aromatase was always stronger in the cells of the cryptorchid horse in comparison to those of the stallion. Therefore, it seems likely that naturally occurring cryptorchidism in the horse may be associated with the increase in conversion of androgens to estrogens not only in the testis but also in the epididimal duct and the prostate. This is in agreement with previous studies of our own showing the increase in conversion of androgens to estrogens in testes of cryptorchid mice *[Bilinska et al., 2003]*, mice with a partial deletion in the long arm of Y chromosome *[Kotula-Balak et al., 2004a]*, and in patients with Klinefelter's syndrome *[Kotula-Balak et al., 2004b]*. Very interesting results have been obtained by Li et al. *[2001]* using transgenic mice with aromatase overexpression. In these mice an imbalance in sex hormone biosynthesis provoked distinct differences in the morphology of the testis, similar to those observed in the cryptorchid males.

All these data support the concept that estrogens play an important role in modulating the function of the male reproductive system across the species.

MICE MODELS WITH TARGETED DISRUPTION OF AROMATASE OR ESTROGEN RECEPTOR GENES

Although, a direct effect of estrogens on male reproduction has not yet been completely understood, data obtained from rodents clearly show that estrogens are important to maintain reproductive functions in the male *[for review Carreau et al., 2001; 2003; Hess et al., 2001; O'Donnell et al., 2001; Luconi et al., 2002; Cho et al., 2003]*.

To elucidate the role of estrogens in males, several experimental models have been used. Besides the use of knockout mice, ERKO and ARKO *[for review Couse and Korach, 1999]*, treatment with either aromatase inhibitors *[Steele et al., 1987]* or the pure steroidal anti-estrogen ICI 182,780 have been applied *[Lee et al., 2000; Oliveira et al., 2001, 2002, Gancarczyk et al., 2004]*.

The generation of aromatase deficient mice (ARKO) has been described by three groups independently *[Fisher et al., 1998; Robertson et al., 1999; Toda et al., 2001]*. In general, the phenotypes of the different mice appear to be similar. The authors reported that ARKO males are initially fully fertile but their fertility decreases with advancing age with a progressive disruption of spermatogenesis caused by abnormal germ cell development. Stereological analysis showed that sperm from ARKO have a significant reduction in motility. This is associated with Leydig cell hyperplasia, while the number of Sertoli cells is essentially normal. Moreover, ARKO male mice, show impaired sexual behavior *[Toda et al., 2001]*.

The ERa knockout mice were termed aERKO by the Korach group, whereas ERb knockout mice were termed bERKO by the Gustafsson group. The aERKO male mice undergo normal fetal development of both external and internal structures of the reproductive tract *[Eddy et al., 1996]*, while the adult males exhibit several abnormalities and deficiencies that result in infertility. According to Hess et al. *[2000]*, the infertility of the aERKO has been shown to be a result of impaired fluid reabsorption in the efferent ductules. The testicular phenotype of these mice is characterized by seminiferous tubule atrophy and several defects in sperm morphology. Mahato and co-workers *[2001]* showed in aERKO mice that germ cells without ERα can not develop normally. Additionally, it was found that somatic cells of the male reproductive tract require ERa to support production of fertile sperm. In the aERKO model morphological changes in the ventral prostate were also observed *[Eddy et al., 1996; Hess et al., 2001]*. The lumina were enlarged and the glandular epithelium flattened. Moreover, in the aERKO, a significant decrease in the weight of the epididymis and vas deferens were noticed, whereas the seminal vesicles and coagulating glands appeared normal in size.

On the other hand, bERKO male mice that have been generated by Krege et al. *[1998]* appeared to be morphologically normal, and were fully fertile. The testes of bERKO mice are of normal size with no signs of hyperplasia in the prostate lobes. This can suggest that ERb is not essential for normal reproductive function in the male. According to current models the lack of spermatogenic disruption in bERKO mice might be explained by compensation of ERb by the ERa or by nongenomic action of estrogens in the testis. Double ERKO mice share some of the phenotypic characteristics of both aERKO and bERKO mice *[for review O'Donnell et al., 2001]*.

Estrogen sulfotransferase knockout mice (ESTKO) have been developed through targeted disruption of the EST gene. Age-dependent structural and functional alterations were observed in such testes *[Qian et al., 2001]*. The most prominent abnormality is the presence of numerous hypertrophic or hyperplastic Leydig cells, as well as seminiferous tubule damage.

Transgenic mice that overexpress P450arom have been reported by Tekmal et al. *[1996]*. In these mice 50% of them were infertile and the testes were larger than normal in size and frequently, Leydig cell tumors were present.

In a series of experiments, using the anti-estrogen ICI 182,780 to block the effects of endogenous estrogens via ERα or ERb, Hess and co-workers confirmed the hypothesis that

estradiol is an essential hormone for normal male reproduction [for review Hess, 2003]. According to Lee et al. [2000] treatment with ICI 182,780 generates alterations in the mouse reproductive tract that are similar to those found in αERKO mice. Long-term treatment with ICI as reported by Oliveira et al. [2001] results in alterations in the structure and function of the male reproductive tract, including an increase in testicedar weight, luminal dilation of seminiferous tubules, testicular atrophy, and finally, infertility of the rat. In our study, treatment of immature bank voles with estradiol or ICI 182,780 during a two-week-period resulted in alterations of testicular structure that can be explained by the sensitivity of bank voles to estrogens and anti-estrogens especially after exposure to short light cycles [Gancarczyk et al., 2004]. It should be noted that variable sensitivity to estrogens and anti-estrogens has also been found in other species [Spearow et al., 1999].

In humans, it has been shown that the aromatase deficiency (6 cases reported) as well as lack of ERa expression (1 clinical case reported) due to naturally-occurring mutations lead to infertility and decreased sperm number and viability [for review Faustini-Fustini et al., 1999].

BANK VOLES AS A USEFUL MODEL FOR STUDYING THE ROLE OF ESTROGENS IN THE MALE GONAD

In 1988, Kula [1988] suggested for the first time that estradiol plays a stimulatory role in proliferation of spermatogonia during the initiation of spermatogenesis in the rat. In further studies, *Walczak and Kula [1999] and Kula et al. [2001]* confirmed the important role of estradiol in germ cell development that acts through the enhancement of the stimulatory effect of FSH on testicular maturation and contributes to the precocious initiation of spermatogenesis. *In 2000, Ebling et al. [2000]* evoked complete spermatogenesis by administering continuous treatment with 17 beta-E_2 in inherited hypogonadal mouse due to targeted disruption of GnRH encoding gene. *Pentikäinen et al. [2000]* demonstrated that E_2 acts as a germ cell survival factor in the human testis in vitro. It is also important to consider the recent work of *Li et al. (2001)* showing that multiplication of rat fetal germ cells gonocytes in vitro is, in part, under E_2 control. *Saunders et al. [2001]* showed the expression of ERβ in rat gonocytes, while *Chieffi et al. [2002]* confirmed the role of E_2 in inducing proliferation of spermatogonia in the lizard.

Since we found aromatase expression in rat and mouse testicular cells, we extended our experiments to seasonally breeding rodents, the bank voles. It was of interest to show whether the aromatization is dependent on the length of light/dark cycles [Bilinska et al., 2000; 2001; Kotula-Balak et al., 2003; Gancarczyk et al., 2005, in press].

In seasonal breeders, terminal differentiation of the gonads can be retarded or even stopped by changes in the photoperiod [Clarke, 1977]. Although it is generally accepted that gonadal function is under the hypothalamic-pituitary-testicular axis, the actions of gonadotropins within the gonads are additionally modulated by other hormones and factors. There is evidence that amongst field voles from laboratory stocks there are strains which are more or less susceptible to the retarding effects of short photoperiods upon sexual development [Clarke, 1985]. Bank voles seem to be very sensitive to a short photoperiod. Thus, they are a good model for studying the functional a role of estrogens in the physiological recrudescence of testis and especially spermatogenesis. Moreover, the reproductive cycle of the bank voles can be easily mimicked under laboratory conditions by subjecting the animals to different light cycle regimes. According to Craven and Clarke [1982], the inhibitory effect of a short photoperiod on testicular androgen biosynthesis in a wild population of voles could be explained by an increase in secretion of melatonin by pineal gland which modifies the hypothalamic-pituitary-testicular axis, controlling, therefore, functions of the gonads. Melatonin inhibition of GnRH release brings about a decrease in plasma LH, FSH and PRL levels and subsequent decrease in LH receptors. During the short light regime, a decrease in the levels of gonadotropins lowers production of testosterone together with a decrease in the number of its own receptors [Tähkä and Rajaniemi,1985; Tähkä et al., 1997]. Recently, it has been confirmed that inhibition of testosterone biosynthesis takes place at two different stages which seem to be the most sensitive to various light regimes. First, the cAMP-dependent transport of cholesterol through the inner mitochondrial membrane, presumably at the level of StAR protein, and second, the expression and activity of cytochrome P450 enzymes [Stocco et al., 1998]. Kruczek [1986] also showed the seasonal effect on sexual maturation on female bank voles, while, very recently, *Galas et al. [2005]* reported an inhibitory effect of photoperiod on cultured granulosa cells of the bank vole females reflected by differences in progesterone secretion between long and short day control and hormone-treated cultures. In our earlier study, similar inhibitory effects of photoperiod reflected by a low aromatase immunoreactivity and a decrease in androgen and estrogen secretion was also noticed in bank vole Leydig cells in vitro [Gancarczyk et al., 2003], both, in control and hormone-stimulated cultures. Moreover, the length of light regimes appeared to have a profound influence on aromatization and antioxidant capacity of LH, IGF-I, and prolactin- stimulated bank vole testicular cells [Gancarczyk et al., 2005, in press].

In young bank voles that were reared under short light cycles immunostaining for aromatase is confined to Leydig cells. In males that were kept under long day conditions germ

cells in seminiferous tubules were also positively stained.

In mature bank voles the strongly positive immunostaining for aromatase is also found in seminiferous tubules, and particularly in spermatocytes and spermatids. These results corroborate our previous studies showing aromatase expression and immunolocalization in various germ cells of adult rat *[Levallet et al., 1998]*, inside cytoplasmic droplets of bank vole elongated spermatids *[Kotula-Balak et al., 2003]*, and in human ejaculated spermatozoa *[Rago et al., 2003; Lambard et al., 2003]*. The latter authors suggest that aromatase may be linked with the induction of sperm motility. By and large the localization of aromatase in testicular cells suggests a possible role for estrogens in the testis.

As mentioned above, the effect of E_2 supplementation or its deprivation on the induction or disruption of spermatogenesis has been studied in bank voles that were kept in different light regimes *[Gancarczyk et al., 2004]*. A low dose of E_2 caused acceleration of the onset of spermatogenesis in immature males, especially in those submitted to short light cycles, whereas a high dose of E_2 or of the anti-estrogen ICI 182,780 induced defects in testicular structure, an increase in the number of apoptotic germ cells, and impaired reproductive function *(Fig. 3)*. It is interesting that the positive role of E_2 in the recrudescence of spermatogenesis was earlier reported in the seasonal breeders, the ground squirrel and Siberian hamster *[Pudney et al.,1985; Pak et al., 2002]*. Dose-dependent effects of E_2 on spermatogenesis have also been shown in a series of experiments by the group of Sharpe *[Atanassova et al., 1999, 2000]*.

In order to improve our understanding of estrogen action within the testis and the putative roles of estrogens in the bank vole gonad, immunohistochemical detection of ERα and ERβ was performed. Our results indicate that Sertoli and germ cells of this animal exhibit not only immunoreactivity for aromatase but also for ERβ, whereas immunolocalization for ERα is restricted to Leydig cells as it was also shown in human and rat Leydig cells *[Saunders et al., 1998]*. Thus, it appears that ERα and ERβ positive staining is co-expressed with immunoreactivity for aromatase. More precisely, the distribution pattern of aromatase is roughly similar to the sum of ERα and ERβ. Sections of the bank vole testis that had been double labeled for aromatase and ERβ revealed that 70-80% of the aromatase immunopositive cells were also positive for ERβ *[Bilinska et al., 2000; 2001]*. In other studies on the rat *[Van Pelt et al., 1998]*, the high testicular expression of ERβ was also found in Sertoli cells and germ cells. Therefore, the ERβ is suggested to play a major role in mediating estrogen action in the regulation of spermatogenesis. On the other hand, the immunoexpression of ERα only in Leydig cells may suggest that estrogens exert an autocrine action mediated by ERα on Leydig cell function. Irrespective of age and length of the day, Leydig cells and peritubular-myoid cells did not express any positive
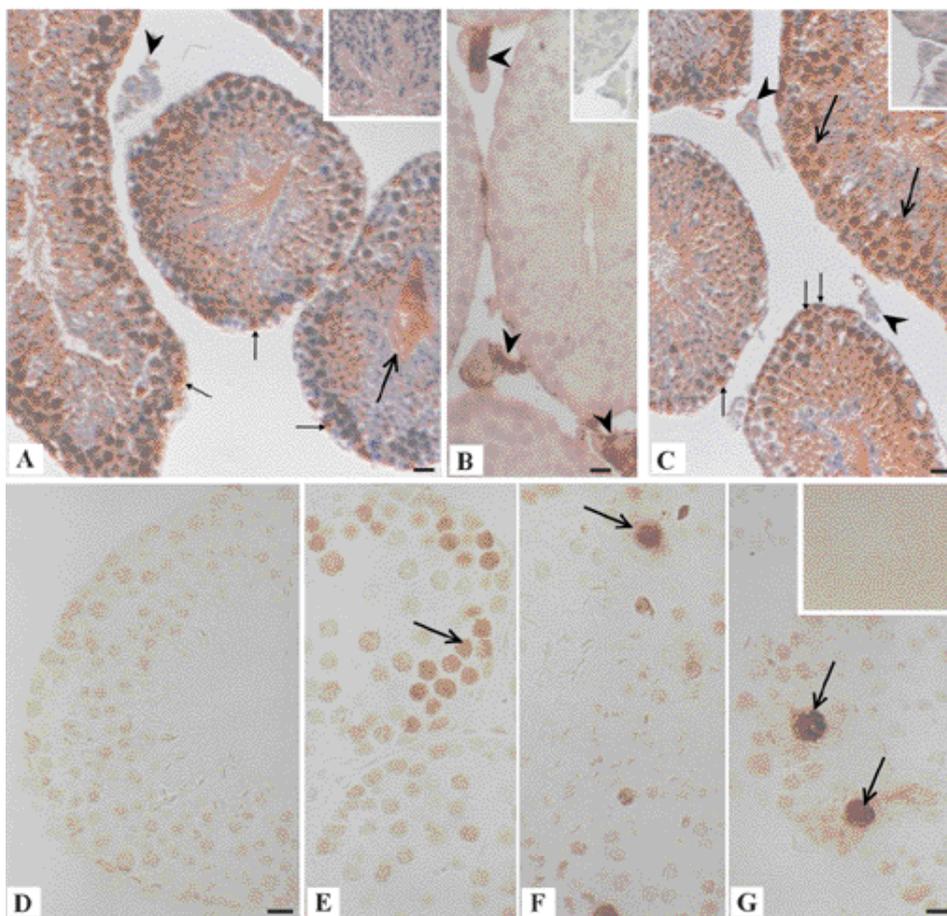
Figure 3: Bank vole testicular sections (18L:6D), (A-F). Immunoexpression of aromatase (A), ERα (B), and ERβ (C). Inserts represent respective negative controls. Leydig cells – large arrows, Sertoli cells – small arrows, germ cells – arrowheads. Bars represent 10 µm.
Visualization of apoptotic germ cells by the TUNEL method (D-F). No apoptotic cells visible after a low dose of estradiol (D) Apoptotic germ cells after a high dose of estradiol (E-G). Insert represents control reaction. Bar represents 10 µm

immunoreactivity for ERβ. Therefore, the presence of ERβ in male germ cells agrees well with the concept that these cells represent a target for estrogens.

The widespread expression of aromatase and ERβ in testicular cells of the bank vole seems to be consistent with a proposed role for estrogens in regulation of spermatogenesis [Kula, 1988; Sharpe, 1997; Walczak and Kula, 1999; Kuiper et al., 1998; Ebling et al., 2000; Kula et al., 2001; for review Carreau et al., 1999; O'Donnell et al., 2001; Luconi et al., 2002]. The expression of ERβ in various stages of germ cells as well as in Sertoli, but not in Leydig cells, suggests that estrogens directly affect germ cells during testicular development and spermatogenesis. This is in agreement with earlier observations of the group of Hess et al. [2001; 2003] and Van Pelt et al. [1998] on rat testicular cells. More precisely, the latter authors observed the age-dependent expression of ERβ in rat Sertoli cells and A type of spermatogonia, however, they did not find any positive signal in other types of germ cells. Thus, the findings regarding the localization of ERβ in bank vole testicular cells are partly in contradiction with ERβ expression in fetal, immature and adult rat testicular cells.

According to Wilson and McPhaul [1996] ERβ immunolocalization closely resembles that of ARs. This agrees well with our observation that Sertoli cells from sexually mature bank voles kept under long light cycles are immunopositive for ARs, aromatase, and ERβ. It is a remarkable coincidence that androgen receptors are not only expressed in the majority of sites at which ERs are expressed (germ cells are a notable exception) but also aromatase expression appears at many of the same sites. It can be envisaged, therefore, that the local balance between estrogen and androgen action could be finally regulated. Moreover, there are some interesting data which suggest that the balance between androgen and estrogen action is of special importance [for review Sharpe, 1998]. As it was mentioned above, developmental abnormalities of the male reproductive system can be induced by exposure to either an anti-androgen or an estrogen. Also, gynaecomastia in males can be caused by either too much estrogen or too little androgen [Bulun et al., 1997].

Androgen and estrogen action has also been studied in the male urogenital tract. Many cell types in this tract possess both, ARs and ERs. Data of Goyal et al. [1998] provide strong evidence that not only androgens but also estrogens play an important role in modulating the function of the epithelial cells from goat efferent ductules. It corroborates earlier studies showing that estrogens are important for the maintenance of structural and functional integrity of the male reproductive tract, since the epithelium of the efferent ductules has been found to contain higher levels of ERs than other parts of the reproductive tract. The current data from the literature and the results of our own work support the concept that estrogens play an important role in modulating the function of the male gonad, especially germ cells development, as well as sperm maturation in the epididymis. The fact that elongating spermatids contain aromatase before and during their passage through the efferent ductules, together with the high levels of ER expression within these ductules, suggest that estrogen produced by the sperm may act on these tissues to mediate estrogen-dependent target genes [Hess et al., 2000].

It is clear now that in addition to the traditionally known role of androgen in mediation of testicular function, estrogen is also involved in its regulation. The extensive distribution of ERα and ERβ on other sites of the male reproductive system and throughout the body makes it possible that new and unexpected functions may be attributed to the "female hormone" in the male.

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XENOESTROGENS AND MALE REPRODUCTION

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WHY XENOESTROGENS?

Over the last 50 years a rapid increase in the prevalence of male reproductive disorders is observed. The environmental and life-style changes associated with developing industry and agriculture are considered to be responsible for that. In Poland the incidence of testicular germ cell tumours (GCT) is about 6-times higher in industrially developed regions in comparison to ecologically unpolluted areas *[Didkowska et al., 2002]*. Some of the authors have pointed at the detrimental effect of the exposure to synthetic progestins used since 1960s in pregnant women with the high miscarriage risk, as contraceptives or as a part of chemicals used in pregnancy tests *[Goldman and Bongiovanni, 1967; Czeizel et al., 1985]*. However, a meta-analysis performed by *Raman-Wilms [1995]* found no association between such exposure and male external genitalia malformations.

A hypothesis emerged that the exposure to environmental endocrine disrupting chemicals (xenobiotics) may be a reason for the increasing frequency of reproductive abnormalities *[Skakkebaek et al., 1998; Andersson and Skakkebaek, 1999; McLachlan, 2001]*. This hypothesis is confirmed by the growing number of reports on the male reproductive system anomalies in wild living animals, including snails, fish, amphibians, birds and mammals *[Bryan et al., 1989; Willingham and Crews, 1999; Oehlmann et al., 2000; Vos et al., 2000]*. The fact that reproductive problems are seen even in polar bears indicate that contamination with endocrine disrupters may be a global problem *[Vos et al., 2000]*. Most of the xenobiotics which harmfully influence the male reproductive system exert at least partly the action of natural estrogens and thus they are called xenoestrogens *[Toppari et al., 1995; Toppari et al., 1996; Blair et al., 2000]*.

PREVALENCE OF REPRODUCTIVE SYSTEM ANOMALIES IN MEN

An increase in the incidence of male reproductive system anomalies has been shown in several reports from different countries after the Second World War. The real existence of the problems with the health of male reproductive system is strengthen by demonstrated reports that not only the frequency of congenital anomalies, but also the related disturbances such as cryptorchidism, epididymal anomalies, testicular germ cell tumours (GCT) and the decrease in the number of sperms in ejaculate, appeared to be rising at the same time *[Carlsen et al., 1992; Skakkebaek et al., 2001; Skakkebaek et al., 2003]*.

Skakkebaek et al. [2001] have suggested that the spectrum of male reproductive problems may have the same etiology. They proposed the existence of a new clinical syndrome – the testicular dysgenesis syndrome (TDS), which comprises the wide range of developmental retardations of the testes, including disturbed organogenesis (dysgenesis) and cryptorchidism. The authors proposed that both male infertility (oligo- and azoospermia) and testicular GCT result from TDS. The justification is that in all of these disturbances immature seminal tubules with undifferentiated Sertoli cells, Sertoli-cell-only tubules, intratubular microliths and tubules containing *carcinoma in situ* (CIS) were detected. The structural lesions of the testes suggest that the function of both Sertoli and Leydig cells is impaired and are involved in reproductive system anomalies both during fetal life and in adulthood. Moreover, disturbed internal gonadal milieu may promote neoplastic changes of germ cells. All the disorders included in TDS are risk factors for GCT development. The coexistence of intersex condition with GCT has been reported since years but only scarce clinical material was available. In our own study, which included 40 patients with gonadal

dysgenesis and 6 patients with true hermaphroditism CIS was detected in 55% of the cases with gonadal dysgenesis, 10% of the cases with androgen insensitivity, 17% of the cases with unilateral GCT and 33% of the patients with undescended testes [Slowikowska-Hilczer et al., 2001]. The incidence of neoplastic lesions was the highest in patients with gonadal dysgenesis (65%). Among them the highest risk occurred in patients with partial (91%) and mixed gonadal dysgenesis (77%) [Slowikowska-Hilczer et al., 2003], and less frequently in gonads with pure gonadal dysgenesis (- 23%). This indicates that germ cell neoplastic changes appear in a milieu of relatively well-developed testis structure. Testis structure by itself predisposes to the initiation of germ cell neoplasia. In our other epidemiological approach, the presence of neoplastic changes of germ cells appeared to be independent on the structural and numerical aberrations of sex chromosomes which were generally considered to be a cause of gonadal dysgenesis and GCT. It is observed that the risk of GCT is higher in brothers than in sons of the patients with GCT which indicates less genetic but more environmental influences [Rapley et al., 2000; Rapley et al., 2003].

ENDOCRINE DISRUPTING CHEMICALS WITH ESTROGENIC EFFECTS – XENOESTROGENS

Xenoestrogens may in some aspects mimic the action of natural sex hormone 17b-estradiol and bind to estrogen receptor (ER), despite their different chemical structure. There is a growing number of evidence that estrogens play a role in the development and physiology of male reproductive tract [for review Korach, 1994; Carreau et al., 2003]. Estrogen may play a regulatory role for the initiation of spermatogenesis as a stimulatory factor on spermatogonia multiplication and as a factor which enhances FSH effects on the initiation of spermatogenesis [Kula, 1988; Ebling et al., 2000; Kula et al., 2001; Walczak-Jedrzejowska et al., 2005]. Recently, the presence of functional ER has been demonstrated in differentiating male external genitalia, which indicates a possible novel role of estrogens in the regulation of the development of these sex structures [Jesmin et al., 2002; Crescioli et al., 2003]. It has been established that estrogens influence growth and maturation of bones, and that they are necessary in achieving epiphyseal closure and peak bone mass in the human male [Carani et al., 1997; Faustini-Faustini et al., 1999; Rochira et al., 2001]. Estrogens have effects throughout the brain. Regulation of the serotonergic system appears to be linked to the presence of estrogen-sensitive neurons in the midbrain raphe, whereas estrogens action on the cholinergic function involves induction of choline acetyltransferase and acetylcholinesterase according to a sexually dimorphic pattern. During the period of development, when testosterone is elevated in the male, aromatase and ERs are transiently expressed in the hippocampus, which strongly suggests that this pathway is involved in the masculinization of hippocampal structure and function [for review McEwen and Alves, 1999]. Moreover endogenous estrogens may have a positive [Grumbach and Auchus, 1999] as well as a negative influence on the circulatory system in men [Wranicz et al., 2005].

There is evidence that environmental estrogen-like substances, may lead to many undesirable symptoms both in male and female. The wide phylogenetic distribution of estradiol production and the estrogen signal recognition system in the animal kingdom suggests the possibility that all animals are sensitive to estrogens, whether exopenous or environmental [for review McLachlan, 2001]. In many vertebrate species both ERa and ERb have been found. The third isoform ERg has been cloned in year 2000 from the teleost fish Micropogonias undulates [Hawkins et al., 2000]. The variability in ER isoforms and tissue specific distribution gives the opportunity to the xenoestrogens to exert different actions on the organisms. Nevertheless, although ER has not been found in invertebrate species, there is evidence that estrogen-like chemicals can alter their metabolism and development. The explanation for this can be the existence of a large and still growing, number of receptors with unknown function or ligands, which are called orphan receptors. One of them is steroid/xenobiotic receptor (SXR) which recognizes many xenobiotics and activates different responses [McLachlan, 2001].

Pesticides

An example of the reproductive abnormalities, possibly the results from the xenoestrogen action on the wild living animals, is the demasculinization of alligators in Lake Apopka in Florida [Guillette et al.,1994; 1999]. These reptiles have decreased phallus size, abnormal gonads and altered sex hormone levels possibly due to the endocrine-disrupting effects of DDT (dichloro-difenylo-trichloroethan) and the related organochlorine pesticides. DDT is the first and the most known pesticide, which can act as an estrogen agonist [Welch and Conney, 1969] or anti-androgen [Laws et al., 1995]. It was discovered in 1939 by Paul Müller as a very effective insecticide. It quickly became the most widely used pesticide in the world. However, in the 1960s, it was discovered that DDT caused birth defects in animals and humans. DDT is now banned in many countries, but it is still used in some developing regions to prevent malaria and other tropical diseases by killing mosquitos and other disease-carrying insects. In North-Eastern Brazil, one of the poorest regions, where 50% of the population lives in favellas, mosquito and rodent reproduction is extended. DDT and other insecticides are widely used by the inhabitants, in addition to broad agricultural pesticide application. The incidence of genital malformations in male newborns is

dramatically high in this region – 94/10000/year *[Sampaio et al., 2005]*. Pesticide use has been increased 50-fold since 1950 and 2.5 mln tons of industrial pesticides are now used each year. The most widely used pesticides today are nonpersistent organophosphates, including glyphosphate, which is currently the world's most used herbicide. The pesticides shown to have estrogenic or anti-androgenic properties include chlordecone, dieldrin, vinclozin, endosulfan, toxaphene and linuron *[LeBlanc et al., 1997]*.

Several investigators have shown striking differences in semen quality, GCT rate, cryptorchidism and hypospadias between Denmark and Finland *[Suominen and Vierula, 1993; Adami et al., 1994; Skakkebaek and Keiding 1994; Boisen et al., 2004]*. It has been demonstrated that the concentrations of pesticides in the human breast milk were significantly lower in Finland than in Denmark, suggesting that Danish children are more exposed compared to Finnish children *[Damgaard et al., 2005]*. DDT and some other pesticides are slowly biodegradated, so they can be accumulated in the organisms and influence upon the next generations. Developmental abnormalities may be the result from *in ovo* or *utero* exposure, presumably of maternal origin *[Milnes et al., 2005]*.

Industrial xenoestrogens

Not only pesticides but also different chemicals used in everyday practice and environmental pollutants have been shown to possess estrogen-like bioactivity. They are detergents, organic solvents, paints, combustion products and heavy metals. Bisphenol A, a monomer of polycarbonate plastics and constituent of epoxy and polystyrene resins is widely used in the food packaging industry and dentistry *[Ben-Jonathan and Steinmetz, 1998]*. Detectable amounts of bisphenol A were found in food cans and human saliva after the treatment with dental sealants *[Brotons et al., 1995; Olea et al., 1996]*. Octylphenol, a constituent of alkylphenol polyethoxylates is used as surfactant in detergents, paints and herbicides [Purdom et al., 1994]. Alkylphenols are degradated in plants to stable products, can accumulate within internal organs of fish and birds and thus pass through the food chain to humans. Bisphenol A and octylphenol can act as weak estrogens and influence the reproductive health *[Kuiper et al., 1997]*.

Numerous published reports have linked exposure to heavy metals such as lead, cadmium and mercury with male infertility. Battery workers intoxicated with these metals have defects of spermatogenesis *[Chia et al., 1992]*. It has been found that the exposure to inorganic lead produced inhibition of intratesticular testosterone synthesis in both animals and men *[Rodamilans et al., 1988]*. Treatment with lead and cadmium in animals was associated with the increase in apoptosis of spermatogenic cells *[Awara et al., 2001; Millan et al., 2001]*. Recently, it has been suggested that cadmium is a new environmental estrogen because it can mimic some effects of estradiol *[Garcia-Morales et al., 1994; Stoica et al., 2000]*.

Medical xenoestrogens

Diethylstilbestrol (DES), is a synthetic potent estrogen, which has been widely used in medicine for postcoital contraception, maintenance of high risk pregnancy with imminent miscarriage, suppression of lactation, estrogen replacement therapy and prostate cancer therapy. In agriculture DES was used to stimulate growth of chicken and cattle. DES and its metabolites were present in the meat and excreted to ecosystem. DES has a strong estrogenic effect exposure *in utero* to DES resulted in increased prevalence of male urogenital tract abnormalities, cryptorchidism, GCT and reduced fertility in men *[Gill et al., 1979; Driscoll and Taylor, 1980; Newbold and MacLaclan, 1996; McKinnell et al., 2001]*.

There are also some studies on the environmental contamination with the estrogenic components of commonly used oral contraceptive 17a-ethinyl estradiol *[Belfroid et al., 1999; Lagana et al., 2000]*. Estrogens and their glucuronides have been found in municipal sewage, surface and waste waters in industrial regions.

Neonatal treatment of male rats with high doses of DES or ethinyl estradiol induces a range of developmental abnormalitie such as inhibition of Sertoli cell proliferation *[Atanassova et al., 1999]*, suppression of Leydig cell development and function *[Sharpe et al., 2003]*, abnormal development of the rete testis *[McKinnell et al., 2001]*, efferent ducts *[Fisher et al., 1999]*, epididymis and vas deferens *[Atanassova et al., 2001; McKinnell et al., 2001]*, seminal vesicles *[Williams et al., 2001]* and prostate *[Prins and Birch, 1995; Prins et al., 2001]*. Additionally, DES suppresses expression of the androgen receptor *[McKinnell et al., 2001]*.

Phytoestrogens

Chemicals with some estrogenic activity are present in the environment not only as pollutants, resulting from the developing civilisation, but also as chemicals naturally produced by plants. Some of them are used in the traditional medicine and kitchen, mainly in Asia. Plant compounds with estrogen-like biological activity are called phytoestrogens. There are three main phytoestrogen classes: isoflavones, coumestans and lignans. Genistein, which belongs to the isoflavones group, is of the greatest interest at present. Isoflavones are found in fruits and vegetables, but predominantly in leguminous plants and are especially abundant in soybean. Phytoestrogens may exert both estrogenic and anti-estrogenic effects on metabolism,

depending on several factors, including their concentration, the concentrations of endogenous estrogens and individual characteristics such as gender and reproductive status in women [Adlercreutz and Mazur, 1997].

It was found that isoflavones exert some beneficial effects: 1. inhibit tumor cell proliferation, angiogenesis and cell cycle progression, 2. decrease of total cholesterol and LDL cholesterol, increase of HDL cholesterol and improve endothelial function, 3. increase bone mineral density and 4. decrease frequency of hot flushes [Murkies et al., 1998; Tham et al., 1998]. Currently the increase in plant-based foods consumption, especially soy products, is recommended to increase fibre and antioxidant intakes, replacing sources of saturated fat and cholesterol in the diet.

Nevertheless, phytoestrogens can exert also adverse effects, on male and female reproductive system. These effects were first observed in sheep, which became infertile during feeding with subterranean clover [Bennetts et al., 1946]. It was found that chronic exposure of spermatozoa to high doses of genistein was associated with infertility problems through suppression/inhibition of acrosome reaction and sperm motility [Kumi-Diaka and Townsend, 2001]. Paris et al. [2005] have demonstrated for the first time that phytoestrogens (among others genistein) exhibit some antiandrogenic action.

Mycoestrogens

The dietary estrogens originate not only from plants and meat of animals treated with hormones but also from food contamination with moulds. Consumption of cereal contaminated with *Fusarium sp* has been associated with estrogenic effects in poultry and livestock [Meronuck et al., 1970; Roine et al., 1971]. Zearalenone, a fungal mycotoxin produced by Fusarium, binds to ER [Powell-Jones et al., 1981]. Recently, it was demonstrated that mycoestrogens (zearalenone and its metabolites) are nearly as potent as flutamide, an antiandrogenic medication [Paris et al., 2005]. These indicate that prenatal exposure to phyto/mycoestrogens via vegetarian diet during pregnancy may alter male fetal sex differentiation and further reproductive ability.

MECHANISMS OF XENOESTROGENS' ACTION
Receptor binding

Endocrine disrupting chemicals can bind to the tissue receptor sites of the specific hormone and act as hormone agonists or antagonists. The receptor may behave as a signal integration unit and collect information from growth factors, other nuclear receptors and series of chaperone and co-regulator proteins [for review McLachlan, 2001]. The response depends on the convergence of activating ligands and cellular signals, the multiple receptor isoforms, contributions of co-activators and co-repressors [Muramatsu and Inoue, 2000]. Despite their different chemical structure xenobiotics can influence the same receptors. For instance, cadmium has been demonstrated to activate ERa by interacting with the ligand-binding domain of the receptor [Stoica et al., 2001].

Changes in hormonal metabolism

Xenobiotics can alter hormone homeostasis by interfering with hormone biosynthesis, metabolism and elimination. It was demonstrated that chronic subcutaneous administration of pesticide octylphenol to adult male rats decreased serum FSH, LH and testosterone levels, and increased prolactin [Blake and Boockfor, 1997; Boockfor and Blake, 1997]. The authors observed shrinkage of the testes and the accessory sex organs. Sperm numbers were reduced and the evaluation of sperm morphology revealed marked increase in the proportions of head and tail abnormalities. When octylphenol was administered to the male rats in drinking water no significant alteration was observed in mean serum LH, FSH and testosterone levels and reproductive organ weight. However, an increased frequency of epididymal sperm with tail abnormalities was seen [Blake et al., 2004] and the highest doses of octylphenol decreased epididymal sperm number. *Khurana et al. [2000]* found that exposure of newborn male rats to bisphenol A or octylphenol resulted in delayed and sustained hyperprolactinemia and increased expression of ERs in the anterior pituitary, whereas the hypothalamic ERs were less responsive. Although bisphenol A or octylphenol can act as weak estrogens [Kuiper et al., 1997], it was reported by *Steinmetz et al. [1997]* that the estrogenic effect of bisphenol A was higher *in vivo* than it was expected from the *in vitro* studies. Xenoestrogens do not bind to the sex hormone binding globuline (SHBG) which may explain their large bioeffects even in low concentrations.

Genomic imprinting

It was established also that xenobiotics, directly or through related pathways, play a role in programming or imprinting genes involved in cell proliferation, differentiation or survival. Genes can be either over- or underexpressed or will respond to a later signal, such as other hormone, leading to an altered cell function. A key event in establishing the pattern of gene expression in the cell is the methylation or demethylation of regulatory elements of the gene. The persistent change in gene expression is called genomic imprinting. It is accomplished in epigenetic fashion and leads to the silencing of a gene from one parent. The epigenetic changes in gene function that occur without a change in the sequence of nuclear DNA may be heritable. Descendants of

the cell in which the gene was turned on or off will inherit this activity even if the original stimulus for gene-activation is no longer present. Diffusion of the gene's product to other cells can also make the heritable characteristic spread.

Little is known about the role of estrogens in gene imprinting. Nevertheless, it was found that estrogen-associated signalling pathways may contribute to DNA methylation or demethylation [McLachlan, 2001]. Developmental exposure to estrogens resulted in the persistent overexpression of lactotransferrin in the uterus of female mouse [Nelson et al., 1994; Li et al., 1997]. It has been presented also that developmental exposure to DES can perturb normal uterine development by affecting genetic pathways governing uterine differentiation [Huang et al., 2005]. DDT binds to and activate ERs, but it was found recently that DDT targets also non-ER pathways [Frigo et al., 2005]. DDT and its metabolites stimulate activator protein-1-mediated gene expression through the p38 mitogen-activated protein kinase cascade and in this way induces apoptosis and the expression of the death ligand TNF-a. McLachlan [2001] suggests that cellular imprinting by estrogens may arise through the mechanisms: 1) directly imprinting of the gene probably by the DNA methylation, leading to persistent genetic change, and 2) alteration of signalling pathways at key points in cell differentiation, resulting in altered gene expression.

Genetic aberrations or polymorphism may predispose to augmented effects by environmental factors. In the study of Danish population it was found that a specific Y chromosome haplogroup (hg26) is significantly over-represented in men with reduced sperm counts [McElreavey and Quintana-Murci, 2003]. It was suggested that the factors encoded by genes on this class of Y chromosome may be particularly susceptible to environmental pollutions that cause reproductive disturbances in Denmark. Ogata et al. [2005] suggest that homozygosity for the specific ERa haplotype is responsible for the risine incidence of cryptorchidism in response to endocrine disruptors.

Special attention to developmental influence

Xenoestrogens do not bind to alphafetoprotein (AFP), which is present in the fetal circulation, to inactivate endogenous estrogens of mother's origin influencing the male fetus. Thus, male fetus is not protected against exogenous estrogen-like chemicals [Bonde, 1995]. Andersson and Skakkebaek [1999] warn about the xenobiotic levels in meat from hormone-treated animals, which are probably too high and not safe for prepubertal children and human fetuses. Wisniewski et al. [2003, 2005] found that perinatal (gestation and lactation) exposure of female rats and mice to "natural" phytoestrogen – genistein, in doses common in human diets,

altered masculinisation (smaller anogenital distance and testis size, delayed preputial separation) and decreased testosterone concentration in adulthood. Perinatal exposure to genistein caused also long-term dysfunction in sexual behaviour (less intention to mount, intromit and ejaculate). Moreover, aggressive behaviours were decreased, while defensive increased. Treatment with lower doses of genistein exerted greater effects on phenotypic and behavioural abnormalities. In the face of popularity of soy infant formulas these results are alarming. The authors suggest that the influence of isoflavone exposure during early childhood on reproductive and behavioural health in boys and men should be considered. Phytoestrogens isoflavones bind ERa and ERb and alter the transcription of estrogen-responsive genes [Kuiper et al., 1997]. Phyto- and mycoestrogens can act also as antiandrogens by lowering the androgen receptor nuclear translocation and the intranuclear cluster formation induced by androgens [Paris et al., 2005].

Exposure to exogenous estrogens may diminish production of fetal pituitary gonadotropin FSH by negative feedback mechanism. Lower FSH serum levels may result in decreased rate of Sertoli cells proliferation and disturbed synthesis of substances which create intratesticular milieu and control the activity of germ cells, Leydig cells and peritubular cells within testes. Disturbed biosynthesis of Sertoli cells products may result in disorders of the differentation of male reproductive system, cryptorchidism, formation of testicular neoplastic changes and furthermore infertility and GCT [Skakkebaek et al., 1998]. It has been hypothesized that while in dysgenetic gonads of intersex individuals with Y chromosome material in the karyotype Sertoli cells have an impaired function, their activity may be still strong enough to prevent the primordial germ cells from entering meiosis, as it occurs in the ovary. There may be, however, too few or too poor functioning Sertoli cells to stimulate adequate differentiation of gonocytes into spermatogonia. The primordial germ cells may not have clear signals to differentiate either into female or into mature male germ cells and they therefore kept their fetal characteristics as multipotential gonocytes (stem cell potential).

Nielsen et al. [2000] found that ERa may be involved in the development of Leydig and peritubular cells. The enhanced estrogenic signalling can suppress the biosynthesis of insulin-like 3 hormone (Insl3) by Leydig cells, which is necessary for the development of gubernaculum, and by attenuating the production of androgens, necessary for the regression of the cranial suspensory ligament [Ogata et al., 2005]. Defects of Insl3 action can cause cryptorchidism in male mice, while over-expression in female mice can cause ovarian descent. Recently, Ferlin and Foresta [2005] demonstrated that serum level of Insl3 is decreased in adult men with infertility caused by severe inherited testicular

damage, reflecting the functional status of the Leydig cells.

CONCLUSIONS

Although physiologic action of estrogen in the male reproductive system, bones, brain and other organs is of vital importance, it is necessary to realize that most of endocrine disruptors exert estrogen-like or anti-androgenic activity and due to this are harmful to male reproductive system, both during development and in adulthood. Potentially, endocrine disruptors may have also adverse effects in the subsequent generations. The number of these substances is still growing, whereas the mechanism of their action is still not exactly known. Therefore the responsibility lays on the contemporary human population to control the environmental pollution and try to diminish their influence on the nature.

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Anti-oestrogens in the Treatment of Male Infertility

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SUMMARY

The use of anti-oestrogens for the treatment of idiopathic oligozoospermia has been introduced for almost three decades. The mechanism of their action has been well established and it is mediated through their competitive binding to oestradiol receptors at its target cells, mostly at the hypothalamus as well as at peripheral levels. This action leads to an increase in the gonadotropin secretion and sex steroids and this effect is expected to stimulate spermatogenesis. Today, most of the clinical observations available come from studies on the effect of the two main anti-oestrogens clomiphene citrate (CC) and tamoxifen citrate (TMX) in patients with oligozoospermia. It should be stressed at this point that CC has been extensively tried in the 70's and 80's with TMX being the favorite anti-oestrogen in recent years.

Weak androgens (mesterolone and testosterone undecanoate) have been also introduced for the treatment of idiopathic oligozoospermia (I.O.). These per os given steroids exert satisfactory androgenic activity on their target tissues but have very weak or no effect on gonadotropin secretion. Of these agents, TU administration has been evaluated with encouraging, although not definite, results.

A combination of the anti-oestrogen TMX with TU has been extensively tried with regard to its effect on endocrine and sperm parameters and was found to improve markedly testicular sperm activity without compromising pituitary or Leydig cell function. But most importantly, it has been demonstrated that this combination effects significant beneficial effects when pregnancy was the outcome measure.

By and large, it appears that combination of anti-oestrogens with a weak androgen offers potentially certain advantages over single-agent intervention and its rational, way of development and clinical validation together with some necessary data on pharmacodynamics were presented in detail. This intervention effects a new medical approach towards the treatment of patients with I.O.

INTRODUCTION

The role of gonadal steroids in the male is well established. As the main Leydig cell products, testosterone (T) and oestradiol (E_2), enter the circulation, mostly bind to carrier proteins and bind to their specific receptors to elicit the corresponding response related either strictly to reproductive processes or to various non-gonadal functions at large *[for review Griffin and Wilson, 2003]*. In accordance with this sequence of events, both T and E_2 act at the proper hypothalamic and pituitary centers thus conveying messages from the testes about their everyday secretion. In this manner, the gonads regulate central kisspeptin and GnRH secretory activity and pituitary gonadotropin synthesis and release *[Aparicio, 2005; Murphy, 2005; Conn et al., 1981]*.

However, in addition to the normal ligands (T and E_2), other less or non-oestrogenic molecules have been demonstrated to posses a binding affinity to gonadal steroid hypothalamic receptors blocking endogenous (native) oestrogens' attachment to their respective receptors, thus creating a factitious image of low steroid activity. In response to this false message, the hypothalamic-pituitary complex is activated with an increase of GnRH pulses and subsequent rise in FSH/LH secretion.

This property of certain substances termed anti-oestrogens has been exploited diagnostically and therapeutically from the late 60's with dynamic tests of gonadotropin reserve in cases of reproductive abnormalities of central origin and with medical intervention for boosting spermatogenesis or inducing ovulation in appropriate cases *[Kotoulas et al, 1994; Beck et al., 2005]*. On both occasions, the central event was the deceit of E_2 hypothalamic receptors.

Of a similar design, although through a different mode of action, inhibitors of aromatase activity reduce oestrogen availability thus creating a false impression of impaired gonadal secretion which leads to a rise of central

gonadotropin activity. As in the previous case, aromatase inhibitors have been used both diagnostically and therapeutically in both sexes, although to a very limited scale [Clark and Sherins, 1989].

Today, most of the clinical observations available in the human and particularly in men, come from studies on the effect of the two main anti-oestrogens clomiphene citrate (CC) and tamoxifen citrate (TMX) in patients with oligozoospermia. It should be stressed at this point that CC has been extensively tried in the 70's and 80's with TMX being the favorite anti-oestrogen in use in recent years. A relative disadvantage of CC is its native estrogenic activity causing a SHBG rise, which in its turn, reduces free T availability [Adamopoulos et al., 1981].

Another class of agents has been introduced for the treatment of idiopathic oligozoospermia (I.O.), that of the weak androgens mesterolone and testosterone undecanoate (TU). These per os given steroids exert satisfactory androgenic activity on their target tissues but have very weak or no effect on gonadotropin secretion. Of these agents, mesterolone has been properly assessed in a WHO sponsored trial [World Heath Organization, 1989], whereas, TU administration has been evaluated with encouraging, although not definite, results [Push, 1989].

The combination of anti-oestrogens with a weak androgen offers potentially certain advantages over single-agent intervention and its rational way of development and clinical validation together with some necessary data on pharmacodynamics are presented in this review.

ANTI-OESTROGENS

Tamoxifen

Tamoxifen (TMX) is an non-steroidal triphenylethylene-based agent which displays a complex spectrum of oestrogen antagonist and oestrogen agonist-like pharmacological effects (Figure 1a). After oral administration, TMX is absorbed rapidly with maximum serum concentrations attained after approximately 4-7 hours and circulates highly bound to serum albumin (>99%). Its metabolism is effected by hydroxylation, demethylation and conjugation, giving rise to several metabolites which have a similar pharmacological action to that of the parent compound and thus contribute to the therapeutic effect. Excretion occurs primarily via the faeces and an elimination half life of approximately 7 days has been calculated for the drug itself, whereas that of N-desmethyltamoxifen, the principal circulating metabolite, is 14 days. Steady-state concentrations are achieved after about 4 weeks of treatment and serum concentrations are dose related.

TMX was introduced three decades ago as an empiric treatment of I.O. because of its stimulatory action on

gonadotropin secretion and its postulated direct effect on Leydig cell function and 5a-dihydrotestosterone production in seminiferous tubules and epididymis [Comhaire, 1976; Willis et al., 1977; Vermeulen and Comhaire, 1978; Zalata et al., 1995; Smals et al., 1980].

The overall effect of TMX on spermatogenesis is stimulatory, resulting in an average twofold increase in spermatozoa concentration but with no marked changes in motility and morphology [Buvat et al., 1983; Kotoulas et al., 1994].

Clomiphene citrate

Clomiphene citrate is also a triphenylethylene derivative in which the ether side-chain moiety is essential for the antioestrogenic activity (Figure 1b). It is orally active and undergoes hepatic metabolism. Its excretion occurs primarily in the faeces and to a lesser extend in the urine and its biological half life is approximately 5 days. It was the first antioestrogen agent to be used for the treatment of I.O. However the initial surge of enthusiasm has gradually subsided due to the non-satisfactory results in proper clinical trials [Scottish Infertility Group, 1982; World Health Organization, 1992]. One of the main shortcomings of CC administration appears to be a significant, SHBG-induced reduction of T bioavailiability [Adamopoulos et al., 1981]. This marked change is caused by the oestrogenic activity of CC.

Aromatase inhibitors

Aromatase is an enzyme responsible for the final step in oestrogen synthesis, namely the conversion of the androgens, androstenedione and testosterone into oestrone and oestradiol respectively. Aromatase belongs to the group of cytochrome P-450 enzymes, which are involved in the biosynthesis of aldosterone, cortisol and androgens and it is found in peripheral tissues such as muscle, fat and liver.

The degree of selectivity of an aromatase inhibitor for the aromatase enzyme influences its ease of use and the tolerability profile. Of the agents belonging to this group, anastrozole is the most selective of the third-genaration aromatase inhibitors, having no significant effect upon basal or ACTH-stimulated cortisol or aldosterone levels.

The clinical use of aromatase inhibitors in I.O. has been very limited and this is probably due to the non-satisfactory results in the few clinical trials conducted so far [Clark and Sherins, 1989; Teris and McCallum, 2002; Raman and Schlegel, 2002; Mitwally and Casper, 2003].

WEAK ANDROGENS

Testosterone undecanoate (TU)

TU is a testosterone ester with a long fatty acid side chain

(Figure 1c). It is absorbed in the lymphatics of small intestine and partially escapes first pass hepatic metabolism. After a single oral dose of TU, maximal T levels are attained in about 5 hours and fall gradually to baseline by 8 hours. TU is a relatively weak androgen and it does not suppress basal and stimulated pituitary gonadotropin and Leydig cell function *[Adamopoulos et al., 1995]* while it can improve sperm morphology and the functional sperm fraction (FSF) in men with I.O. *[Push et al., 1989]*. It should be noted however, that there are no concrete data available on the direct effect of TU on epididymis and accessory glands in humans.

1a: Tamoxifen citrate

1b: Clomiphene citrate

1c: Testosterone undecanoate

Figure 1: Molecular structure of
a) Tamoxifen Citrate
b) Clomiphene Citrate
c) Testosterone Undecanoate

Mesterolone

Mesterolone is a non-aromatizable, 5a-reduced testosterone ester, which has been tried as a monotherapy for I.O. but without a marked beneficial effect on sperm

parameters *[Scottish Infertility Group, 1984]*. As in the case of TU, pituitary suppression is either minimal or absent in men treated with agent. However, it should be noted that in a large multi-center study organized by the World Health Organization *[1989]*, this compound marginally failed to achieve a statistically significant improvement in terms of cumulative life table pregnancy rates ($19\pm6\%$ in verum vs $11\pm5\%$ in placebo treated).

COMBINATION OF ANTIOESTROGENS AND WEAK ANDROGENS

A combination treatment with TMX and TU has been proposed as a new therapeutic option in men with I.O. This approach was based on the concept of the two hormone effect for initiation and maintenance of spermatogenesis - FSH and T *[Jegou, 1993; Sharpe, 1994]* and the fact that in I.O. men some definite and demonstrable abnormalities of endocrine activity have been observed at one or more steps of hormone secretion and action which may compromise spermatogenesis *[Adamopoulos and Nicopoulou, 1998]*. In this approach, the central effect of TMX is complemented by the peripheral action of TU, mainly on accessory glands, as a guarantee against potential disturbances of androgen synthesis, metabolism and/or transport, which have often been observed in men with I.O. *[Glass and Vigerski, 1980; Adamopoulos et al., 1984; Giagulli and Vermeulen, 1988]*.

The effects of the combination of the two agents on basal or stimulated pituitary and testicular endocrine activity was firstly evaluated in a short-term administration protocol *[Adamopoulos et al., 1989]* and subsequently in a long-term one *[Adamopoulos et al., 1995]*. A properly controlled clinical trial followed short after and demonstrated that TMX (10 mg b.i.d.) and TU (40 mg t.i.d.) given for a period of six months increased markedly some conventional sperm parameters and improved important functional indices such as aniline and acrosine in treated men when compared with single agent treatments or placebo *[Adamopoulos et al., 1997]*. The results of this trial were not only confirmed but further strengthened by a more recent prospective, randomized, placebo-controlled trial *[Adamopoulos et al., 2003a]*.

This new study recruited a relative large number of men with I.O. (n = 212) distributed in active and placebo treatment groups. Moreover, a group of normozoospermic men were followed-up during investigation and treatment for their partner's problem as a control population for
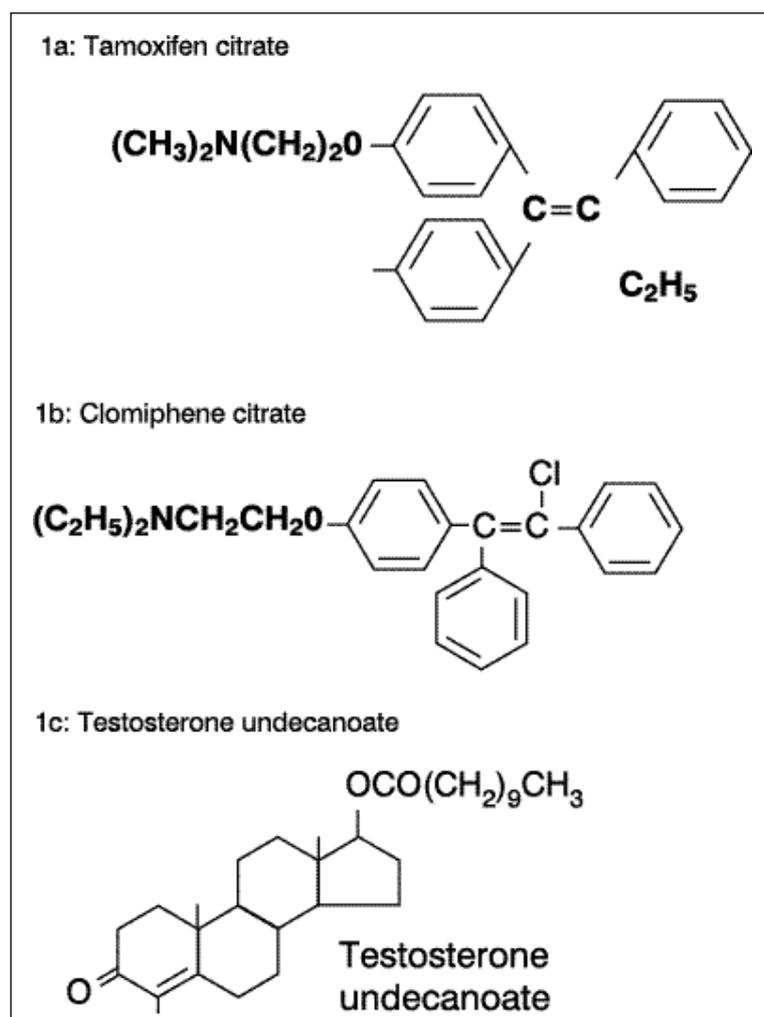
assessment of a regression to the mean effect. Patients were treated with TMX + TU (10 mg b.i.d. + 40 mg t.i.d., respectively) or placebo for a six month period, following which a fair number of men were followed-up for an additional 3 month post-treatment period. The latter subgroup was considered as on drugs' influence due to the long lasting TMX and its metabolites biological effect. Outcome measures for this study were the incidence of pregnancy, testicular volume and changes of sperm parameters.

A distinct characteristic of the study was its holistic approach since intervention was restricted not only to the male patients but also included couple counseling. The latter approach signified that investigation and treatment of the female partners, if there was a problem detected, was a part of the study.

The results of the trial were as follows:

a) the cumulative number of pregnancies after treatment was markedly higher in the active treatment group than the placebo group (35 vs 11, corresponding to a pregnancy incidence of 33.9% and 10.4%, respectively) (Figure 2),

b) Total testicular volume was significantly increased in active treatment recipients *(Table 1)*,

c) sperm concentration, as well as progressive motility, morphology and mean functional sperm fraction markedly improved starting from the third month on active treatment *(Tables 1, 2)*.

It should be noted here that the response to treatment was independent of the severity of the problem *[Adamopoulos et al., 2005a]*.

No serious side effects of active treatment were reported apart from a mild and transient liver enzyme elevation in a few cases and a slight increase in body weight during therapy in some men.

An interesting phenomenon related to variations in reproductive activity at large and sperm production in particular, was observed in this study. Using a >100% increase over basal values of FSF as an outcome measure, it has been demonstrated that when treatment started in autumn or winter seasons, the response to TMX + TU was higher than in the other two seasons *[Adamopoulos et al., 2005b] (Figure 3)*. It is reminded, that in this part of the world, autumn and winter months are characterized by low photoperiod time, low temperatures and high humidity. In practical terms, this observation implies that autumn and winter months offer a better chance for successful treatment.
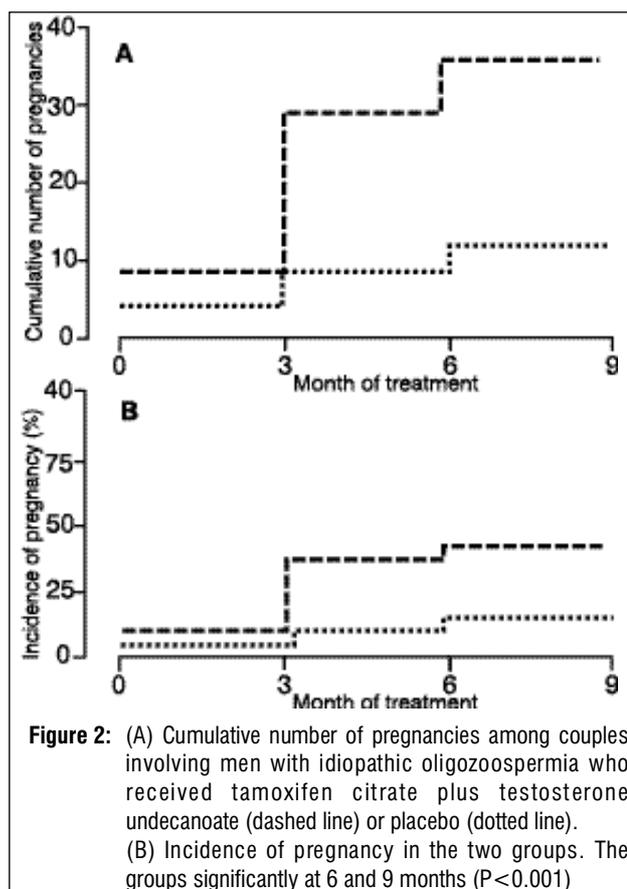

Figure 2: (A) Cumulative number of pregnancies among couples involving men with idiopathic oligozoospermia who received tamoxifen citrate plus testosterone undecanoate (dashed line) or placebo (dotted line).
(B) Incidence of pregnancy in the two groups. The groups significantly at 6 and 9 months (P<0.001)

Table 1

	Sperm variables and total testicular volume in oligospermic men 3 months after the end of treatment								
	Basal evaluation			Final Evaluation			Off-treatment evaluation		
	FSF				FSF			FSF	
Group	Total count (X10^6 cells/mL)	(X10^6 cells/ mL)	TTV (mL)	Total count (X10^6 cells/mL)	FSF (X10^6 cells/mL)	TTV (ML)	Total (X10^6 cells/mL)	(X10^6 cells/ mL)	TTV (ML)
Active treatment (n=21)	22.8(16.3,29.4)	3.5(1.9,5.6)	32.3±9.5	67.0(43.4,96.0)	6.47(2.9,7.6)	37.1±10.6	61.6(30.6,102.0)	13.8(3.9,17.4)	34.1±5.6
Placebo (n=11)	27.5(20.9,47.4)	3.6(0.9,5.3)	27.0±7.8	34.7(18.0,67.4)	4.6(1.3,12.9)	30.0±8.1	26.9(23.6,38.9)	3.8(2.7,5.7)	28.2±2.8

Note: Data are medians (25th, 75th) or means (±SD). FSF=functional sperm fraction; TTV=total testicular volume

Adamopoulos. Tamoxifen and androgens in oligozoospermia. Fertil Steril 2003.

Table 2

Functional sperm fraction and percentage change as a result of treatment

Group	FSF at baseline (X10⁶ cells/mL)	3-month evaluation		6-month evaluation	
		FSF(X10⁶ cells/mL)	Change %	FSF(X10⁶ cells/mL)	Change %
Active treatment (n=106)	3.0(1.0, 6.2)	8.3(3.9, 18.9)	176.4±51.3	16.3(6.3, 25.7)	443.3±64.7[c]
Placebo(n=106)	3.8(0.9, 10.1)	4.1(1.7, 11.9)	7.8±0.6[a]	4.4(1.4, 12.3)	11.6±2.1[a]
Normozoospermic men (n=82)	41.7(21.7, 65.8)	40.3(24.2, 66.0)	3.8±1.70[a,b]	44.5(29.7, 73.4)	6.7±2.7, 8.9

Note: Data are medians (25th, 75th percentile) or means (±SD). FSF=functional sperm fraction

[a] $p<.001$ vs active treatment
[b] $p<.001$ vs placebo
[c] $p<.001$ vs 3-month value

Adamopoulos. Tamoxifen and androgens in oligozoospermia. Fertil Steril 2003.
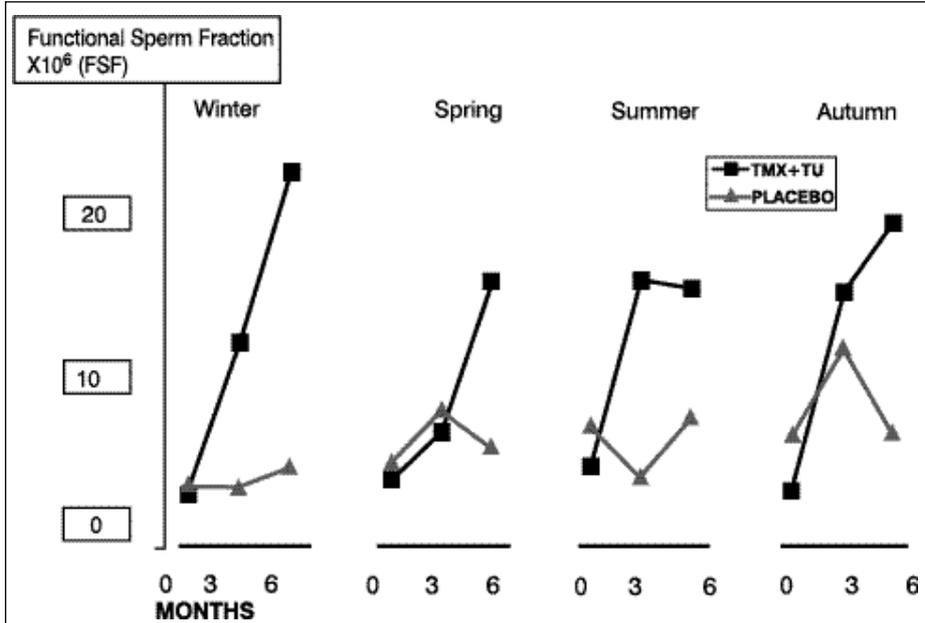

Figure 3: Seasonal variation in treatment response.

followed by a relapse to pre-treatment sperm conditions, usually within a course of 6-8 weeks. Obviously additional courses are in order should need be and after a reasonable interval of a few months.

In view of these conditions and particularly the long duration of treatment, defining suitable criteria for patient selection is of paramount importance. In this context, a number of potentially suitable indices as gonadal volume, testicular cytology or histology and serum FSH and/or inhibin-B concentrations have been exploited *[Adamopoulos, 2000]*. Each of them has been evaluated in different clinical settings, with FSH administration as the intervention agent *[Foresta et al., 1998, 1999; Adamopoulos et al., 2003b]*.

With regard to the TMX + TU combination we have recently conducted an assessment of pre- and post-treatment testicular volume, functional sperm fraction (FSF: a product of total sperm number x % of good motility x % of good morphology/104), and also pre-treatment FSH and inhibin-B concentrations in 50 men with I.O. Assessment of these data showed that of the variables examined the best predictive value for successful (more than 100% increase of FSF at the end of 6 months of treatment) was the basal concentration of inhibin-B and the ratio of inhibin: FSH before treatment *(Billa et al, 2004)*. Based on these findings an inhibin:FSH ratio greater than 2.8 was considered a good predictive index for successful response to TMX + TU (doubling of FSF).

SELECTION CRITERIA FOR TREATMENT

Medical intervention for I.O. should be administered for adequate period usually defined as one and preferably two spermatogenic cycles equal to three to six months of treatment. This period of time is necessary to demonstrate the effectiveness of any medical treatment on sperm parameters but does not restrict its duration to this time-limit. On the contrary, once a successful response has been observed treatment could be continued until pregnancy occurs, but not for more than 9-12 months and always under periodic control of liver enzymes. As it is obvious, prolongation of treatment is necessary for two reasons: a) improvement of sperm parameters does not guarantee immediate conception and b) withdrawal of medication is

GENERAL COMMENTS

Idiopathic oligozoospermia is a multi-factorial in aetiology syndrome with many of its causative factors not recognized or understood. The main reason for this scandalous situation is the lack of solid background information on the myriad of particular processes regulating spermatogenesis and sperm maturation, but also the very simplistic, if not naive, procedures involved in the investigation of their deviations from normality. Because of this situation, the chances for a successful therapeutic intervention are to a large extent fortuitous. Single agent treatment may successfully improve specific problems as is the case of impaired motility with administration of weak androgens, kallikrein, anti-oxidants or carnitine [Kessopoulou et al., 1995; Push, 1989; Lenzi et al., 2003, 2004; Keck et al., 1994]. On the other hand, a combined agent regimen affords better chances to be effective since it addresses more than one of the many specific problems of the process. One may use the paradigm of two persons fishing in a pool with muddy waters using one or two separate hooks respectively. It appears that TMX + TU treatment is a double hook approach. Its effectiveness is irrespective of the initial qualitative or quantitative sperm parameters as shown in our experience [Adamopoulos et al., 2005a]. Moreover, by using FSH and inhibin-B measurements, a reasonable prediction for a successful outcome might be made. Finally, long-term use toxicity or other important side effects have not been reported so far. The only unexplored aspect of this treatment is that of chromosome Y microdeletions which may be forwarded to the male offspring. This possibility is currently under investigation by our group. However, it should be emphasized that this is a common feature of all conceptions resulting from sperm of men with I.O. irrespective of the type of treatment (natural, IUI or IVF-ICSI).

Last, but by no means least, is the financial aspect of the combination treatment in comparison to assisted reproduction techniques. Indeed the former type of treatment has been estimated to be a number of times lower than the latter. Therefore, for the lower socioeconomic classes of the population in the west or for couples in the developing countries this treatment offers a realistic low cost opportunity for procreation. Moreover, it provides a reasonable chance to people who have no access to assisted reproduction technology or for ethical or religious reasons do not accept it, to try a "normal", acceptable and affordable way to procreation. Finally, this approach, unlike the assisted reproduction techniques, addresses the problem only of the suffering part and limits treatment to the needy person in the couple.

The last comment is of a rather professional nature and relates to the clan of Andrologists, Endocrinologists and all Reproductive Medicine specialists all over the world. As it is evident, an important tool in the therapeutic armamentarium for I.O. raises enormously the overall prestige and other benefits of the subspecialties and their faithful servants.

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ROLE OF ESTROGENS ON BONE IN HUMAN MALE

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INTRODUCTION

In women, bone physiology, pathophysiology and epidemiology are well understood while in men, bone pathophysiology remains a poorly understood phenomenon even though some steps forward have been made in recent years [Rochira et al., 2005]. Particularly, remarkable advances have been provided in the knowledge of the role of sex steroids on growth and skeletal maturation [Rochira et al., 2001], since it was established that estrogen is necessary to achieve epiphyseal closure and peak bone mass also in the human male [Faustini-Faustini et al., 1999]. Soon after, the importance of estrogen on bone in men has been recognized during adulthood and aging too [Amin et al., 2000; Falahati-Nini et al., 2000; Khosla, 2002].

As in women, susceptibility to osteoporosis and osteoporotic fracture in men is determined by the entity of both skeletal accrual at puberty (peak of bone mass) and subsequent bone loss. In both sexes, sex steroids are known to be critically involved in the developing of human skeleton, with androgens traditionally assumed to drive the acquisition of bone mass in men [Faustini-Faustini et al., 1999]. In the last decade, the development of animal models of estrogen insufficiency (either estrogen resistant or aromatase deficient) [Couse and Korach, 1999], together with the description of estrogen resistance and aromatase deficiency, due to naturally occurring inactivating mutations of the estrogen receptor (ER) [Smith et al., 1994] or of aromatase gene respectively in human males [Morishima et al. 1995; Carani et al., 1997; Bilezkian et al., 1998; Herrmann et al., 2002; Maffei et al., 2004], opened new perspectives on estrogen physiology in men [Grumbach and Auchus, 1999; Rochira et al., 2001]. In males testosterone comes from Leydig cells production in the testis, while circulating estrogens originate from androgen aromatization by the aromatase cytochrome-P450 enzyme in the testis (80%) and in peripheral tissues (20%). Estrogens act by binding to their nuclear estrogen-receptors (ER α and β) [Enmark and Gustafsson, 1999], or by a non-genomic pathway involving a plasma-membrane ER interaction [Levin, 1999].

Estradiol is synthesized in a number of extragonadal sites and acts as a circulating hormone, but its effects are mainly due to the local production rate (paracrine or intracrine factor). Thus, circulating estrogens reflect the metabolism of estrogens formed in these extragonadal sites, whose biosynthesis depends on a circulating source of androgenic precursors [Davison et al., 2005; Simpson et al., 2005].

In bone, the activity of local aromatase leads to a major source of estrogen production responsible for the maintenance of mineralization, although this is extremely difficult to prove due to sampling problems [Simpson et al., 2000; 2005]. Accordingly, bone tissue express aromatase in osteoblasts and chondrocytes [Oz et al., 2000], and aromatase activity in cultured osteoblasts is quite similar to that present in adipose stromal cells [Shozu and Simpson, 1998]. These findings could have probably also considerable clinical implications [Simpson et al., 2000].

ROLE OF ESTROGENS IN THE DEVELOPING SKELETON

Bone clinical features of patients with congenital estrogen deficiency are osteopenia or osteoporosis at a various degree together with tall stature, a history of continuing linear growth into adulthood, unfused epiphyses, delayed bone age, eunuchoid proportions of the skeleton and progressive genu valgum [Smith et al., 1994; Morishima et al., 1995; Carani et

al., 1997; Bilezikian et al., 1998; Herrmann et al., 2002; Rochira et al., 2002; Maffei et al., 2004; Rochira et al., 2005]. No skeletal abnormalities were reported in a young boy with aromatase deficiency diagnosed during infancy [Deladoëy et al., 1999; Bouillon et al., 2004].

Traditionally, hormones, particularly sex steroids have been thought to be responsible for both closure of epiphyses and cessation of growth at the end of puberty [Ohlsson et al., 1993; Van der Eerden et al., 2003]. Overall, in both sexes androgens, rather than estrogens, have been considered as the main hormones involved on pubertal growth, and the pubertal growth spurt was supposed by due to testicular androgens in boys and adrenal androgens in girls [Pescovitz, 1990]. On the other hand both sex steroids have been reported to stimulate epiphyseal maturation and accretion of bone mineral mass [Smith and Korach, 1996].

Accordingly, since the peak of height velocity occurs when estradiol levels are lowest, but quite similar in both boys and in girls [Klein et al., 1994], it has been suggested that androgens may partly act on cartilage after their conversion into estrogens in boys [Bourguignon, 1988; Blanchard et al., 1991].

By these findings it was proposed that a biphasic effect of circulating estrogens may operate on growth plates and skeletal maturation: low levels, typical of prepubertal stage, stimulate the growth plate, whereas increasing concentrations, as observed during late puberty, inhibit this process by inducing a terminal differentiation of chondrocytes and leading to epiphyseal closure [Cutler, 1997; Balestrieri et al., 2001]. In this view, male pubertal growth spurt seems to be an estrogen-dependent process [Faustini-Faustini et al., 1999; Grumbach and Auchus, 1999].

The recent description of estrogen treatment effect on a young boy affected by aromatase deficiency agrees with the concept that a low dose of estradiol could prevent the occurrence of skeletal morphological alterations reported in adult patients with congenital estrogen deficiency, miming the physiological prepubertal circulating levels, when started in a significantly earlier stage of skeletal development. In fact, the young boy presented osteopenia and low BMD and delayed bone age with open epiphyses, without other skeleton abnormalities [Bouillon et al., 2004]. Evidence on this biphasic effect of estrogen on bone growth has come also from other clinical studies performed in boys with idiopathic delay of puberty [Caruso-Nicoletti et al., 1985; Cutler, 1997], in patients affected by complete androgen insensitivity syndrome (CAIS) [Zachmann et al., 1986] and in excessively tall girls [Zachmann et al., 1975].

In addition, no abnormalities of both early growth and pubertal development were reported in congenital estrogen-deficient adult men as judged by their history. Thus, it was hypothesized that pubertal growth spurt does not occur in men with congenital estrogen deficiency, remaining longitudinal growth a linear process during puberty and adulthood as it happens in the prepubertal age.

Based on this evidence, it is clear that estrogen replacement in an adult male with aromatase deficiency should be prescribed in order to achieve two main short or medicine term goals: first, the closure of the epiphyses and second, the attachement of the peak of bone mass both of trabecular and cortical bones [Carani et al., 1997; Bilezikian et al., 1998; Balestrieri et al., 2001].

Accordingly, in a man with aromatase deficiency the initial treatment with high dose of transdermal estradiol (0.47 mg/Kg daily) for six months followed by a low dose one (0.24 mg/Kg daily) resulted in a complete epiphyseal closure [Carani et al., 1997; Rochira et al., 2000], being inefficacious in the estrogen-resistant man [Smith et al., 1994]. By contrast, in adult aromatase-deficient men, a long-term high-dose testosterone treatment alone was ineffective in improving bone maturation [Carani et al., 1997; Maffei et al., 2004].

In this view, a high-dose of estradiol should be therefore preferred to a lower one for starting estrogen replacement therapy in aromatase-deficient adult men, in order to achieve the epiphyseal closure. Then, by our experience, when the ephiphyseal closure is achieved, the dosage should be reduced in order to preserve a normal BMD and to maintain both serum estradiol and gonadotropins in the normal range [Rochira et al., 2000; Balestrieri et al., 2001].

Biliezikian et al. also achieved a complete epiphyseal closure in the other aromatase deficient adult man, starting with a low-dose of conjugated estrogens, and progressively increasing it [Bilezikian et al., 1998], but this second regime of treatment is compromised by high estradiol levels.

In addition, low dose treatment with should be preferred in cases of young boys [Bouillon et al., 2004] in evolutive phases of puberty for two reasons. First, a high dosage may lead to an accelarated epiphyseal closure and growth arrest with impaired final adult height. Second, a low dosage is more physiologic for a pubertal boy and it stimulates bone elongation and bone thickness [Caruso-Nicoletti et al., 1985; Cutler, 1997].

The young boy with aromatase deficiency described by Bouillon continued his linear growth during several years of treatment; he presented delayed closure of the epiphyses but his skeleton changes were similar to those occurring in normal pubertal growth. In this case low-dose estrogen treatment increased bone age and improved axial and peripheral BMD; further, periosteal diameter continued to expand during estrogen therapy, supporting the hypothesis of an essential contribution of a major stimulatory effect of low-dose estrogen to periosteal pubertal growth [Bouillon et al.,

2004].

Along with skeletal maturation and progressive epiphyseal closure, the mineralization of the growing skeleton increases rapidly during puberty, especially in cancellous bone *[Faustini-Faustini et al., 1999; Bouillon et al., 2004].*

In normal male puberty, the increase in bone diameter and cortical thickness, due to an accelerated periosteal apposition, were traditionally considered as a result of the stimulatory effects of androgens associated with the lack of the inhibitory effects of estrogens. In both sexes, during prepubertal period, periosteal apposition leads to an increase in bone width, but when the pubertal growth starts, the bone diameter continues to increase in males, whereas in girls this process seems to be inhibited. Thus, this gender-specific pattern during pubertal growth should be linked mainly to differences circulating estrogens rather than androgens *[Seeman, 2001].*

In adult men with estrogen deficiency estrogen treatment resulted in an increase of BMD at lumbar spine and femoral neck in a dose dependent fashion even when the treatment was started in adulthood *[Rochira et al., 2000; Maffei et al., 2004],* whereas a long-term high-dose testosterone treatment alone was ineffective. This supports the concept that even though bone accrual failed at puberty in these patients it could be corrected by the starting estrogen treatment during adulthood.

Estrogens role on skeletal maturation becomes clearer if we consider that accelerated growth, advanced bone age, and probably a short final stature constitute the clinical features of the aromatase excess syndrome in both sexes *[Stratakis et al., 1998],* an opposite phenotype of that of aromatase deficiency *[Rochira et al., 2001, 2002].*

These findings clearly demonstrate that androgens alone are not able to complete bone maturation; furthermore, epiphyseal closure does not develop without estrogens and low-dose estradiol treatment probably is a critical contribution to periosteal pubertal growth, even in the male.

ROLE OF ESTROGEN ON THE MATURE SKELETON

During adulthood, sex steroids are considered necessary in order to maintain bone mass in both sexes. The prevailing view among physicians was that androgens maintain bone mass in males as estrogens do in females *[Finkelstein et al., 1996].* In the congenital estrogen deficiency we can constantly observe a severe osteopenia or osteoporosis *[Smith et al., 1994; Carani et al., 1997; Bilezikian et al., 1998; Herrmann et al., 2002; Maffei et al., 2004],* thus androgens alone are probably not able to promote a normal skeletal mineralization. On the other hand, the efficacy of estrogen

treatment in normalizing the bone mineral density (BMD) in the aromatase deficiency has been established *[Carani et al., 1997; Bilezikian et al., 1998; Rochira et al., 2000; Herrmann et al., 2002; Maffei et al., 2004].* In order to maintain a normal BMD and hormones within the normal range it has been suggested that the minimal amount of exogenous estrogen may be 0.24 mg/Kg daily (transdermal estradiol) without inducing undesirable side effects, such as gynecomastia and loss of libido. This dose is lower than that commonly used in postmenopausal women which resulted in bone health. It could be supposed that the amount of estrogen needed to maintain normal bone mineralization is similar in both sexes and the threshold for estradiol serum levels, below which we may expect an impairment in bone health, probably does not differ between males and females *[Rochira et al., 2000 Balestrieri et al., 2001].*

Furthermore, estrogen replacement therapy in men should be continued lifelong, because even after an increase in BMD has been achieved, the lowering of estradiol led to bone loss in an aromatase-deficient man suggesting an anabolic role on male bone for estrogens *[Rochira et al., 2000].* Apart from congenital estrogen deficiency, studies in male to female transsexuals suggested a positive effect of estrogen treatment on BMD *[Reutrakul et al., 1998; Van Kesteren et al., 1998].* Moreover, a strict correlation between bioavailable estrogens and BMD has been documented in elderly *[Greendale et al., 1997]* and in adult men *[Khosla et al., 1998].* Finally, a major risk of vertebral fracture has been established in men with low bioavailable circulating estrogens *[Barret-Connor et al., 2000].* These data suggest that estrogens are responsible at least in part for the bone actions which were classically ascribed to androgens, even though a pure androgen action on bone has been suggested by several data *[Amin et al., 2000; Szulc et al., 2001; Goderie-Plomp et al., 2004; Lorentzon et al., 2005; Rochira et al., 2005].*

UNRESOLVED ISSUES

Traditionally, it has been suggested that the sexual dimorphism of bone structure could be explained on the basis of different concentrations of circulating androgen between the two sexes. In fact, bone mass in men is higher than in women and male bone is also stronger than that of females *[Bonjour et al., 1991]* and these gender differences in both cortical and cancellous bone mass are related to bone size in humans *[Loro et al., 2000; Seeman, 2001].*

Paradoxically, nowadays we know more on estrogens and less on androgens role on bone pathophysiology. Undoubtedly androgens have a direct action on bone since the androgen receptor is expressed in bone tissue *[Vanderschueren et al., 2004],* but it remains to establish in detail what are the specific direct actions of androgens on

bone.

Androgens may account for the sexual dimorphism existing in both bone size and strength [Seeman, 2001] and one of the most important actions of androgens occurs at puberty in boys and it may explain the formansion of larger and stronger bones in men. Probably androgens exert their action on periosteal size at puberty [Duan et al., 2003].

It remains to establish whether there is a difference in bone periosteal apposition between the two sexes during adulthood and in particular during aging. In fact a lot of studies have provided data for a greater periosteal apposition in men then in women [Seeman, 2001; Duan et al., 2003], but some evidence suggests that postmenopausal periosteal apposition occurs in women too [Ahlborg et al., 2003] whereas others studies failed to show a gender based pattern of periosteal accretion [Riggs et al., 2004].

In conclusion, several lines of evidence support the view that estrogens are required and mediate part of the actions of androgens on the bone at puberty and also regulate bone homeostasis during adulthood and aging in men.

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Estrogens and Coronary Artery Disease in Men

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INTRODUCTION

Coronary artery disease (CAD) is one of the most common diseases affecting populations in the XXI century, therefore the exact knowledge of its pathogenesis, that might result in new way of treatment, constitutes the main challenge of contemporary cardiology. Clear differences in the clinical course of CAD between men and women have been observed for years. Lower incidence of CAD in premenopausal women led to the conclusion that female sex hormones, including estradiol (E_2) are responsible for this difference. This assumption led to the wide use of estrogen replacement therapy in postmenopausal women, between others as a means to decrease the risk of CAD. Nevertheless, the results of recent studies have negatively verified this approach [Hulley et al., 1998]. Even though the role of sex hormones in the pathogenesis of CAD was postulated years ago, little is known about the link between sex steroids secretion or action and CAD spectrum in men.

Estrogens are produced by the ovary and are traditionally considered as exclusively female sex hormones. For years it has been accepted that these hormones have no biological effects in men or are capable of inducing malfunctioning of the testes. However, it has now been established that estrogens are produced in the testes, adipose tissue, brain, adrenals, liver, breast, and hair. Daily production rates and serum concentration of E_2 in men are higher than those in postmenopausal women.

BASIC FINDINGS

The initial observations indicating that E_2 may be physiologically important for male gonadal function were presented at the end of 80's [Kula, 1988] and were confirmed recently [Ebling et al.,2000; Kula et al., 2001]. The classical view considering estrogens as an exclusively female hormone was definetely modified after the discovery of estrogen receptors (ER) in the male. Moreover, papers on transgenic mice with ER gene deprivation (ER knock out - ERKO) or with knock out of the gene coding aromatase, which is necessary for testosterone to estrogen conversion (ARKO), were published in 90-ties [Eddy et al., 1996]. Subsequently, the first studies on patients with inherited mutations of the gene encoding aromatase showed that in men, as in male animals, estrogens are mainly responsible for pubertal spurt in bone growth and epiphyseal closure after puberty [Carani et al., 1997]. More recent data indicate a possible role of estrogens and ER in the cardiovascular system function. Two types of estrogen receptors have been identified in men: ERα and ERβ [Mosselman et al., 1996]. It is currently known that both ERα and ERβ can be found in the cardiovascular system. In a 31-year-old man with inherited mutation of ERα, premature CAD was documented, suggesting a possible preventive role of estrogens for CAD [Sudhir et al., 1997]. Furthermore, as in women, a nongenomic influence of estrogens on cardiovascular system has been reported in men. In patients with inherited mutation of ERα resulting in the lack of estrogen activity, a rapid (within 5 minutes) relaxation of brachial artery after administration of E_2 was observed pointing to nongenomic, ER-independent action of this hormone in men [Goetz et al., 1999]. This is related to the stimulation by E_2 of the calcium-dependent potassium channels in the vascular smooth muscles.

Further chapters of this paper will deal with clinical correlations to ascertain a potential role of estrogens in the pathogenesis of ischemic heart disease in men.

ESTROGENS AND THE METABOLIC RISK FACTORS FOR CAD

Lipid profile

It has been documented that physiological levels of E_2 may influence serum lipoprotein concentration in healthy men *[Dai et al., 1994; Shono et al., 1996]*. Nevertheless, the results of these studies are contradictory. Shono et al. *[1996]* demonstrated that E_2 negatively correlates with the levels of low density lipoproteins fraction of cholesterol (LDL), suggesting a beneficial effect of this steroid. *Kiel et al. [1989]* correlated blood E_2 level with lipid profile both in healthy men and in men with angiographically confirmed CAD and showed a plausible, positive correlation between E_2 levels and the levels of high density lipoprotein fraction of cholesterol (HDL) in both groups of men. The same study showed, however, an unfavourable (positive) correlation between blood levels of E_2 and total cholesterol concentration. Other investigators did not find any correlations between E_2 and serum lipoprotein concentration in healthy men *[Duell and Bierman, 1990; Tchernhof et al., 1997]*.

In our prospective study of 111 men with a mean age of 55 years and angiographically confirmed CAD, unfavourable correlations between E_2 and total cholesterol, LDL and triglycerides were found. A significant positive correlation between E_2 blood levels and total cholesterol (p=0.005), LDL (p=0.004), total cholesterol/HDL ratio (p=0.02) and triglycerides (p=0.03) were present. All lipid factors are considered as important measures of atherosclerotic milieu. These correlations remained significant after adjustment for clinical covariates like age, hypertension, diabetes or prior hypertension in the multivariate logistic regression model. No associations were found between levels of the blood lipids and testosterone or dehydroepiandrosterone (DHEA) levels. These results indicated a possible role of E_2 in promoting the development of atherogenic lipid milieu in men with CAD *[Wranicz et al., 2005]*.

Our studies have not supported the findings on the reported cases of young men lacking E_2 due to an inherited mutation of the gene encoding aromatase, where the unfavourably increased levels of LDL were associated with decreased levels of HDL *[Morishima et al., 1995; Carani et al., 1997; Bileizikian et al., 1998]*. Supplementation of E_2 in these patients resulted in the normalization of lipoprotein profile *[Carani et al., 1997; Bileizikian et al., 1998]*, indicating a favourable effect of estrogens. This discrepancy can be explained by the profound differences in the clinical material. Thus, while in men with inherited estrogen deficiency increased susceptibility of ER to estrogen can be present since ER are not saturated, this unique prepubertal-like state is not present in adult men with normal secretion of E_2 *[Wranicz et al., 2005]*. Therefore, it seems that observations in men with inherited estrogen deficiency (due to mutation of the gene encoding aromatase), representing physiology during development, may have limited application to clinical practice representing pathology, where estrogens are available and act through functional ER.

Obesity and glucose levels

Overweight and obesity are well recognized risk factors for CAD in both men and women. The distribution of fatty tissue in humans shows sexual dimorphism. Females in the reproductive period of life tend to show gynoidal distribution of adipose tissue (hips, buttock), while androidal distribution predominates in men and in postmenopausal women. The former phenotype, with predominant androidal (trunk or visceral) obesity, is related to a higher risk of diabetes, insulin resistance, CAD and breast cancer development.

The relationship between estrogen deficiency and development of visceral obesity may be founded on the grounds that both in females and in males with inherited estrogen deficiency, androidal obesity with coexisting insulin resistance, hypercholesterolemia and hypertriglyceridemia were observed *[Morishima et al., 1995; 1997; Bileizikian et al., 1998]*. *Mendoza et al. [1983, 1986]* showed higher E_2 levels in healthy but obese men as compared to healthy men with normal body weight. These authors concluded that the profile of endogenous sex hormones observed in obese men (high E_2, low testosterone and high E_2/testosterone ratio) is similar to the one found in young postinfarction men, suggesting a possible unfavourable role of E_2, predisposing for CAD.

Coexistence of obesity and diabetes is frequent and unfavourable for the cardiovascular system. Nevertheless, the results of the few studies assessing a role of E_2 in the metabolism of glucose are contradictory. There are data showing that in healthy men E_2 levels negatively correlate with basal blood level of glucose *[Shono et al., 1996]*, while other authors did not confirm these results. Tchernhof et al. *[1997]* did not find any relationship between E_2 and glucose levels or the degree of visceral obesity (detected by computerized tomography). On the other hand, in men with angiographically documented CAD, unfavourable correlation between E_2 and impaired glucose tolerance was observed *[Dai et al., 1994]*.

ENDOGENOUS ESTROGENS VERSUS THE OCURRENCE, CLINICAL STAGE AND PREDICTION OF CAD IN MEN

Data regarding correlations between estrogens and CAD are also contradictory. Higher levels of E_2 and low testosterone/E_2 ratio were observed in men with CAD as compared to healthy controls, appearing together with signs of metabolic syndrome [Dunajska et al., 2004]. In the study of Philips et al. [1983, 1996] higher levels of E_2 were found in men with CAD than in healthy men. Girndt et al. [1980] documented higher E_2 levels in men with angiographically documented CAD as compared to healthy subjects. Srzednicki et al. [1990] also found significantly increased E_2 levels in patients with stable and unstable CAD.

Several other studies did not support the association between higher E_2 and CAD. Goldberg et al. [1987] and Lichtenstein et al. [1987] showed that blood E_2 did not differentiate group of healthy men and patients with CAD. Also Hauner et al. [1991] did not find any difference in E_2 levels between men with angiographically documented CAD and in subjects with no coronary atherosclerosis on angiography. The lack of correlation between E_2 and CAD is also supported by a study of Barrett-Connor and Khaw [1988] who evaluated a cohort of over 1000 healthy men during 12 years follow up and did not find E_2 levels to be predictive for CAD. Similar results based on over 10 years follow up were prescated by Contoreggi et al. [1990].

INTERVENTIONAL CLINICAL STUDIES

Chronic administration of estrogens in men with male-to-female transsexualism resulted in the reduction of total cholesterol, LDL and Apo-lipoproteins B levels, suggesting a plausible effect of estrogens in men [Sosa et al.,2004].

The influence of E_2 on vascular endothelium, causing blood vessels relaxation, indicates another plausible effect of estrogens. Chester et al. [1995] observed that E_2 provoked in vitro relaxation of coronary arteries what was less expressed in arteries taken from men than from women. The direct effect of E_2 on coronary arteries was also studied. Both relaxation and no influence were reported [Blumenthal et al., 1998; Reis et al., 1998]. In a randomized, double-blind, placebo controlled, cross-over study on 30 healthy men, vasodilation was not increased by E_2 administration [New et al., 2000]. On the other hand, intracoronary injection of E_2 in men undergoing coronary angioplasty significantly attenuated vasoconstriction by reducing endothelin levels [Lee et al., 2002].

"The Coronary Drug Project" research group (1973), a clinical study with administration of estrogens in men with CAD after myocardial infarction, was stopped before completion of the study due to increased incidence of mortality and subsequent myocardial infarctions after estrogen administration as compared to the controls. Similarly, in men with male-to-female transexualism treated with estrogens and anti-androgens orally venous thrombo-embolism was found as one of the most frequent side effects [Van Kesteren et al., 1997]. Recent clinical experience based on the hormonel replacement therapy in postmenopausal women and studies with administration of oral hormonal contraceptives to women showed an increase in thrombotic events related to the estrogenic component [Hulley et al., 1998]. Of course it can not be excluded that there may exist a difference between the effects of long-term therapy, which may represent the unfavourable effects of estrogen [Hulley et al., 1995; The Coronary Drug Project Reaserch Group, 1973; Van Kesteren et al., 1997] and the short term effects that may account for the non-genomic stimulation of vascular dilation [Chester et al., 1995; Blumenthal et al., 1998; Lee et al., 2002].

Much less is known about the influence of estrogen therapy in men on the biochemical risk factors of CAD, that are other than lipids. Thus it was observed that estrogen administration results in a decrease of homocystein levels [Giltay et al., 2003; Giri et al., 1998]. On the other hand, a favorable effect of estrogens on procoagulation activity was also showed. A decrease in plasminogen activation inhibitor and fibrinogen levels as well as a decrease in platelet activating factor activity were seen after estrogen treatment [Giri et al., 1998].

SEX–SPECIFIC DIFFERENCES IN ESTROGEN ACTION ON VASCULAR ENDOTHELIUM

In 2003, Gong et al. have shown that in contrast to women in whom estrogens generate nitric oxide (NO) production in the vascular endothelium, this effect was not seen in men. NO is responsible for vascular dilation and inhibits lipoprotein oxydation, monocyte adhesion to the endothelium, as well as production of the inflammation mediators. All these mechanisms of NO action prevent development of arteriosclerosis. Endothelial NO synthase (eNOS) is stimulated by HDL. However, according to Gong et al. [2003] eNOS is stimulated only by HDL separated from females. Female HDL is a carrier of E_2 and acts together with E_2 in the vascular endothelium stimulating eNOS through HDL receptor, called scavenger receptor class B type I which is, localised in the sinuses of vascular endothelium. As HDL separated from males is not a carrier of E_2 it does not stimulate eNOS in men. This difference may explain the lack of the relaxation effect of estrogen administration on coronary

blood vessels *[Reis et al., 1998; New et al., 2000]* or lack of correlations between blood E_2 concentration and the severity of CAD spectrum in men *[Goldnerg et al., 1987; Lichtenstein et al., 1987; Hauner et al., 1991; Barret-Connor and Khaw, 1998; Contoreggi et al., 1990]*.

CONCLUSIONS

Although in man with inherited mutation of gene encoding ERa (estrogen resistance) the occurrence of premature CAD was documented, and in men with estrogen deficiency (inherited lack of aromatase) an unfavourable lipid milieu was reported, the main body of both epidemiological and interventional studies suggests that in men estrogens may either not influence or may promote the develpoment of CAD. It is possible that the beneficial effect of estrogen administration on the lipid milieu in patients with estrogen deficiency, is limited only to this unique clinical situation. Thus, in the absence of endogenous estrogens an increased susceptibility of ER to estrogens should be present due to unsaturation of ER. This prepubertal-like state may represent a plausible effect of estrogen on the cardiovascular system during development, which may not fully apply to clinical pathology where estrogens are present in circulation and act through ER.

There may exist a sex defferences in the response to estrogens action as well as the discrepancy between short-term non-genomic effects and those of a long-term exposure to estrogens. Several reports are available indicating that estrogen administartion may have an unfavourable effect not only on blood lipid profile but also on venous thrombo-embolism in both sexes. In this context the role of estrogens in the regulation of cardiovascular system gains a special importance but further studies are needed.

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