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Expression of estrogen receptors α and β in paratesticular tissues in boys operated on for unilateral cryptorchidism between the 1st and 4th years of life

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The aim of this study was to assess the expression of estrogen receptors α and β in paratesticular tissues in a group of boys with and without cryptorchidism, and evaluation of karyotypes, localization, morphology and the major length of the undescended testes.

Material/Methods: Fifty boys (1–4 years old) with unilateral cryptorchidism were evaluated. Fifty healthy boys within the same age range, with inguinal hernia, served as a control group. Measurements concerning expression of ERα ERβ receptors were preformed using monoclonal mouse antibodies against human receptor α and β.

Results: In the mesothelial layer, the expression of ERα was higher in the patients group with undescended testes and it was statistically significant (p=0.04). There was no difference in the expression of ERβ in this layer between groups. In the stromal cell layer there was statistically significant higher expression of ERβ (p<0.05) in the group of patients with undescended testes.

Conclusions: There was no difference between expressions of ERα in stromal cell layer. In the endothelial layer there was no difference in expression of ERα and ERβ. In the smooth muscle layer there was no expression of ERα in either group. The expression of ERβ in the smooth muscle layer was nearly identical in both groups. Undescended testes were generally found in the superficial inguinal pouch (n=46). The major lengths of the undescended testes were smaller in comparison to the testes positioned normally. In 9 of the cases the testes had different shape, and turgor deficit, and epididymides were smaller, dysplastic and separated from the testis.

key words: estrogen receptor • cryptorchidism • gonadal function • orchidopexy • testis descent

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Background

There is growing evidence from clinical and epidemiological studies for an increasing incidence of male reproductive disorders (ie, cryptorchidism). The etiology of undescended testis is still surrounded by much controversy. Approximately 90% of cases of cryptorchidism occur spontaneously or from unknown causes. The process of descending of the testes is one of the most important factors in spermatogenesis in mature testis. This suggests that environmental or lifestyle, rather than genetic, factors are plausible causes [1]. Indeed, mutations in the Insl3 or LGR8 *loci* do not seem to represent a frequent cause of human cryptorchidism [2].

A broad expression of estrogen receptors (ERs) in the testis suggests an important role of estrogens in regulating testicular cell function and reproductive events. Estrogen is a key regulator of growth and differentiation in a broad range of target tissues – the reproductive tract, mammary gland, and the central nervous and skeletal systems [3,4]. Estrogen is also known to be involved in many pathological processes such as breast and endometrial cancer, and osteoporosis [5,6]. The major source of endogenous estrogen in men is adipose tissue, but the receptor proteins (ERα and ERβ) are localized in most cell types in the testis in concordance with a physiological role for estrogen in testicular development and function [7]. The presence of an estrogen binding receptor protein – ERα – was first reported in 1962 [8]. In 1996, an additional estrogen receptor – ERβ – was cloned from rat prostate [9]. ERβ were cloned from many species, including humans [10,11]. ERα and ERβ belong to the superfamily of nuclear receptors and specifically to the family of steroid receptors that act as ligand-regulated transcription factors [12,13]. ERα and ERβ have different biological functions and different phenotypes [14]. Abnormal estrogen action has been implicated as a possible cause for sporadic cryptorchidism in humans [15]. Animal studies support the human correlations. In mice, *in utero* exposure to estradiol induces cryptorchidism [16–19]. Estradiol is known to inhibit androgen production, either by limiting the development and growth of Leydig cells, or by directly inhibiting the activities of several steroidogenic enzymes involved in testosterone synthesis [20]. Estradiol is produced not only by the mother, but also in significant amounts by Sertoli cells [21,22]. In addition, testes concentrate estradiol as much as 10- to 50-fold higher than in peripheral blood [23]. Despite the above facts, the intra-abdominal position of the testes in estrogen-treated mice is due to the absence of Insl3 hormone, but not of androgens. Estrogens block the first phase of testicular descent (transabdominal descent), whereas androgens control only the second, inguinoscrotal, phase [24–26]. The first phase of typical testicular descent takes place between the 10th and 15th weeks of human gestation [27]. This occurrence is independent of androgen levels, as the process has been found to transpire in both animals and humans with complete androgen insensitivity, and is believed to be influenced by AMH (anti-Müllerian hormone) and insulin-like hormone 3 (INSL3) [28,29]. INSL3 is secreted by Leydig cells shortly after the onset of testicular development, and controls the thickening of the gubernaculum anchoring the testis to the inguinal region [30]. Disruption of the INSL3 gene in mice results in bilateral intra-abdominal testes [25,31]. In humans, it was found that only 1.9% of the cases of cryptorchidism were caused by INSL3 gene mutations, and that the mutations of the INSL3 receptor on the whole were uncommon [32,33]. The second, or inguinoscrotal, phase of testicular descent occurs between the 26th to 40th weeks of gestation [27]. During this phase, the testes migrate through the inguinal canal and across the pubic region to the scrotum. The testis and epididymis then remain within the diverticulum of the peritoneum, which elongates within the gubernaculum [34]. Furthermore, the gubernaculum, growing out of the abdominal wall, might be under the control of Hox genes – a group of genes that determines the basic structure and orientation of an organism. Disruption of some Hox genes in mice has been shown to lead to cryptorchidism, but the relevance of this observation is debatable in human studies of cryptorchidism [35]. On the other hand, there is much clinical evidence that shows reduced androgen action to be associated with undescended testes [36].

In the present study we assessed expression of estrogen receptors α and β in paratesticular tissues in a group of boys with and without cryptorchidism. We evaluated the karyotypes of these boys, as well as the position, morphology and diameters of the undescended testes.

Material and Methods

Study population

Fifty boys aged 1–4 years (median=2,4 y.) with unilateral cryptorchidism, and without previous human chorionic gonadotropin treatment, were evaluated. All of them underwent orchidopexy in 2010. Abnormal karyotype, as well as the presence of any endocrine disorders, and hormonal drugs intake, constituted grounds for exclusion from the study. Prior to their orchidopexy, all of the subjects had their karyotypes (to exclude chromosomal abnormalities) evaluated. During the actual orchidopexy, the gubernaculum samples were collected, the position and morphology of the testes were evaluated, and their diameters were measured.

Control group

Fifty healthy boys aged 1–4 years (median=2,1 y.), admitted to the Pediatric Surgery Department for planned inguinal hernia repairs in 2010, served as controls. All boys in the control group had their testes in the scrotum. Their karyotypes were also determined prior to their procedures. The samples of the diverticulum of the peritoneum were collected during herniotomy.

Data were collected from patients admitted to the Pediatric Surgery Department for either a planned orchidopexy or hernia repair. All parents of the patients gave informed consent for both clinical and histological follow-up. Tissue samples – gubernaculum and diverticulum of peritoneum – were collected during planned surgeries. Measurements of expression of ER α ER β receptors were preformed using monoclonal mouse antibodies against human receptor α, (Monoclonal Mouse Anti-Human Estrogen Receptor α, clone 1D5, of IgG1 kappa isotype), and β (Monoclonal Mouse Anti-Human Estrogen Receptor β clone PPG5/10), respectively. In further stages we used anti-mouse antibody combined with biotin and peroxidase, and visualized by using FLEX+ Mouse, High pH (K8002/K8012) for alpha

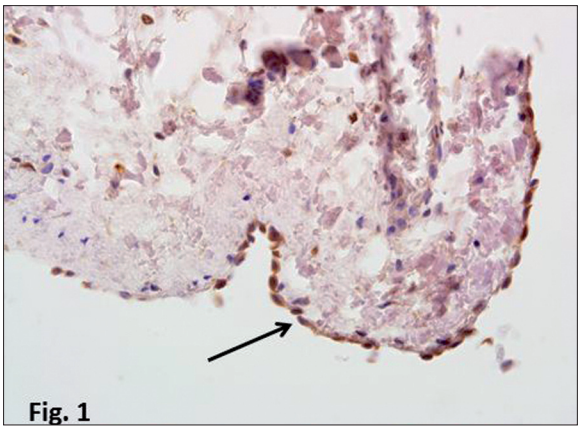

Figure 1. Strong nuclear Er alpha receptor expression in the mesothelial cells (cryptorchidism). Magn 200×.

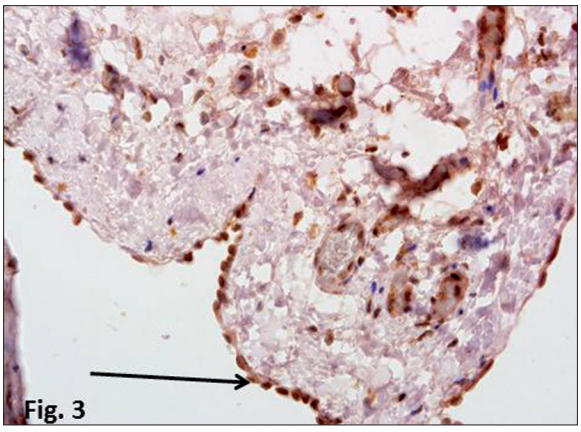

Figure 3. Strong nuclear Er beta expression within the mesothelial cells (control group). Magn. 200×.

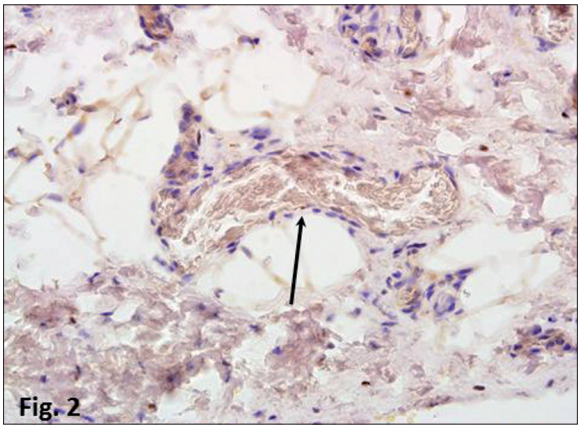

Figure 2. Weak Er alpha expression in the endothelial cells of the small blood vessels (control group). Magn 400×.

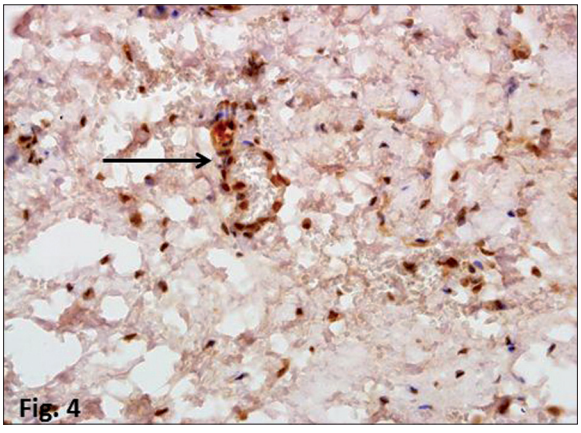

Figure 4. Strong nuclear Er beta immunoexpression in the endothelial cells of the blood vessels (cryptorchidism). Magn. 400×.

receptors and DAKO LSAB+/HRP, (K 0679) and DAKO EnVision+/HRP (K4004 and K4006) for beta. Sections were counter-stained with haematoxylin. The results were expressed as the percentage of positive cells with a strong positive receptor staining and labeled as follows: negative (–) with ⩽10% of positive cells, positive (+) with 11% to 50% of positive cells, and highly positive (++) with ⩾51% positive cells, in a set of 10 random fields under 20× magnification.

The study was approved by the local Ethics Committee as an audit of a clinically agreed-upon protocol of investigation and treatment.

Statistical analyses

Statistical analyses were carried out using Statistica 10.0 StatSoft. To compare observed data we used chi-square test and Pearson's correlation. P values less than 0.05 were considered significant.

Results

There was no statistically significant difference in the age distribution of the 2 groups. All boys had karyotypes 46XY. The undescended testes were mainly localized in the inguinal canal (n=46), but in 2 of the instances were located in the external ring of the inguinal canal, while 2 more subjects had theirs in the abdominal cavity. The overall lengths of the undescended testes differed from 0.8 cm to 2 cm, and in most cases were found to be smaller in comparison to the testes positioned normally (mean 1cm and mean 1.5 cm, respectively). In 9 of the cases of cryptorchidism, the testes had different shape (drop-like), and the epididymides were small, dysplastic and separated from the testis.

We measured expression of Erα and ERβ in paratesticular tissues: in the mesothelial layer, stromal cells, endothelial layer, and smooth muscle layer.

In the mesothelial layer there was a statistically significant ($p=0.04$) difference in the expression of ERα. The expression of ERα was higher in undescended testes. In 71% of the cases of the cryptorchidism we found a high expression of the ERα. In the inguinal hernia group the strong expression (++) were found only in 18% of the cases. We also found that in 32% of the inguinal hernia patients there was no ERα expression at all. There was no difference in the expression of ERβ in the mesothelial layer between the 2 groups.

In the stromal cell layer there was statistically significant higher expression of ERβ ($p<0.05$) in undescended testes. In these cases, 62% of patients with undescended testes had

a strong ERβ expression. There was no statistically significant difference between expressions of ERα in stromal cell layer between the 2 groups.

In the endothelial layer there was no statistically significant difference in expression of ERα and ERβ between the 2 groups. In the both groups, expression of the ERα and ERβ in most of the cases were only positive (+).

In the smooth muscle layer, there was no expression of ERα in both groups. The expression of ERβ in the smooth muscle layer was nearly identical in the group of boys with inguinal hernia and in boys with unilateral cryptorchidism: no expression in 7 and 10 cases, normal expression in 32 and 34 cases, and high expression in 11 and 6 cases, respectively.

Figures 1–4 shows the expression of the alpha and beta receptors in the particular layers in both groups.

Discussion

In this study we focused on ERα and ERβ expression in paratesticular tissues of cryptorchid testes. There are few reports about the relationship between ERs and spermatogenic failure in cryptorchidism [37,38]. It was shown that testosterone level was lower and estradiol level was higher in the cryptorchid than in normal testes by radioimmunological analysis of testicular tissue [37,39]. Studies in rodents have revealed that ERα is predominantly expressed in the pituitary, uterus, ovary, mammary gland, testis, epididymis and kidney; whereas ERβ is predominant in hypothalamus, prostate, lung, and bladder [40]. In our study we found expression of ERα and ERβ in the mesothelial layer, stromal cells, and the endothelial layer of paratesticular tissues of normal and undescended testes. In the smooth muscle layer there was no expression of ERα and nearly identical expression of ERβ. Mizuno et al showed increased expression of ERα in cryptorchid testes, suggesting that estradiol level was increased in the cryptorchid testes because estrogens upregulate the expression of the ERα gene in most mammalian tissues [37,41]. We also observed higher expression of ERα in the mesothelial layer of paratesticular tissues of undescended testes. In our study we found also higher expression of ERβ in the stromal cell layer of paratesticular tissue in undescended testes. Excess intratesticular estrogens inhibit spermiation [37,42]. An estrogen excess can decrease testicular androgen production by lowering the activity of steroidogenic enzymes that convert progesterone to testosterone [43]. The estrogens also act as permanent organizing agents during male fetal development, and as reversible regulators in adult life. Strauss et al. have mentioned the importance of androgen-estrogen balance for male fertility and reproductive tract function. Using transgenic male mice that express human aromatase, they demonstrated that chronic imbalance in the androgen-estrogen ratio leads to severe abnormalities in the development, structure, and function of mouse Leydig cells [37,44]. In vertebrates, estradiol also inhibits Leydig cell precursor development. By reducing the number or volume of Leydig cells in the developing testis, testosterone production is compromised, with impaired masculinization (undescended testis, hypospadias) and spermatogenic progression [45]. In estrogen-treated mice, the intra-abdominal position of the testes is due to the absence of Insl3 hormone, but not of androgens. Estrogens block the first phase of testicular descent (transabdominal descent), which is controlled hormonally by Insl3 [30]. As a possible alternative mechanism, other authors suggested that *in utero* exposure to diethylstilbestrol can probably induce resistance to AMH, which is responsible not only for the apoptosis of the Müllerian ducts, but also plays a role in testicular descent [46]. In contrast to ERβ mutant mice, testicular descent in ERα mutant mice was not affected by *in utero* exposure to estradiol. The transabdominal descent appeared to be completed [1]. Insl3 transcription was fully restored in the absence of ERα, but not in the absence of ERβ, even in the presence of saturating levels of exogenous estrogens, strongly suggesting that ERα mediates Insl3 down-regulation and subsequent cryptorchidism upon exposure to xenoestrogens *in utero* [1]. Estradiol inhibits testicular descent via ERα by acting directly on fetal Leydig cells. This fact correlates with ERα expression in Leydig cells. ERα is expressed in fetal Leydig cells until birth, whereas ERβ is present in gonocytes, Sertoli cells, and Leydig cells, and this receptor subtype appears to remain in these cells until birth [1,47]. It has been shown that endogenous estrogen physiologically inhibits steroidogenesis via ERα by acting directly on fetal Leydig cells [1,48]. ERα-deficient mice display higher levels of testicular testosterone due to hypertrophy of fetal Leydig cells, and increased expression of Star, Cyp17a1, and P450scc genes.

Unilateral cryptorchidism carries an increased risk of infertility in adulthood [32]. Up to 30% of men operated on in childhood for unilateral cryptorchidism are likely to be subfertile in later life [49]. Men who undergo an operation for bilateral cryptorchidism are more affected – up to 54% are infertile according to their semen and hormonal analysis [50]. The position of the testes at the time of orchidopexy is also important. In fact, a lack of fertility has been reported in men who underwent bilateral abdominal orchidopexy in childhood [51]. In our study, mean diameters of undescended testes were smaller in comparison to the normally developing ones (1×0.5 cm and 1.5×0.8 cm, respectively).

Testicular size and sperm density are positively correlated to germ-cell status in the cryptorchid testes in childhood [52]. Estrogenic exposure may, through ERα, inhibit the activation of Insl3 and steroidogenic genes in fetal Leydig cells.

If estrogen underlies sporadic cryptorchidism, then it is likely that these effects are mediated by smaller doses localized to the correct target tissue at the precise time, thus achieving maximal effect. It may be possible that a small excess of free estradiol at the right developmental stage may have a strong impact on testicular descent [30]. The main argument against estrogens as potential factors in impaired testicular descent is the lack of persistent Müllerian structures in affected humans [30].

Conclusions

Our results show that estrogens are potential mediators of cryptorchidism, and the inhibitory effects of estrogens on testicular descent may be mediated via ERα and ERβ.

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Statement

Authors do not declare any conflict of interest.

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