First International Journal of Andrology
ANDROLOGIA

ORIGINAL ARTICLE

Oestrogen receptor alpha gene polymorphisms relationship with semen variables in infertile men

A. Zalata[1], H. A. Abdalla[1], Y. El-Bayoumy[2] & T. Mostafa[3]

1 Department of Medical Biochemistry, Faculty of Medicine, Mansoura University, Mansoura, Egypt;
2 Department of Dermatology & Andrology, Faculty of Medicine, Mansoura University, Mansoura, Egypt;
3 Department of Andrology & Sexology, Faculty of Medicine, Cairo University, Cairo, Egypt

Keywords
Hormones—male infertility—oestrogen—polymorphism—semen

Correspondence
Prof. Dr. Taymour Mostafa, Department of Andrology & Sexology, Faculty of Medicine, Cairo University, Cairo 11562, Egypt.
Tel. +2 01005150297;
Fax: +2 23654133;
E-mail: taymour1155@link.net

Accepted: April 8, 2013

doi: 10.1111/and.12123

Summary

This study aimed to assess the association of oestrogen receptor alpha (ER-α) gene polymorphisms and semen variables in infertile oligoasthenoteratozoospermic (OAT) men. In all, 141 men were grouped into fertile men ($n = 60$) and infertile OAT men ($n = 81$). They were subjected to assessment of semen analysis, acrosin activity, serum reproductive hormones and genotyping of ER-α gene. Frequencies of p and x alleles in ER-α gene PvuII and XbaI polymorphisms were more prevalent among fertile men compared with infertile OAT men. Presence of P and X alleles was associated with increased incidence of male infertility for genotypes PP, XX compared with genotypes pp and xx (OR = 2.8; 95% CI: 2.36–6.97; $P = 0.001$ and OR = 4.1, 95% CI: 1.49–11.39; $P = 0.001$, respectively). The mean of semen variables and sperm acrosin activity were significantly higher in cases associated with pp than PP and in xx than XX genotypes of ER-α gene. Mean levels of all serum reproductive hormones demonstrated nonsignificant differences in different ER-α genotypes except oestrogen that was elevated in PP and XX ER-α gene genotypes. It is concluded that as oestrogen is concerned in male gamete maturation, ER-α gene polymorphisms might play a role in the pathophysiology of male infertility.

Introduction

The traditional view of oestradiol as the 'female' hormone and of testosterone as the 'male' hormone has been challenged due to the increased interest in elucidating the role of oestrogens in males. Additionally, the increased interest in the role of oestrogen in male reproduction stems from various reports that exposure to oestrogens in the environment may have detrimental effects on the male reproductive development and/or health (Foster *et al.*, 2008; Zalata *et al.*, 2008; Hofny *et al.*, 2010).

Oestrogen receptors (ERs) are members of the nuclear receptor (NR) superfamily that mediates the pleiotropic effects of oestrogen in a diverse range of developmental and physiological processes playing an important role in mediating oestrogen action on target tissues (El-Shafei *et al.*, 2011; Filipiak *et al.*, 2013). Two subtypes of ERs are known; ER-α encoded by the ESR1 gene on chromosome 6 and ERβ encoded by the ESR2 gene on chromosome 14 (Enmark *et al.*, 1997). ER-α, the first identified and the most abundant one, is found in all human reproductive tissues. ER-α knockout male mice resulted in impaired spermatogenesis and sperm production because of testicular atrophy and dilation of efferent ductules due to inhibited fluid resorption (Hewitt & Korach, 2003; Carreau *et al.*, 2010).

Kukuvitis *et al.* (2002) suggested that ESR1 PvuII and XbaI polymorphisms have an effect on azoospermic or idiopathic severe oligozoospermic men. Guarducci *et al.* (2006) showed that specific allelic combinations of ER-α, which confer a stronger oestrogen effect, may negatively influence human spermatogenesis. Su *et al.* (2010) added that polymorphisms of oestrogen-related genes jointly confer susceptibility to human spermatogenic defect at the pre-receptor, receptor and post-receptor levels. Lately, Guido *et al.* (2011) added a role for E_2/ERs in human sperm physiology in modulating sperm metabolism and detrimental effects related to the pathophysiology of varicocele association where Wang *et al.* (2011) pointed to that spermatogenic arrest may be related to a complex

series of disorders in cell signal transduction involving androgen receptor (AR) and ER-α.

This study aimed to assess the association of ER-α gene polymorphisms and semen variables in infertile men.

Materials and methods

This study included 141 Egyptian men after IRB approval and informed consents. They were grouped into healthy fertile men ($n = 60$) and infertile oligoasthenoteratozoospermic men (OAT) ($n = 81$). Fertile men were healthy volunteers that achieved conception within 1 year with normozoospermic semen parameters. Inclusion criteria included the same ethnic origin (Caucasians). Exclusion criteria were varicocele, hormonal therapy, hypogonadism, smoking, Y chromosome deletions and karyotype abnormalities.

All men were subjected to history taking, clinical examination and semen analysis. Semen analysis was carried out twice 10 days apart after 4–5 days of sexual abstinence using computer-assisted semen analysis (Autosperm) (Fertipro, Beernem, Belgium) according to WHO guidelines (2010). Sperm morphology was evaluated by phase contrast microscope. Spermatozoa were separated using Sil-select gradient (Fertipro N.V., Industriepark Noord, Beernem, Belgium), and the purified spermatozoa were used for assessment of acrosin activity assessment. In addition, serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), prolactin (PRL) and oestrogen (E_2) were estimated in addition to genotyping of ER-α.

Acrosin activity assessment

Gelatin-covered slides were prepared by spreading 20μl 5% gelatin (Merck, Darmstadt, Germany) in distilled water on the slides that were then air-dried, stored at 4 °C overnight, fixed and washed in phosphate-buffered saline (Zalata *et al.*, 2012). Purified spermatozoa were diluted 1 : 10 in PBS containing 15.7 mM α-D-glucose. Semen samples were smeared on prepared slides and incubated in a moist chamber at 37 °C for 2 h. The halo diameter around any 10 spermatozoa in the ejaculate was measured in phase contrast with an eyepiece micrometer. The halo formation rate was calculated/slide as the percentage of spermatozoa showing a halo evaluating 100 spermatozoa. Acrosin activity index was calculated by multiplying the halo diameter X the halo formation rate.

Reproductive hormones estimation

Blood samples were used to separate sera for FSH, LH, PRL by enzyme-linked immunosorbent assay where serum total T and E_2 were estimated by enzyme immunoassay (Diagnostics Systems Laboratories, Webster, TX, USA).

Genotyping of ER-α

DNA was extracted from EDTA anti-coagulated blood for PCR amplification (Cai *et al.*, 2003). Reagents: (i) PCR-Master-Mix Y (PeQLab Biotechnologie GmbH, Erlangen, Germany). 2x PCR-Master-Mix Y with 1.25U Taq DNA polymerase/25 μl, 40 mM Tris–HCl (pH 8.55 at 25 °C), 32 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.02% Tween 20, 0.4 mM dNTPs mix (dATP, dCTP, dGTP, dTTP); (ii) sterile double-distilled water; (iii) two primers (Eurofins MWG Operon, Ebersberg, Germany).

Forward primer: 5′- CTGCCACCCTATCTGTATCTTT TCCTATTCTCC – 3′.

Reverse primer: 5′- TCTTTCTCTGCCACCCTGGCGT CGATTATCTGA – 3′.

Oligonucleotide primers were stored in the form of aliquots at −30 °C until the time of use. Stock solution was diluted with sterile water to reach l0 pmol μl^{-1}. Thermal cycler was used for amplification. The following mix was prepared for each sample: 25 μl 2× PCR-Master-Mix Y, 30 pmol forward primer (3 μl), 30 pmol reverse primer (3 μl), 3 μl template DNA, 16 μl sterile double-distilled water to reach 50 μl. This mix was put in a thin wall PCR microcentrifuge tube. The tube was mixed gently and centrifuged at 1957 ***g*** for 10 s. Amplification was performed with initial denaturation at 94 °C for 3 min, 36 cycles of denaturation at 94 °C for 45 s then annealing at 61 °C for 45 s then extension at 72 °C for 2 min and final extension at 72 °C for 7 min.

Restriction endonuclease PvuII analysis: 0.5 μl (6 U) of the restriction endonuclease PvuII (Mbiotech Inc., Seoul, Korea), 10 μl amplified PCR product, 2.0 μl buffer B [6 mM Tris–HCl (pH 7.5 at 37 °C), 6 mM MgCl2, 50 mM NaCl, 1 mM DTT] and 7.5 μl sterile distilled water were used. The reaction mixture was incubated in 37 °C water bath for 16 h then subjected to agarose gel electrophoresis (8 μl restriction analysis + 2 μl loading dye) using 2% agarose gel containing 2 μl 10 mg ml^{-1} ethidium bromide for 1 h at 70 V. In agarose gel of digested products of ER-α gene by PvuII restriction enzyme, PP genotype indicated absent PvuII restriction site from both alleles giving one band at 1.30 kb, pp genotype indicated presence of PvuII restriction site on both alleles, giving two bands at 0.85, 0.45 kb and Pp genotype indicated presence of PvuII restriction sites on one of the two alleles, giving three bands at 1.30, 0.85, 0.45 kb (Fig. 1).

Restriction endonuclease XbaI analysis: 1 μl (10 U) of the restriction endonuclease XbaI (Fermentas Inc, Glen Burnie, MD, USA), 10 μl amplified PCR product, 2.0 μl 10 × buffer tango [33 mM tris-acetate (pH = 7.9), 10 mM

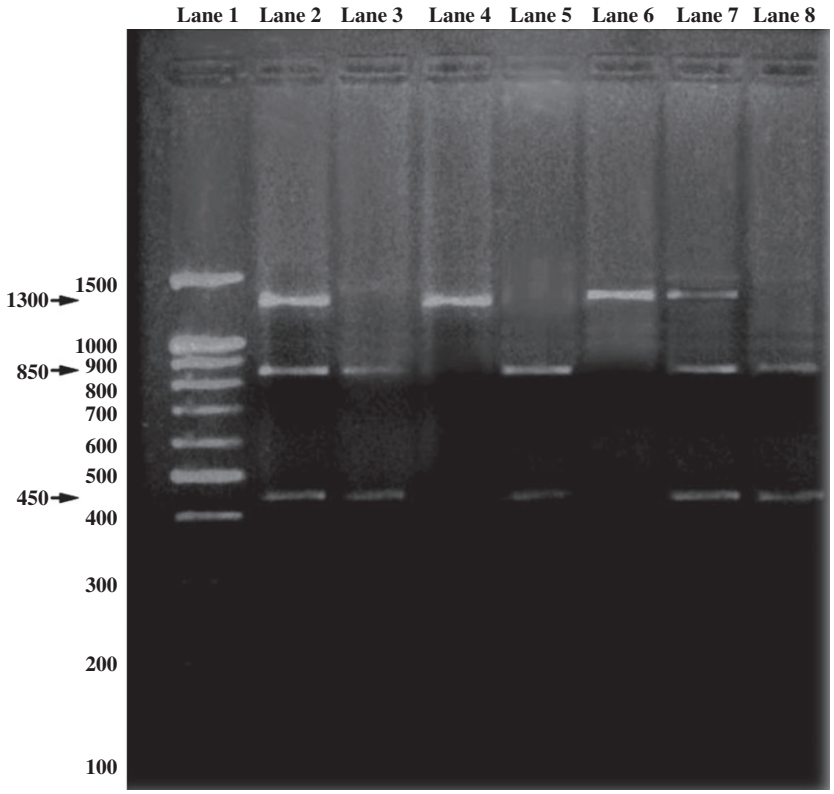

Fig. 1 Agarose gel of digested products of ER-α gene by PvuII restriction enzyme. PP genotype indicated absence of PvuII restriction site from both alleles giving one band at 1.30 kb (lanes 4, 6). pp genotype indicated presence of PvuII restriction site on both alleles, giving two bands at 0.85 kb, 0.45 kb (lanes 3, 5, 8). Pp genotype indicated presence of PvuII restriction sites on one of the two alleles, giving three bands at 1.30, 0.85, 0.45 kb (lanes 2, 7). Lane 1: DNA marker (100 bp DNA Ladder).

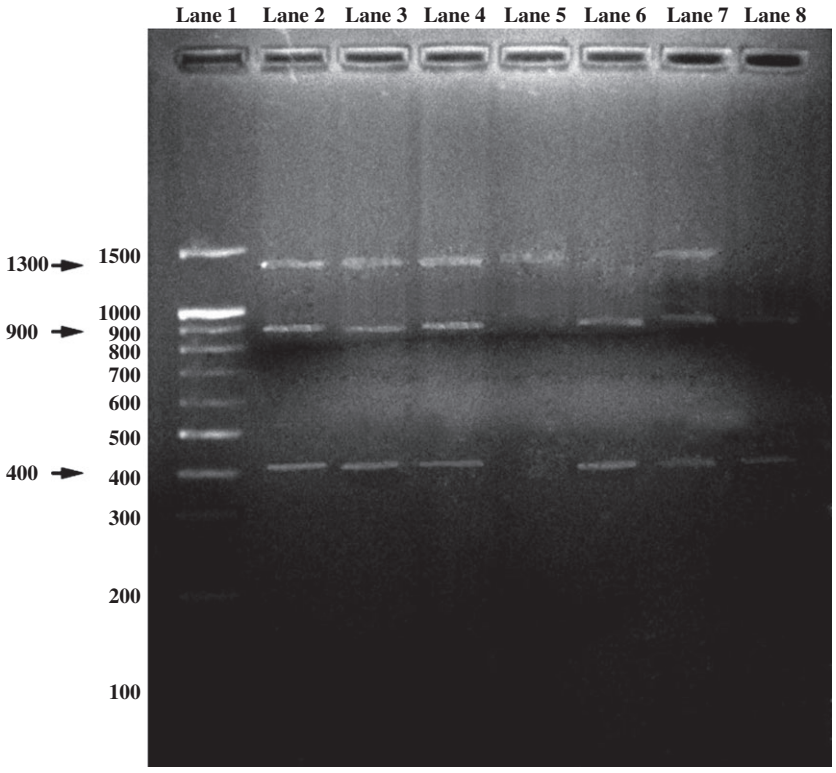

Fig. 2 Agarose gel of digested products of ER-α gene by XbaI restriction enzyme. XX genotype indicated absence of XbaI restriction site from both alleles giving one band at 1.30 kb (lane 5). xx genotype indicated presence of XbaI restriction site on both alleles, giving two bands at 0.90, 0.40 kb (lanes 6, 8). Xx genotype indicated presence of XbaI restriction site on one of the two alleles, giving three bands at 1.30, 0.90, 0.40 kb (lanes 2, 3, 4, 7). Lane 1: DNA marker (100 bp DNA ladder).

Table 1 Semen parameters of fertile and infertile men (median, range)

	Fertile men (n = 60)	OAT men (n = 81)
Sperm count (10^6 ml^{-1})	70.4 (64.0–76.8)	8.15 (6.3–9.3)[a]
Sperm motility (%)	61.0 (59.0–63.0)	12.0 (9.2–20.0)[a]
Sperm velocity (μm s^{-1})	79.6 (73.9–80.3)	36.2 (26.9–38.0)[a]
Sperm linear velocity (μm s^{-1})	62.3 (60.3–63.9)	20.3 (13.4–22.2)[a]
Sperm linearity index (μm s^{-1})	82.0 (77.0–84.5)	60.3 (52.2–64.0)[a]
Sperm normal morphology (%)	64.0 (62.0–64.0)	2.0 (2.0–4.0)[a]
Acrosin activity index	12.3 (11.1–12.4)	1.2 (1.0–1.6)[a]

[a]Significant difference compared with the controls.

magnesium acetate, 66 mM potassium acetate, 0.1 mg ml^{-1} (BSA)] and 18 μl sterile distilled water. The reaction mixture was incubated in 37 °C water bath for 16 h then subjected to agarose gel electrophoresis (8 μl restriction analysis + 2 μl loading dye) using 2% agarose gel containing 2 μl 10 mg ml^{-1} ethidium bromide for 1 at 70 V. In agarose gel of digested products of ER-α gene by XbaI restriction enzyme, XX genotype indicated absence of XbaI restriction site from both alleles giving one band at 1.30 kb, xx genotype indicated presence of XbaI restriction site on both alleles, giving two bands at 0.90, 0.40 kb, Xx genotype indicated presence of XbaI restriction site on one of the two alleles, giving three bands at 1.30, 0.90, 0.40 kb (Fig. 2).

Statistical analysis

It was carried out using SPSS program version 17 (SPSS Inc., Chicago, IL, USA). The data were expressed as median and range. Mann–Whitney test was used for comparisons, Spearman rank correlation coefficient was used to study the relation between variables and chi-square test for frequency tables where odds ratio calculated the outcome in two groups. $P < 0.05$ was set as significant.

Results

The estimated variables of the investigated groups were represented in Table 1. Frequencies of ER-α gene PvuII polymorphisms demonstrated that p allele was more prevalent among fertile men compared with infertile OAT men where 31.7% of fertile and 19.8% of OAT men were homozygous and 45% of fertile men and 39.5% of OAT are heterozygous for this allele. The presence of P allele was associated with increased risk of infertility compared with genotype pp (OR = 2.8; 95% CI: 2.36–6.97; P = 0.05). Frequencies of ER-α gene XbaI polymorphisms demonstrated significant increase in x allele distribution among fertile than OAT men where 33.3% of fertile men and 21.0% of OAT men were homozygous and 53.5% of fertile men and 44.4% of OAT men are heterozygous for this allele. The presence of X allele was associated with increased risk of infertility compared with genotype xx (OR = 4.1, 95% CI: 1.49–11.39; P = 0.001) (Table 2).

The median of the sperm count, sperm linear velocity, linearity index, normal sperm morphology, acrosin activity index was significantly higher in individuals with pp than PP genotype of ER-α gene PvuII polymorphisms and also significantly higher in xx than XX genotype of ER-α gene XbaI polymorphisms. Median serum E_2 level was significantly increased in individuals with pp compared with those with PP and in xx genotypes of ER-α gene PvuII polymorphisms compared with those with XX genotypes of ER-α gene XbaI polymorphisms (Tables 3 and 4).

Discussion

The present study revealed that ER-α gene polymorphisms at the restriction sites PvuII (pp genotype) and XbaI (xx genotype) were associated with significant

Table 2 Polymorphisms of ER-α PvuII and XbaI genes in the studied groups (n = 141)

	Fertile men (n = 60)	OAT men (n = 81)	OR	95% CI	P
ER-α PvuII					
PP	14 (23.3%)	33 (40.7%)	2.8	2.36–6.97	0.001[a]
Pp	27 (45%)	32 (39.5%)	1.4	0.61–3.26	>0.05
pp	19 (31.7%)	16 (19.8%)	1	ref	
P (chi-Square test)	<0.05[a]				
ER-α XbaI					
XX	8 (13.3%)	28 (34.6%)	4.1	1.49–11.39	0.001[a]
Xx	32 (53.4%)	36 (44.4%)	1.3	0.59–2.95	>0.05
xx	20 (33.3%)	17 (21.0%)	1	ref	
P (chi-Square test)	<0.01[a]				

[a]Significant.

Table 3 Tested variables associated with polymorphisms of ER-α PvuII and ER-α XbaI genes (median, range)

	ER-α PvuII genes			ER-α XbaI gene		
	PP (n = 47)	Pp (n = 59)	pp (n = 35)	XX (n = 36)	Xx (n = 68)	xx (n = 37)
Sperm count (10^6 ml^{-1})	14.6 (9.1–18.6)	17.1 (9.6–55.9)	50.4 (15.3–63.2)[a,b]	9.6 (7.8–16.6)	18.7 (10.4–59.7)[c]	50.4 (16.3–69.9)[c,d]
Sperm motility (%)	30.0 (20–37.0)	31 (21.5–57.0)	53 (26.9–59.0)[a,b]	24.0 (11.7–30.3)	32.0 (17.2–57.0)	53.0 (31.1–58.9)[c,d]
Sperm velocity (μm s^{-1})	43.4 (37.5–66.8)	61.3 (39.7–69.9)[a]	70 (44.4–78.4)[a]	41.8 (33.1–54.4)	62.4 (38.0–69.9)[c]	73.9 (61.5–75.5)[c,d]
Sperm linear velocity (μm s^{-1})	22.4 (21.2–38.3)	35.8 (22.7–54.4)	52.6 (31.8–60.8)[a,b]	22.3 (20.0–33.9)	35.9 (23.0–55.2)	52.6 (35.8–60.3)[c,d]
Sperm linearity index	64 (59.6–71.8)	74 (67.6–78.9)[a]	77 (68.1–83.4)[a]	62.7 (51.8–70.2)	76.9 (70.4–79.2)	74.0 (64.2–83.7)
Sperm normal morphology (%)	6 (2.0–12.0)	10 (4.0–62.0)	60 (6.5–62.0)[a,b]	4.0 (2.0–10.0)	12.0 (4.0–62.0)[c]	60 (8.3–62.0)[c,d]
Acrosin activity index	2.3 (1.3–5.1)	3.1 (1.3–9.8)[a]	9.7 (2.9–11.4)[a,b]	2.0 (1.2–3.4)	3.1 (1.3–9.8)[c]	9.7 (4.7–12.3)[c,d]
Serum FSH (mIU ml^{-1})	8.6 (8.2–9.9)	7.1 (6.4–8.8)	8.3 (6.4–8.9)	9.2 (8.4–11.4)	8.4 (6.5–9.1)	8.3 (6.4–8.9)
Serum LH (mIU ml^{-1})	6.4 (6.1–7.3)	6.4 (5.7–6.5)	6.4 (5.6–7.5)	6.5 (6.2–7.7)	6.3 (5.4–6.4)	6.4 (6.3–7.5)
Serum PRL (ng ml^{-1})	5.4 (4.5–6.4)	6.4 (5.4–6.6)	6.4 (4.7–7.4)	6.4 (5.2–7.4)	6.4 (5.1–6.4)	5.4 (4.7–6.4)
Serum T (ng ml^{-1})	6.0 (5.1–6.7)	6.8 (5.9–7.9)	7.3 (6.0–8.3)	5.9 (4.7–6.8)	6.8 (5.9–7.9)	7.2 (5.9–8.4)
Serum E_2 (pg ml^{-1})	37 (32.3–44.4)	34 (33–37.0)	28 (24.0–36.0)[a,b]	36.0 (31.6–44.3)	35.0 (33.0–37.0)	29 (27.0–33)[c,d]

[a]Significant difference compared with PP group.
[b]Significant difference compared with Pp group.
[c]Significant difference compared with XX group.
[d]Significant difference compared with Xx group.

increase in seminal sperm count, sperm linear velocity, linearity index, normal sperm morphology and acrosin activity index. Possible explanations of how human male fertility is affected by the intronic PvuII and XbaI polymorphisms of the ER-α gene could include the following:

1 Intronic polymorphism may be in linkage disequilibrium with exon alteration that affects ER protein function (Goessl *et al.*, 1997).

2 Intronic changes in gene sequence may affect other genes by influencing the transcription and/or stability of their mRNA (Kobayashi *et al.*, 1996).

3 Some introns contain regulatory sequences that are enhancers affecting levels of expression through transcriptional regulation (van Duijnhoven *et al.*, 1996).

4 Some polymorphisms in the genes coding for these receptors may change the expression of the receptors modifying the effect of oestrogen (Kinoshita & Chen, 2003).

The increased frequency of pp genotype and xx genotype in fertile men is in agreement with Kukuvitis *et al.* (2002) reporting in a sample of Greeks, a significantly lower frequency of ER-α xx genotype among azoospermics or idiopathic severe oligozoospermia. They suggested that ER alpha and AR gene play significant role in male fertility where a synergy might exist between unfavourable genotypes of these two genes. Corbo *et al.* (2007) added in a sample of healthy Italians that ER-α gene xx and pp genotypes are associated with fertile states. *In vitro* studies declared that enhancer activity differs among ER-α gene haplotypes, the highest being associated with ER-α gene xp haplotype and the ER-α gene *x allele (Maruyama *et al.*, 2000). This difference suggested that presence of ER-α gene xx and pp genotypes could increase ER-α gene function, which affects oestrogen biological action and/or its role on the reproductive efficiency.

ER-α gene polymorphisms at the restriction site ER-α xx or pp genotypes were associated with significant increase in sperm count, sperm motility parameters. Lazaros *et al.* (2010) reported that men with pp and xx genotypes had higher sperm count, while those with Pp, PP and Xx, XX genotypes had lower sperm count. As oestrogen regulates the reabsorption of luminal fluid in the epididymal head, reduced sperm production is thought to be a consequence of impaired fluid resorption within the efferent ducts of the testis (Eddy *et al.*, 1996). Safarinejad *et al.* (2010) reported that men with ER-α PvuII TT, ER-α XbaI AA genotypes had significantly lower values for sperm density, sperm motility and sperm normal morphology suggesting a protective effect for infertility in the presence of ER-α PvuII TC, ER-α XbaI AG genotypes. Lately, Lee *et al.* (2011) suggested that oestrogen-related genes mainly regulate sperm concentration and motility, but none of the oestrogen-related genes were associated with sperm morphology.

Regarding sperm motility, the existence of ERs on the sperm membrane and its midpiece suggests a role of oestrogens in male gamete motility (Solakidi *et al.*, 2005). Oestrogens can regulate mitochondrial function by increasing nuclear respiratory factor-1 (NRF-1) expression.

Specifically, oestradiol stimulates mitochondrial function through a genomic mechanism of ER action involving direct ER-α and ER-β interaction with an oestrogen response element in the NRF-1 promoter. It has been suggested that ER-α polymorphisms can increase mitochondrial activity via NRF-1 transcription in ejaculated spermatozoa presenting them with high motility (Mattingly *et al.*, 2008). In their study, Lazaros *et al.* (2010) associated PvuII and XbaI polymorphisms with sperm motility only in oligozoospermics where men with Pp, pp and Xx, xx genotypes had higher sperm motility compared with those with PP and XX genotypes.

Increased acrosin activity index was associated with ER-α gene polymorphisms at the restriction sites XbaI xx and PvuII pp genotypes. Previous data obtained from both mice and humans showed that oestrogens are positively involved in sperm capacitation and acrosome reaction where the existence of ER-α at the upper post-acrosomal sperm head region is relevant for a role of oestrogens in male gamete maturation and function (Mattingly *et al.*, 2008).

Oestradiol levels were demonstrated to be significantly higher in men with PP and XX genotypes of ER-α gene compared with other genotypes. Safarinejad *et al.* (2010) reported that fertile or infertile with ER-α Pvull TT, ER-α XbaI AA genotypes had significantly higher serum levels of free oestradiol. It has been reported that ER-α gene polymorphisms may modulate the effect of oestradiol on CYP19 expression (Kinoshita & Chen, 2003). Aromatase is required for synthesis of oestrogens from C19 steroids and is present in Leydig cells, Sertoli cells, spermatocytes and spermatids where a targeted disruption of the CYP19 gene that encodes aromatase causes a decline in sperm numbers and loss of male fertility (Carreau *et al.*, 1999; Robertson *et al.*, 1999).

It is concluded that as far as oestrogen is concerned in male gamete maturation, ER-α gene polymorphisms might play a role in the pathophysiology of male infertility.

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