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PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA

PROGRAMA DE MESTRADO EM GENÉTICA

NÚCLEO DE PESQUISAS REPLICON

D I S S E R T A Ç Ã O D E M E S T R A D O

ANÁLISE MOLECULAR DO GENE DO RECEPTOR DE ANDRÓGENOS EM HOMENS COM SUSPEITA DE INFERTILIDADE

CAROLINE OLIVEIRA DE ARAÚJO MELO

- MESTRANDA -

GOIÂNIA – GOIÁS – BRASIL

- JANEIRO DE 2010 -

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DISSERTAÇÃO APRESENTADA AO PROGRAMA DE MESTRADO EM GENÉTICA DA PONTIFÍCIA UNIVERSIDADE CATÓLICA DE GOIÁS COMO REQUISITO PARCIAL E OBRIGATÓRIO PARA A OBTENÇÃO DO TÍTULO DE MESTRE EM GENÉTICA.

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10,0 (DEZ PONTOS)

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Drª. Daniela de Melo e Silva / MGene – PUC Goiás
(presidente orientadora)

.....
Dr. Aparecido Divino da Cruz, PhD – MGene – PUC Goiás
(membro interno)

.....
Drª. Menira Borges de Lima e Souza - UFG
(membro externo)

“Se você pensa que pode, ou sonha que pode, comece. Uma jornada de mil milhas inicia-se com o primeiro passo. A diferença entre o possível e o impossível está na determinação de cada um. Sabem o que é impossível? É aquilo que ninguém fez, até que alguém o faça”.

Johann Wolfgang von Goethe

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RESUMO

Andrógeno é um termo genérico geralmente utilizado para descrever um grupo de hormônios esteroides sexuais. Os andrógenos são produzidos no homem primariamente pelos testículos. No entanto, algumas pequenas quantidades são também produzidas pelos ovários nas mulheres e pelas glândulas adrenais, em ambos os sexos. Os andrógenos são responsáveis pela diferenciação sexual masculina durante a embriogênese na 6ª ou 7ª semana de gestação, desencadeando o desenvolvimento dos testículos e pênis em fetos masculinos e é dirigido pelo fator determinante testicular, o gene SRY (região determinante do sexo no cromossomo Y), localizado no braço curto do cromossomo Y. A diferenciação da genitália externa masculina em pênis, escroto e uretra peniana ocorre entre a 9ª e 13ª semana de gravidez e requer concentração adequada de testosterona e a conversão para um outro andrógeno mais potente, a di-hidrotestosterona (DHT), através da ação da 5 α- redutase em tecidos alvos. As ações da testosterona e DHT requerem a presença dos receptores androgênicos funcionais. O gene *AR* é uma proteína codificada para o gene localizado no Xq11.2-q12. Ele abrange mais de 90 kb e codifica pra a proteína que funciona como um hormônio esteroide que ativa o fator de transcrição. O AR, como outros membros da superfamília de receptores nucleares, tem três domínios principais: o AR é caracterizado por uma estrutura modular consistindo de quatro domínios funcionais: o domínio N-terminal (NTD), um domínio de ligação ao DNA (DBD), a região de dobradiça, e um domínio de ligação ao ligante (LBD). Mutações no gene *AR* causam a Síndrome de Insensibilidade aos Andrógens ligada ao cromossomo X (AIS) caracterizada pela insensibilidade androgênica, que afeta o desenvolvimento sexual adequado tanto na embriogênese quanto na puberdade. Como uma desordem genética, o AIS apresenta um problema e um fardo para as pessoas afetadas e suas famílias e um grande desafio médico para os provedores de saúde. Essa resposta prejudicada aos andrógenos resulta na incapacidade ou redução da capacidade do receptor de andrógeno (AR) de transativar os genes responsivos aos andrógenos em células–alvo, e leva à diferenciação e desenvolvimento anormais da genitália masculina interna e externa, e assim, levando ao pseudo-hermafroditismo masculino.

Palavras-chave: andrógenos, pseudo-hermafroditismo, infertilidade, gene *AR*

ABSTRACT

The androgen is a generic term usually applied to describe a group of sex steroid hormones. Androgens are produced primarily by a male's testes. However, some small amounts are also produced by the ovaries in females and by the adrenal gland, in both sexes. Androgens are responsible for the male sex differentiation during embryogenesis at the 6^{th} or 7^{th} week of gestation , triggering the development of the testes and penis in male fetuses and is directed by the testicular determining factor, the gene SRY (sex determining region on Y chromosome), located on the short arm of chromosome Y. The differentiation of male external genitalia in penis, scrotum and penile urethra occurs between the 9^{th} and 13^{th} weeks of pregnancy and requires adequate concentration of testosterone and converting this to another more potent androgen dihydrotestosterone (DHT), through the action of 5α –reductase in target tissues. The actions of testosterone and DHT require the presence of functional androgen receptors, which, after the connection with these hormones, activate the transcription of specific genes in target tissues. The *AR* gene is a protein coding gene located at Xq11.2-q12. It spans over 90 kb and codes for a protein that functions as a steroid-hormone activated transcription factor. The AR, like other members of the nuclear receptor superfamily, has three major functional domains: the AR is characterized by a modular structure consisting of four functional domains: an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a ligand (androgen-) binding domain (LBD). Mutations in the *AR* gene cause the X-linked Androgen Insensitivity Syndrome (AIS) characterized by androgen unresponsiveness, which affects the proper male sexual development both at embryogenesis and puberty. As a genetic disorder, AIS presents a problem and a burden to the affected people and their families and a major medical challenge for the health providers. This impaired response to androgen results from the incapacity or reduced capacity of the androgen receptor (AR) to transactivate androgen-responsive genes in target cells, and leads to abnormal differentiation and development of male internal and external genitalia, and thus leading to male pseudohermaphroditism.

Keywords: androgens, pseudohermaphroditism, infertility, AR gene

INTRODUÇÃO

A presente dissertação teve como objetivos, o de avaliar pacientes com infertilidade idiopática, atendidos no Hospital das Clínicas de Goiânia-GO, no ano de 2006; o de analisar 62 aviadores agrícolas que foram expostos a agrotóxicos e finalmente, o de avaliar uma paciente que apresentava o sexo genético 46, XY e que foi atendida em um serviço público de genética. Nesse sentido, esse estudo foi dividido em três capítulos, conforme descritos, resumidamente, a seguir.

O primeiro capítulo da dissertação se trata dos pacientes com infertilidade idiopática que procuraram atendimento no Hospital das Clínicas do município de Goiânia-GO. Os éxons 1 e 4 do gene do Receptor de Andrógeno (RA) foram analisados para verificar se havia deleção em algum deles associando tais alterações com dados clínicos e laboratoriais dos pacientes.

O segundo capítulo teve como objetivo avaliar uma paciente atendida no Laboratório de Citogenética Humana e Genética Molecular - LaGene. Após ser detectado um cariótipo 46,XY, foi realizada também a técnica de PCR (Reação em Cadeia da Polimerase) para verificar se havia alguma deleção dos éxons do gene *RA,* já que foi confirmado, pela técnica de FISH (Hibridização Fluorescente *In situ*) que a paciente apresentava o gene.

O terceiro capítulo teve como objetivo principal analisar deleções em cinco éxons do gene do RA de 62 pilotos agrícolas, que foram expostos a agrotóxicos por, no mínimo, três anos. Foram, também, selecionados cinco pacientes, aleatoriamente, cada um deles apresentando deleção de um dos éxons estudados, para a realização da técnica de FISH, a fim de verificar se a deleção de alguns dos éxons supracitados tinha alguma influência na hibridização da sonda específica para o gene *RA* (LSI Androgen Receptor SpectrumOrange (Xq12).

CAPÍTULO 1

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EVOLUTION AND TECHNOLOGY

The association between male infertility and androgen receptor: No evidence of mutations in androgen receptor among Brazilian patients

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ABSTRACT. The androgen receptor is encoded by a single-copy gene located in the long arm of the X chromosome (Xq11-12); it consists of eight exons and encodes an intracellular transcription factor that belongs to the steroid/nuclear receptor superfamily. Disturbances in the function of the androgen receptor can lead to several forms of male pseudohermaphroditism, such as androgen insensitivity syndrome (AIS), which can lead to infertility. Infertility affects around 20% of couples, and in half of the cases it is a male problem. Seventy male patients with idiopathic infertility were selected; data was obtained on age, drinking and smoking habits, occupation and family history. The mean age of the patients was 37 years old (standard deviation = 12.3); 44% were azoospermic, 33% were oligozoospermic and 24% did not not have alterations in the spermogram. Our objective was to evaluate a possible association between male infertility and mutations in the androgen receptor gene based on the presence or absence of exons 1 and 4 of this gene. These two exons were tested by polymerase chain reaction (PCR), and their products were separated in 1.5% agarose gels. In this study we found that azoospermic patients clearly presented higher mutation rates on exons 1 and 4 of the AR gene, when compared to other alterations that also lead to infertility, such as oligozoospermia and teratozoospermia. So, we conclude that patients who do not produce sperm have a higher number ofmutations in the androgen receptor gene when compared to those who only have an impaired sperm production. The molecular analysis showed that there was no correlation between alterations on the spermogram and mutations on the two exons analyzed and no association between alterations on the spermogram and the frequency of alcohol drinkers and smokers.

Key words: Male infertility; Androgen receptor; Chromosome X; Exon 1; Exon 4

INTRODUCTION

The androgen receptor (AR) is encoded by a single-copy gene located on the long arm of the X chromosome (Xq11-12), which consists of eight exons and encodes an intracellular transcription factor that belongs to the steroid/nuclear receptor superfamily (Lubahn et al., 1988). The sequencing of the human AR gene (*AR*) has allowed researchers to examine the effect of *AR* mutations on the development of the normal male phenotype, including the development of spermatozoa (Gottlieb et al., 2005).

The AR has four main domains: the amino-terminal activation domain (TAD), comprising exon 1; the DNA binding domain (DBD), comprising exons 2 and 3; the hinge domain and the ligand-binding domain (LBD), comprising exons 4 to 8 (Brinkmann and Trapman, 2000). The receptor is activated when androgen binds to the C-terminal ligand-binding domain (LBD), triggering a cascade of molecular events, including interactions between LBD and TAD, and the recruitment of transcriptional coactivators (Lim et al., 2000).

The development of the male phenotype and the initiation of spermatogenesis, events that lead the production of male gametes, are dependent on cellular phenomena that respond to androgens. The main androgens in mammals are testosterone and dihydrotestosterone, and the action of both is mediated by the androgen receptor (Domenice et al., 2002). Androgen insensitivity syndrome (AIS) is caused by numerous mutations of the androgen receptor gene (*AR*), and it is the major genetic cause of male pseudohermaphroditism. The phenotype may range from partial AIS (PAIS) with ambiguous genitalia to complete AIS (CAIS) with female genitalia. (Yong et al., 2003). Recent studies have shown dual post-meiotic roles for the androgen receptor during male germ cell differentiation, which consists of terminal differentiation of spermatids and their release from the seminiferous epithelium. Further, progression of spermatids to elongation steps is sensitive to Sertoli cell AR function (Holdcraft and Braun, 2004).

AR has two polymorphic sites in exon 1, characterized by different numbers of CAG and GGC repeats resulting in many extensions of polyglutamine and polyglycine in the amino-terminal domain of the AR protein which seems to modulate androgen receptor function. The number of CAG and GGC repeats can range from 10 to 15 and 4 to 24, respectively, in normal men. Expansion of the CAG repeat above the normal

range has been found to be associated with adult onset of spinal and bulbar muscular atrophy (Ferlin et al., 2004).

Infertility is the inability of a couple to conceive after one year of attempt. This dysfunction can affect around 20% of couples, and its cause is multifactorial, involving hormonal factors, factors related to age, and even psychological causes (Uehara et al., 2001). Idiopathic male infertility, accounting for 40% of all male infertility cases, is postulated to have a genetic basis (Gottlieb et al., 2005). Stress, genetic mutations and chromosome abnormalities can cause disturbances in chemical events that occur during spermatogenesis (Lopes et al., 1998 and Sailer, 1995).

Infertile men have several nuclear alterations, including an abnormal structure of chromatin, chromosomes with microdeletions and DNA breaks (Fuentes-Mascorro, 2000).

This study evaluated Brazilian patients to determine the association between male infertility and the androgen receptor gene, based on the presence or the absence of exons 1 and 4 of this gene. The present study is only an initial research into the causes of idiopathic male infertility.

PATIENTS AND METHODS

Seventy male patients with idiopathic infertility were evaluated. All these patients lived in Goiânia, Goiás, and the clinical data were obtained at the first medical visit to the Serviço de Reprodução Assistida of Hospital das Clínicas de Goiânia, GO in the year of 2006. Such clinical data included age, drinking and smoking habits, occupation and family history. As a control group, we selected 25 healthy men, with at least, one biological child.

Molecular analysis

The AR gene is located in the Xq11-12 region. DNA samples obtained from peripheral blood lymphocytes and semen were submitted to polymerase chain reaction (PCR) amplification, employing primers targeting exons 1 and 4 of the AR gene. The sense primer of exon 1 was synthesized as follows: 5’ CGG GTT CTC CAG CTT GAT GCG 3’. The antisense primer was 5’ GCT CCC ACT TCC TCC AAG GAC AAT TAC 3’. The sense primer of exon 4 was 5’ ACA CTA CAC CTG GCT CAA TGG 3’ and the antisense was 5’ CGG AAG CTG AAG AAA CTT GG 3’. The final PCR included buffer 10X, 50 mM MgCl2, 500 U Taq DNA polymerase, oligonucleotides (10

μM), specific for exons 1 and 4 of the androgen receptor (AR) gene, 25 mM of each dNTP and Milli-Q H2O to obtain a final volume of 25 μL. PCR was performed in a GeneAmp PCR system 9700 thermocycler (Perkin-Elmer, USA) under the following conditions: initial denaturing step at 95°C for 3 min, followed by 35 cycles of denaturation, annealing and extension (95° for 1 min, 56° for 1 min and 72° for 1 min, respectively). A final extension at 72°C for 5 min was also performed. For analysis of the products obtained by PCR, the material amplified was submitted to electrophoresis on 1.5% agarose gels and stained with 5 μg/mL ethidium bromide. A visual record of the gels was made with a videodocumentation system (Image Master VDS® - Amersham Pharmacia Biotech, USA).

Statistical analysis

We examined if age was significantly different for the azoospermic and oligozoospermic patients. To test this hypothesis, Student's t-test was used to determine a possible association between the presence or absence of exons 1 and 4 and infertility status and also todetermine the relation between the number of patients that showed alterations on the spermogram and the frequency of alcohol drinkers and smokers.

RESULTS

The mean age of the patients was 37 years (SD = 12.3 years) and the mean age of the control group was 40 years (SD = 10.5 years). All of the healthy men showed both exons 1 and 4.

We found more azoospermic patients (43.63%) related to other alterations on the spermogram, as shown in Table 1. This study also showed that azoospermic patients reported drinking and smoking habits, as shown in Table 2. As shown in Table 3, the number of mutations in azoospermic patients was higher (66.6% in exon 1 and 56.25% in exon 4) than in oligozoospermic patients (11.1% in exon 1 and 18.75% in exon 4).

Table 1. Spermogram results and the number of patients with sperm alterations.

Diagnosis at spermogram	Number of patients	Percentage (%)
Azoospermia	24	43.63
Oligospermia	18	32.72
Others[1]	13	23.63
Total	55	100

[1]Term corresponding to the combination of oligospermia/teratospermia and oligospermia/asthenospermia.

Table 2. Number of patients who showed alterations on spermogram and the frequency of drinkers and smokers.

Diagnosis at spermogram	Number of drinkers	Percentage (%)	Number of smokers	Percentage (%)
Azoospermia	8	50	1	25
Oligospermia	6	37.5	2	50
Others[1]	2	12.5	1	25
Total	16	100	4	100

[1]Term corresponding to the combination of oligospermia/teratospermia and oligospermia/asthenospermia.

Table 3. Number of patients who showed alterations on spermogram and mutations in exons 1 and 4 of the AR gene.

Diagnosis at spermogram	Number of patients with mutation in exon 1	Percentage (%)	Number of patients with mutation in exon 4	Percentage (%)
Azoospermia	6	66.6	9	56.25
Oligospermia	1	11.1	3	18.75
Others [1]	2	22.2	4	25
Total	9	100	16	100

[1]Term corresponding to the combination of oligospermia/teratozoospermia and oligospermia/asthenospermia.

In this study we found that azoospermic patients clearly had higher mutation rates in exons 1 and 4 of the AR gene, when compared to other alterations that also lead to infertility, such as oligozoospermia and teratozoospermia. We can conclude that patients who do not produce sperm have a higher number of mutations in the androgen receptor gene when compared to those who only have an impaired sperm production, as in oligozoospermic and teratozoospermic patients. There was no difference in age between the azoospermic and oligospermic patients (P = 0.007).

DISCUSSION

Although we found in the present study that azoospermic patients have much higher mutation rates in exons 1 and 4 of the AR gene, compared to other changes that also lead to infertility, such as oligozoospermia and teratozoospermia, statistical analysis showed that there was no correlation between the diagnosis of spermogram and mutations in exon 1 (P = 0.26) and 4 (P = 0.39) of the androgen receptor gene. Such findings could be explained by the small sample size of the group analyzed.

We also did not find any correlation between the diagnosis from the spermogram and alcohol drinkers and smokers (P = 0.30 and P = 0.24, respectively), although it has been reported that cigarette smoking affects sperm DNA integrity (Kunzle et al., 2003).

Eskenazi et al. (2003) performed a study to evaluate the relation between seminal volume and drinking and smoking habits, and they concluded that the sperm quality is decreased in men who have these habits during their life.

Cigarette smoke has mutagenic properties, having been associated with an overall reduction in semen quality, and specifically a reduction in sperm count and motility and an increase in number of abnormal cells. However, we could not find a statistically significant difference between spermogram results and lifestyle (alcohol and smoking), probably due to the small sample size or to the amount of cigarettes or alcohol used by our patients, since we did not quantify such variables in our protocols.

A number of recent studies have examined the possible link between the length of the CAG repeat that codes for the polyglutamine tract in exon 1 of AR and male infertility. Those studies found that the length of the polyglutamine repeat is inversely proportional to the degree of normal functionality of AR. This observation has led to the hypothesis that longer polyglutamine tracts may possibly be considered a risk factor for male infertility. Other studies did not find this association. Studies on Asian and European populations have suggested that different environmental influences may be the cause of these very different findings (Gottlieb et al., 2005).

Yong et al. (2003) reported that most described mutations in the carboxy-terminal domain of AR, which includes exons 4 to 8, can lead to a defect in androgen bindng and the loss of receptor function. Genetic examinations of male infertility revealed many loci in the carboxy-terminal domain of AR that are associated with male infertility. On the other hand, not all of the mutations in the carboxy-terminal domain that cause reduction in sensitivity to androgens result in infertility, which could be the case in our study.

Reported here are preliminary findings in men who presented with idiopathic infertility. Therefore, definitive data on the role of AR mutations in male infertility are not yet available, and additional studies in well-defined populations of infertile men of different ethnic origins should be performed. In our case, more extensive investigations, with other exons could be necessary to confirm the initial findings, since mutations in the AR gene are not yet investigated in the Brazilian population which shows admixture, making it difficult to divide into ethnic groups.

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CAPÍTULO 2

ANDROGEN INSENSITIVITY SYNDROME (AIS)

Challenges in clinical and laboratory diagnosis of AIS: a case report

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ABSTRACT

Introduction: The androgen is a generic term usually applied to describe a group of sex steroid hormones. Androgens are responsible for the male sex differentiation during embryogenesis at the 6^{th} or 7^{th} week of gestation , triggering the development of the testes and penis in male fetuses and is directed by the testicular determining factor, the gene SRY (sex determining region on Y chromosome), located on the short arm of chromosome Y. The differentiation of male external genitalia in penis, scrotum and penile urethra occurs between the 9^{th} and 13^{th} weeks of pregnancy and requires adequate concentration of testosterone and converting this to another more potent androgen dihydrotestosterone (DHT), through the action of 5α –reductase in target tissues.

Case Presentation: This report was aimed to provide a genetic diagnosis of a teenager girl presenting a male karyotype to determine the extension of the mutation which affected the *AR* gene. A 15-years old female patient was referred to our laboratory for genetic testing due to precarious breast development and primary amenorrhea. Physical exam, karyotype testing and molecular analysis of the AR gene were critical to make the correct diagnosis of Complete Androgen Insensitivity Syndrome (CAIS).

Conclusion: The sex determination and differentiation depend on a cascade of events that begins with the establishment of chromosomal sex at fertilization and ends with sexual maturation at puberty leading subsequently to fertility. Mutations affecting the *AR* gene may cause either complete or partial AIS. The case reported here is consistent with a CAIS, misdiagnosed at birth, and consequently raised socially and educationally as a female. It is fundamental that health care providers understand the importance of properly diagnosing a newborn with ambiguous genitalia. Furthermore, a child with a pseudo-hermaphrodite phenotype should always undergo adequate endocrine and genetic testing for a definite diagnosis before gender is assigned and surgical interventions are carried out.

Our results revealed that we must be very careful in the selection of the genetic tools that should be applied to the diagnosis for the AIS.

INTRODUCTION

The androgen is a generic term usually applied to describe a group of sex steroid hormones. Androgens are produced primarily by a male's testes. However, some small amounts are also produced by the ovaries in females and by the adrenal gland, in both sexes. Androgens are responsible for the male sex differentiation during embryogenesis at the 6^{th} or 7^{th} week of gestation , triggering the development of the testes and penis in male fetuses and is directed by the testicular determining factor, the gene SRY (sex determining region on Y chromosome), located on the short arm of chromosome Y. The differentiation of male external genitalia in penis, scrotum and penile urethra occurs between the 9^{th} and 13^{th} weeks of pregnancy and requires adequate concentration of testosterone and converting this to another more potent androgen dihydrotestosterone (DHT), through the action of 5α –reductase in target tissues [1]. The actions of testosterone and DHT require the presence of functional androgen receptors, which, after the connection with these hormones, activate the transcription of specific genes in target tissues.Thus, any abnormality in the production or action of androgens in a fetus 46,XY between the 9^{th} and 13^{th} weeks of pregnancy cause incomplete masculinization, resulting in male pseudohermaphroditism . In man, androgens also begin the complex process of puberty, affecting the development of facial, body, and pubic hair, deepening of the voice, and muscle development. Physiologically, androgens regulate the spermatogenesis, and help to maintain male reproductive functions [2].

The *AR* gene is a protein coding gene located at Xq11.2-q12. It spans over 90 kb and codes for a protein that functions as a steroid-hormone activated transcription factor (figure 1). The AR, like other members of the nuclear receptor superfamily, has three major functional domains: the AR is characterized by a modular structure consisting of four functional domains: an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a ligand (androgen-) binding domain (LBD) [3].

The complete form of AIS is relatively rare. In childhood, the most common clinical presentation is the presence of bilateral inguinal hernia. Individuals not diagnosed during childhood are detected after puberty because of primary amenorrhea. Patients with the complete form of AIS have female external genitalia, absence or thinning of pubic hair and absence of uterus [4].

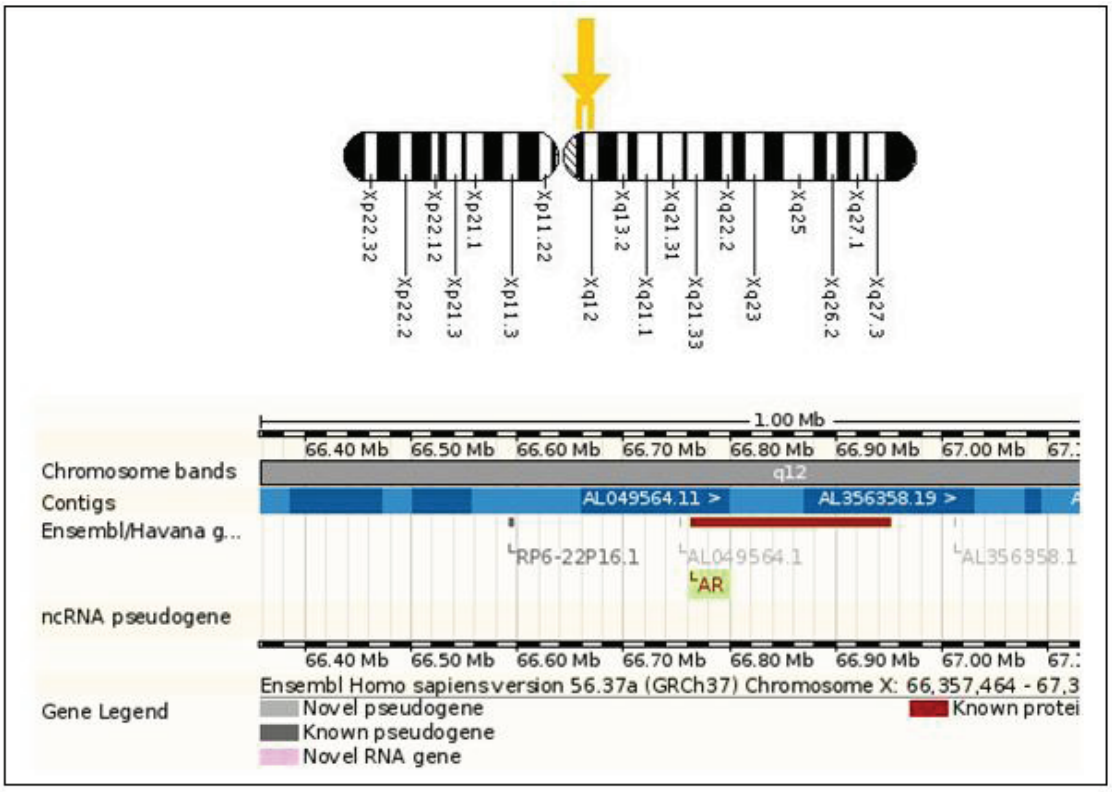

Figure 1. Cytogenetic Location of *AR* gene: Xq11.2-q12 and Molecular Location on the X chromosome: base pairs 66,680,598 to 66,860,843

The AR NTD is relatively long and displays the most sequence variability among nuclear receptors. It is very flexible and displays a high degree of intrinsic disorder. In addition, the NTD has a variable number of homopolymeric repeats, the most important of which is a polyglutamine repeat that ranges from 8 to 31 repeats in normal individuals, with an average length of 20 base pairs [5].

The DBD is centrally located in the AR and it is the most conserved region within the nuclear receptor family. This region has nine cysteine residues, of which eight of them are involved in forming two zinc fingers, and a C-terminal extension. The first zinc finger, most proximal to the NTD, determines the specificity of DNA recognition, whereas residues in the second zinc finger are involved in AR dimerization. Two AR monomers in a head-to-head conformation bind as a homodimer to AREs, that are direct or indirect repeats of the core consensus 5'TGTTCT3', or more complex response elements with diverse arrangements of AREs. The C-terminal extension is important for the three-dimensional structure of the DBD and it plays a role in mediating the AR selectivity of DNA interaction [6].

The hinge region has long been considered to be a flexible linker between the DBD and LBD in the AR. More recently, however, this region was shown to be involved in DNA binding as well as AR dimerization. It was suggested that the hinge region also acts to attenuate transcriptional activity of the *AR* gene [7].

Mutations in the *AR* gene cause the X-linked Androgen Insensitivity Syndrome (AIS) characterized by androgen unresponsiveness, which affects the proper male sexual development both at embryogenesis and puberty. As a genetic disorder, AIS presents a problem and a burden to the affected people and their families and a major medical challenge for the health providers. This impaired response to androgen results from the incapacity or reduced capacity of the androgen receptor (AR) to transactivate androgen-responsive genes in target cells, and leads to abnormal differentiation and development of male internal and external genitalia, and thus leading to male pseudohermaphroditism [1].

Most of mutations on the *AR* gene result in a condition collectively known as Androgen Insensitivity Syndrome (OMIN #300068). Depending on the extent of the AR defect, the phenotype can vary, distinguishing three forms, known as Complete (CAIS), Partial (PAIS), and Mild (MAIS) forms of Androgen Insensitivity Syndrome. All forms of AIS are X-linked traits inherited as a recessive disorder. Despite a normal male karyotype (46,XY), individuals affected with CAIS – also known as Testicular Feminization Syndrome, have female external genitalia, blind vagina, absent uterus and female adnexa, female breast development, and abdominal or inguinal testes. Partial androgen insensitivity results in hypospadias and micropenis with gynecomastia [8]. Patients usually come to medical attention in the neonatal period because of inguinal hernia and/or ambiguous genitalia or at puberty because of primary amenorrhea associated with normal breast development and reduced pubic hair. In contrast, PAIS is heterogeneous condition and covers a wide spectrum of undervirilization situations resulting in different degrees of ambiguous external genitalia, with a range from an almost complete form to phenotypic males with isolated azoospermia [9].

The current case report was aimed to provide a genetic diagnosis of a teenager girl with normal male karyotype using FISH and PCR in order to determine the nature and the extension of the mutation which affected the *AR* gene.

CASE PRESENTATION

In 2009, a 15-years old female patient was referred to the Laboratory of Human Cytogenetics and Molecular Genetics (LaGene) in Goiânia-GO (Brazil) for gentic testing due to precarious breast development and primary amenorrhea. Her medical history included the clinical information of abdominal mass removal as a newborn. The tissue removed was referred as an umbilical hernia. Her G-band karyotype revealed a diploid set of chromosomes, including 22 pairs of homologous autosomes and 1 pair of sex chromosomes, compatible with a 46, XY male chromosome complement.

The geneticists of the laboratory concluded that the mass withdrawal from the abdomen of the patient was, in fact, testes and that the patient had a condition known as cryptorchidism, a reproductive change characterized by a failure of the displacement of one or both testes to the abdominal cavity to the scrotum.

We performed the methods of PCR and FISH to verify mutations of the exons 1, 4, 6, 7 and 8 of the AR gene and to detect the AR gene, respectively. We prepared a culture of T-lymphocytes in the medium RPMI 1640, supplemented with 20% of bovine fetal calf and 2% of phytohemagglutinin. Metaphasic preparations were made by conventional methodology. The use of slides for FISH were prepared with a micropipette, dripped about 15 μL of the material set. Only the slides of good quality (in terms of metaphases), were selected by phase contrast microscope and were subjected to the method of FISH using the probe LSI Androgen Receptor SpectrumOrange (Xq12) (Vysis ®, Abbott Park, Illinois, USA). For PCR, primers were used for the exons 1, 4, 6, 7 and 8 of AR [10].

In situ hybridization with LSI AR probe indicated the presence of the gene in all analyzed cells. However, Genomic DNA extracted from peripheral blood leukocytes assessed by PCR revealed coding sequence abnormalities for the *AR* gene which lacked exons 1 through 7, indicating large deletion spanning over the proximal region of the gene. Figure 2 shows hybridization signals in both interphase and metaphase nuclei.

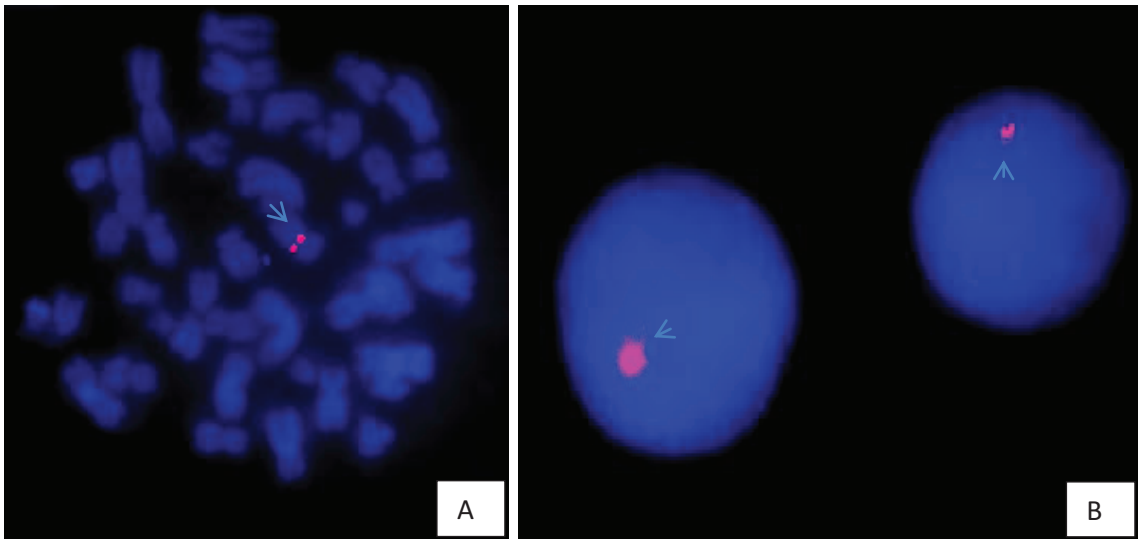

Figure 2. Hybridization signals for the LSI Androgen Receptor probe, indicating the presence of the *AR* gene on the nuclei of a 15-yo patient affected with the AIS.

CONCLUSION

The results reported in this article revealed that we must be very careful in the selection of the genetic testing tools that should be applied to reach the proper diagnosis for the AIS. If a child reached its teenager years undiagnosed due to clinical challenges presented by an ambiguous genitalia it further complicates because there could be several genetic events subjacent of that outcome, ranging from total or partial deletion of the gene to point mutation that efficiently silence the gene and therefore leads to AIS. Here we reported that FISH alone was not able to properly diagnose our patient, despite the proximal deletion within the AR observed by the PCR (Figure 3).

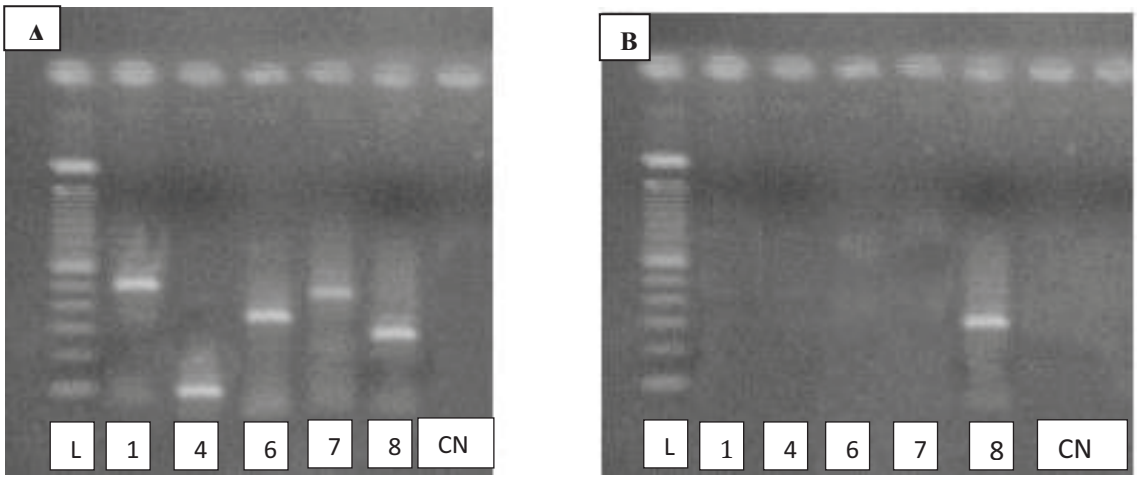

Figure 3. . Images of the PCR products on a 2% agarose gel. The first image (A) shows a gel of a normal patient presenting the five exons analyzed. The second image (B) shows a gel of the analyzed patient, who presented only the exon 7. L- Ladder of 100 bp; 1- Exon 1 with a molecular weight of 528 bp; 4- Exon 4 with a molecular weight of 172 bp; 6- Exon 6 with a molecular weight of 378 bp; 7- Exon 7 with a molecular weight of 516 bp; Exon 8- Exon 8 with a molecular weight of 289; CN- Negative control.

This result could be explained by the size of the probe (380 kb) used, which was bigger than the *AR* gene (90 kb), indicating that the deletion of some exons within the gene was not large enough to prevent probe hybridization (Figure 4) [11].

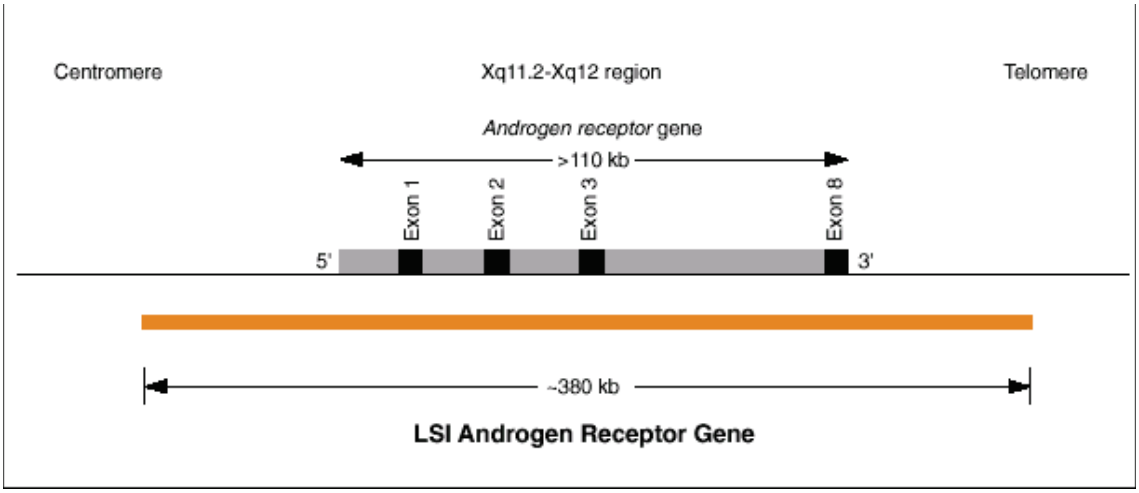

Figure 4. Map of probe and exons amplified by PCR to ilustrated the problem with FISH + and lack of exons 1-7 on the AR gene.

Thus, in the present case, the PCR assay was able to close the diagnosis for our patient with previous history of abdominal mass removal as a newborn who had a

chromosomally normal male karyotype. However, due to complex chromosome aberrations or other genomic mutations, another molecular tools to detect and define DNA mutations, such as DNA sequencing, may be required to reach appropriately the diagnose cases of AIS.

The sex determination and differentiation depend on a cascade of events that begins with the establishment of chromosomal sex at fertilization and ends with sexual maturation at puberty leading subsequently to fertility. Mutations affecting the *AR* gene may cause either complete or partial AIS. The case reported here is consistent with a CAIS, misdiagnosed at birth, and consequently raised socially and educationally as a female. It is fundamental that health care providers understand the importance of properly diagnosing a newborn with ambiguous genitalia. Prompt evaluation of both clinical and genetic findings is crucial to permit proper gender assignment and detection of life-threatening conditions [12]. Furthermore, a child with a pseudo-hermaphrodite phenotype should always undergo adequate endocrine and genetic testing for a definite diagnosis before gender is assigned and surgical interventions are carried out. Inadequate work-up may result in inappropriate gender assignment in infancy with possible inferences on outcome [13].

The presentation of a patient, and specifically a neonate, with abnormal genital development represents a difficult diagnostic and therapeutic challenge. Referral to a centre with experience in the diagnosis and management of disorders of sexual development is advised where the emphasis should be on psychological and genetic counselling. [14].

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CAPÍTULO 3

PESTICIDES AND HUMAN HEALTH

Occupational Exposure

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Keywords: pesticides, infertility, androgen receptor, PCR.

ABSTRACT. The male reproductive functions are highly sensitive to various physical and chemical agents. Such agents are commonly found in the environment and in some occupational activities. The toxic damage to the testicles can result in reduced production or production of defective sperm and impaired production of androgens. Pesticides are able to operate the hormonal pathways affecting the male reproductive system. The effects can happen at different stages of the cell cycle, and during the disjunction in meiosis, these abnormalities may have deleterious effects on reproduction and progeny. This study was developed by molecular analysis of 62 male subjects who were exposed to pesticides. This work was conceived out at Núcleo de Pesquisas Replicon in the years 2008 and 2009. The collection of blood samples from patients by venipuncture was performed in 2006 and DNA extraction using the kit PurelinkTM Genomic DNA Mini Kit (Invitrogen, USA) was held in 2008. Subsequently, the genetic

material extracted was subjected to PCR and thermal cycling protocol to obtain the amplicons. In this procedure we used four sets of primers concerning exons 1, 4, 6, 7 and 8 of the androgen receptor. We also carried out cell cultures of samples from five patients who presented deletion of at least one exon of the *AR* gene to perform the FISH technique in order to verify if a deletion of only one exon could affected the hybridization of the probe LSI Androgen Receptor SpectrumOrange (Xq12) (Vysis ®, Abbott Park, Illinois, USA). The mean age of patients was 40.70 years (SD = ± 11:53 years). Of the 62 patients, 46.8% (29 individuals) in the questionnaire reported smoking and 91.9% (57 individuals) drink at least two days a week. To evaluate if there was any relationship between the age of the patients, the deletions found in molecular analysis and also the relationship between smoking and alcoholism and mutations and the mutations found it was performance the qui-square test. We found that no significant results between these relationships.

Keywords: chemical agents, male infertility, androgen receptor.

INTRODUCTION

Pesticides are a large group of biologically active chemicals used in pest management. They are able to affect human health and the effects of short-term exposure have been well documented. Small amounts of some of the chemicals used as pesticides can lead to death (Brandt *et al.* 2001), disrupt hormones and reduce the ability to play successful reproduction (Bonde, 2002; Claman, 2004; Sharpe & Irvine, 2004). Additonaly, pesticide exposure has been associated with the development of specific cancers (Fleming *et al.* 2003; Alavanja *et al.* 2004).

According to the Food and Agriculture Organization (FAO) of the United Nations, Brazil is the fourth largest consumer of agrochemicals world-wide (Peres & Moreira, 2007). Several studies have shown a huge amount of health problems related to the management of pesticides in Brazil. Some of these studies show poor adherence to personal protective and safety equipment, one of the main factors that directly influence the vulnerability of farm workers against the harmful effects occupational exposure to pesticides in rural productive processes (Chester *et al*. 1993).

The effects of pesticide exposure on male reproductive system are a topic of great interest for both environmental epidemiology and occupational and reproductive health. In recent years, researchers have been increasingly aware that chemicals can

damage the reproductive function of both humans and animals (Moline *et al.,* 2000).

In human health, there are two types of toxicological effects through a direct, due to poisoning of rural workers, and others through indirect effects, affecting the health of consumers when a food, the residual level is at levels harmful to health, is ingested. In the first case, the effects on health can be acute and chronic. In acute intoxication, the actual exposure damage is apparent in a 24-hour period, while in the chronic injuries result from continuous exposure to low doses of one or more hazardous products. Acute effects are more visible, and the intoxicated symptoms include seizures, vomiting, nausea, among others, while chronic effects may appear weeks, years or decades after the period of use (Soares & Porto, 2007).

Usually the occupational exposure of farm workers is due to the lack of information, resources or compliance to the proper management of hazardous substances. The personal protective equipment (PPE's) tends not to be used during preparation and use of agrochemicals, mostly because they are neither always adequate to the worker's reality nor suitable to the climate conditions in Brazil. The investigation and reporting of pesticide exposure and poisoning are still very poorly documented in Brazil. Difficulties of access of rural workers to health centers and incorrect diagnoses of the symptoms are among the main factors that influence underreporting. Moreover, in most Brazilian states, notification to the systems for surveillance and/or epidemiological follow-up is not compulsory (Peres *et al.* 2007).

The male reproductive functions are highly sensitive to various physical and chemical agents. Such agents are commonly found in the environment and in some occupational activities (Oliva *et al*. 2001). Thirty years ago it was discovered that the male reproductive function may be impaired by environmental agents when pesticide producers and rural workers, who were in contact with the DBPC (1,2-dibromo-3-chloropropane), had severely impaired spermatogenesis, leading to infertility. Since then, several classes of pesticides were classified as toxic to the reproductive tract in experimental animal models (Slutsky *et al.* 1999). The World Health Organization (WHO) estimated that about 20,000 people die and about 750,000 people have chronic effects each year due to the pesticide exposure (Dinham & Malik 2003).

The problems affecting the male reproductive tract have increased in the last fifty years, including the incidence of testicular cancer and some congenital anomalies, such as hypospadias and cryptorchidism (Toppari *et al.* 1996).

The toxic damage to the testicles can result in reduced or defective sperm, and impaired androgen productions (Bonde, 1996). Epidemiological studies in both animals and humans have shown that pesticides can operate by hormonal or genotoxic pathways and, thus, affect spermatogenesis. Although, there is strong scientific evidence that pesticide exposure is toxic to human health only a limited number of epidemiological studies have been published to date (Toppari *et al.* 1996).

In the context discussed previously, the scientific scope for the current research was to determine the occurrence of mutations in the Androgen Receptor (*AR)* gene in workers who were occupationally exposed to environmental agents. We evaluated genomic DNA extracted from peripheral blood leukocytes of agricultural pilots who were occupationally exposed to pesticide vapour to determine the potential association between male infertility and total or partial deletions of the *AR* gene assessed by PCR and FISH using a LSI AR probe to Xq12.

MATERIAL AND METHODS

We aimed to check the main exiting deletions on the AR *gene*. We wanted to verify the associate mutations in the AR with the social habits of the patients and finally, to check, by the methodology of FISH, if the deletions in the androgen receptor gene affect the hybridization of the probe.

Cases and Biological Sampling: study group was comprised of 62 agricultural pilots who were occupationally exposed to pesticides during seasonal field spray for at least three years. All participants answered a life style questionnaire (Annex I) after agreeing to and signing a written informed consent. The current research was previously approved by the Ethics Committee on Research with Humans from the National Commission of Ethics on Research (CONEP) in September 2005, according to the document number 1540/2005-CONEP/CNS/MS. Data relating to patients such as name, age, smoking, occupation, and family history were recorded confidentially on spreadsheets to carry out the statistical analysis. Participants donated voluntarily a total of 10 mL of blood obtained by peripheral venopunction performed by qualified technical personnel. Blood samples were collected in heparin and processed and analyzed during the period of month year to month year, using protocols and quality-

control procedures routinely used at the Pontificia Universidade Católica de Goiás/ Núcleo de Pesquisas Replicon.

DNA Extraction and Quantification: Total genomic DNA was extracted from whole human blood using a DNA extraction kit – Purelink™ Genomic DNA Mini Kit (Invitrogen, USA). DNA extraction followed the protocol suggested by the manufacturer. DNA concentration (ng/μL) for each sample was carried on a GeneQuant™ (Amersham Biosciences, USA) spectrophotometer according to the protocol suggested by the manufacturer.

Polymerase Chain Reaction:. The PCR conditions were prepared for final volume of 25 μL, containing 50 mm $MgCl_2$, 500 U Taq DNA polymerase, 10 mM of each primer set, and 25 mM of each dNTP. The PCR was performed in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystem, Foster City, CA, USA). All samples were subjected to PCR following the thermal protocol described on Table 1. PCR was carried out to amplify exons 1, 4, 6, 7, and 8 of the *AR* gene using the primers previously published by Singh et al (2006). All oligonucleotide sequences and expected size of the amplicons can be found in Table 2.

Table 1. PCR thermal protocol used to amplify the exons 1,4, 6, 7, and 8 of *AR* gene of agricultural workers occupationally exposed to pesticide vapours.

Thermal Protocol	Exons 1 and 4			Exon 6			Exons 7 and 8		
	Temp (°C)	Time (min)	Cycle	Temp (°C)	Time (min)	Cycle	Temp (°C)	Time (min)	Cycle
Initial Desnaturation	95	3	1	94	5	1	94	5	1
Amplification Cycles*	95 56 72	1 1 1	35	94 58 70	1 1 1	35	94 58 72	1 1 1	37
Final Extension	72	5	1	70	5	1	72	5	1
Storage	4	∞		4	∞		4	∞	

Legend: Temp – Temperature; min – minutes; * includes one step of cycling desnaturation, annealing, and extension.

Table 2. List of primers used for amplification of exons of the androgen receptor.

Primer	Sequence (5'-3')	Amplicon (bp)
Ex1	F – CGGGTTCTCCAGCTTGATGCG R – GCTCCCACTTCCTCCAAGGACAATTAC	528
Ex4	F – ACACTACACCTGGCTCAATGG R – CGGAAGCTGAAGAAACTTGG	172
Ex6	F – CCAGCAGGAGAAACAGCAAGC R – GGGGAATGAAGAAGGGAAATGTC	378
Ex7	F – AGGCCCCAAGCACACAGACT R – CCTCCACCCCTTTCACAATATC	516
Ex8	F – GCCACCTCCTTGTCAACCCT R – AGAGGAGTAGTGCAGAGTTA	289

(NCBI 2006, Singh *et al*. 2006). Legend: F – Forward; R – Reverse; pb – base pairs

Gel electrophoresis and Fragment Analysis: The amplicons were separated on 1.5% agarose gels with TBE 1X under an electric field of 10 V/cm for two hours. Subsequently, gels were stained by immersion in a solution of ethidium bromide (10 mg/ml) for 15 minutes under constant agitation. Images were captured using a video-photo documentation system (ImageMaster® VDS, Amersham Pharmacia Biotech, USA).

Fluorescent *in situ* Hybridization Technique (FISH)

We used the FISH technique to verify if the deletion on any exon presented in *AR* gene was able to affect the hybridization of the probe in such gene. For this, we selected five pilots who exhibited a deletion of at least one of the five exons analyzed. All deletions were previously verified by PCR technique, and confirmed by a duplicate PCR.

To confirm, the results, after the selection of the five selected cases, metaphase spreads and interphase nuclei from human lymphocytes were prepared from the five selected cases. In summary, routine cultures of lymphocytes were performed using RPMI 1640 supplemented with 20% bovine serum and 2% phytohemagglutinin. Cell harvest and slide preparations followed conventional methodology. colchicine was used as a mitotic inhibitor and added to cultures after 71h. A 0,075M of KCl was used as the hypotonic solution to swell the cells in order to spread out the chromosomes. After sitting in hypotonic solutions, cells were fixed using Carnoy's fixative (3:1 methanol to glacial acetic acid).

The slides for FISH were prepared with a micropipette, dropping approximately 15 μL of cultured material.

Hybridization was accomplished by using a LSI AR probe (Vysis ®, Abbott Park, Illinois, USA), an orange fluorochrome-labeled oligonucleotide probe, designed to hybridize at Xq12. After this step, the slides were subjected to Hybride (Vysis ®, Abbott Park, Illinois, USA), according to the following schedule; a denaturation step for three minutes at 75°C and annealing for twenty-two minutes at 37°C.

Statistical analysis of molecular data

For the statistical analysis, we used the U test of Mann-Whitney, chi-square ($\chi 2$) of independence and logistic regression.

The Mann-Whitney U test is a nonparametric statistical approach used to compare whether two population means are equal or not and whether they come from the same population. The U-Mann Whitney evaluates if two variables are independent or unrelated to each other by a dependency relationship (Monteiro Filho, 2004; Ayres et al., 2007). In this study, the test was used to verify if the data related to the pilots have an influence on the results of the analysis of exons 1, 4, 6, 7, 8 of *AR* gene.

The statistics behind the Chi-square ($\chi 2$) of independence is used to investigate whether distributions of categorical variables differ from one another.

Logistic regression describes the relationship between a dichotomous response variable and a set of explanatory variables. The explanatory variables may be continuous or discrete.

RESULTS

According to the results of semen analysis, all subjects were normal ($20x10^6$ sperm/mL). The mean age of patients was 40.70 years (SD = ± 11.53 years). Of the 62 patients, it was found that 46.8% (29 individuals) reported in the questionnaire that they smoked and 91.9% (57 individuals) reported that they drink at least three times a week (Table 3).

Table 3. Social habits of the studied 62 patients analysed.

Social Habits	Tabagism		Ethilism	
	(n)	(%)	(n)	(%)
Yes	33	53.2	57	91.9
No	29	46.8	5	8.1
Total	62	100	62	100

Legenda: n – number of patientes, % - percentage

With respect to the molecular analysis of the exons 1, 4, 6, 7 and 8 of *AR* gene was found that 67.8% (42) of the patients had mutations in at least one of the exons and 32.2% (20) of patients did not showed any mutation. In relation to the exon 1, we observed that 24.1% (15) showed alterations and 75.9% (47) had no mutation. On the exon 4, 32.2% (20) showed abnormal and 67.8% had no change on such exon. The same results were observed in the analysis of exon 6. For exon 7, it was observed that 14.5% (9) showed abnormal and 85.5% (53) had no mutation. On exon 8, 8.06% (5) of the patients had mutation and 91.94% (57) had no mutation in such exon. The results of molecular analysis of RA could be seen on Table 4.

Table 4. Results of the molecular analysis of the *AR* gene with respect to the alterations on the studied exons.

Exon	Molecular alteration	
	n	%
1	14	22.58
4	20	32.25
6	18	29.03
7	9	14.51
8	5	8.06

Legend: n – number of patients, % - percentage

We also evaluated the mutation frequencies of the exons 1, 4, 6, 7 and 8 according to the ages, as shown on table 5.

Table 5. Frequency distribution of ages of the patients associated with the molecular analysis of exons 1, 4, 6, 7 and 8 of *AR* gene.

MA/ Age	0 – 22		23 – 44		45 – 66	
	(n)	(%)	(n)	(%)	(n)	(%)
Exon 1						
Presence	4	100	23	67.64	20	83.33
Absence	0	0	11	32.36	4	16.67
Total	4	100	34	100	24	100
Exon 4						
Presence	3	75	22	64.7	17	70.83
Absence	1	25	12	35.3	7	29.17
Total	4	100	34	100	24	100
Exon 6						
Presence	1	25	26	76.47	15	62.5
Absence	3	75	8	23.53	9	37.5
Total	4	100	34	100	24	100
Exon 7						
Presence	4	100	28	82.35	21	87.5
Absence	0	0	6	17.65	3	12.5
Total	0	100	34	100	24	100
Exon 8						
Presence	4	100	31	91.17	22	91.66
Absence	0	0	3	8.83	2	8.34
Total	4	100	34	100	24	100

Legend: MA – Molecular analyze of the exons 1, 4, 6, 7 e 8 of the *AR* gene to verify the presence or not of the exons on the samples; 0 – 22 – age between 0 and 22 years old; 23 – 44 – age between 23 and 44 years old; 45 – 66 – age between 45 and 66 years; n – number of patientes; % - percentage; presence – presented the exon; absence – did not present the exon

For statistical comparison between the mutations of exons analyzed and the classes of age on each patient, it was possible to verify that there was no statistically significant difference. This result could be seen on the table 6.

Table 6. Comparative analysis between the number of deletions, social habits and the age of the patients studied.

	deletions x tabagism	Deletions x ethilism	Deletions x age	Tabagism x ethilism	Tabagism x age	Ethilism x age
n (pairs) =	62	62	62	62	62	62
r (Pearson) =	0.1791	0.2043	-0.0818	-0.1972	0.1339	-0.1317
IC 95% =	-0.07 a 0.41	-0.05 a 0.43	-0.32 a 0.17	-0.43 a 0.06	-0.12 a 0.37	-0.37 a 0.12
IC 99% =	-0.15 a 0.48	-0.13 a 0.50	-0.40 a 0.25	-0.49 a 0.14	-0.20 a 0.44	-0.44 a 0.20
R2 =	0.0321	0.0417	0.0067	0.0389	0.0179	0.0174
t =	1.4102	1.6168	-0.6356	-1.5583	1.0469	-1.0294
GL =	60	60	60	60	60	60
(p) =	0.1636	0.1111	0.5274	0.1243	0.2993	0.3074
Power 0.05 =	0.3996	0.4788	0.071	0.9433	0.2708	0.2276
Power 0.01 =	0.1741	0.2312	0.5002	19.3428	0.095	1.5755

Legend: deletions x tabagism = relation between the number of deletions on *AR* gene and tabagism; deletions x ethilism = relation between the number of deletions and ethilism; deletions x age = relations between the number of deletions and the age of the patiente; tabagism x ethilism = relation between the social habits; tabagism x age = relation between tabagism and the age of the patients; ethilism x age = relation between ethilism and the age of the patients.

For the comparative study of social habits (smoking and drinking) of patients with molecular analysis of the exons (1, 4, 6, 7 and 8) of the *AR* gene it was also possible to observed that, among the patients who smoke, 27.49 % (8) had a mutation on exon 1, 28.58% (8) had a mutation on exon 4, 44.45% (12) had a mutation on exon 6, 17.25% (5) presented mutation on exon 7 and and 13.8% had mutation on exon 8

In relation to alcohol consumption, 27.27% (15) had mutations on exon 1, 35.08% (20) had mutation on exon 4, 29.63% (16) had mutation on exon 6, 14.04% (8) had mutation on exon 7 and 8.78% (5) had mutation on exon 8. All these results may be seen on Table 7.

Table 7. Comparative analysis of the social habits of patients with the molecular analysis of the exons 1, 4, 6, 7 and 8 of RA gene

	Tabagism				Ethilism			
MA	Yes		No		Yes		No	
	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
Exon 1								
Presence	21	72.41	26	78.78	42	73.69	7	100
Absence	8	27.49	7	21.22	15	26.31	0	0
Total	29	100	33	100	57	100	7	100
Exon 4								
Presence	21	72.41	22	66.66	37	64.91	5	100
Absence	8	27.49	11	33.34	20	35.08	0	0
Total	29	100	33	100	57	100	5	100
Exon 6								
Presence	16	55.17	26	78.78	38	66.67	4	50
Absence	13	44.43	7	21.22	19	33.33	4	50
Total	29	100	33	100	57	100	8	100
Éxon 7								
Presence	24	82.75	29	87.87	49	85.97	4	80
Absence	5	17.25	4	12.13	8	14.03	1	20
Total	29	100	33	100	57	100	5	100
Exon 8								
Presence	25	86.20	32	96.96	52	91.23	5	100
Absence	4	13.80	1	3.04	5	8.77	0	0
Total	29	100	33	100	57	100	5	100

Legend: MA – Molecular analysis of exons 1, 4, 6, 7 e 8 of *AR* gene vo verify the presence or absence of them in the sampless; n – number of patients; % - percentage; presence – presence of the exon; absence – absence of the exon.

Samples of five patients with deletion of at least one exon of AR were subjected to FISH technique to verify if any deletion could affected the hybridization of the probe. It was observed that despite the deletion, the probe hybridized to the region of AR *gene*. This could be explained by the size of the probe (380kb) with is much bigger than the *AR* gene (90kb). All results can be seen below:

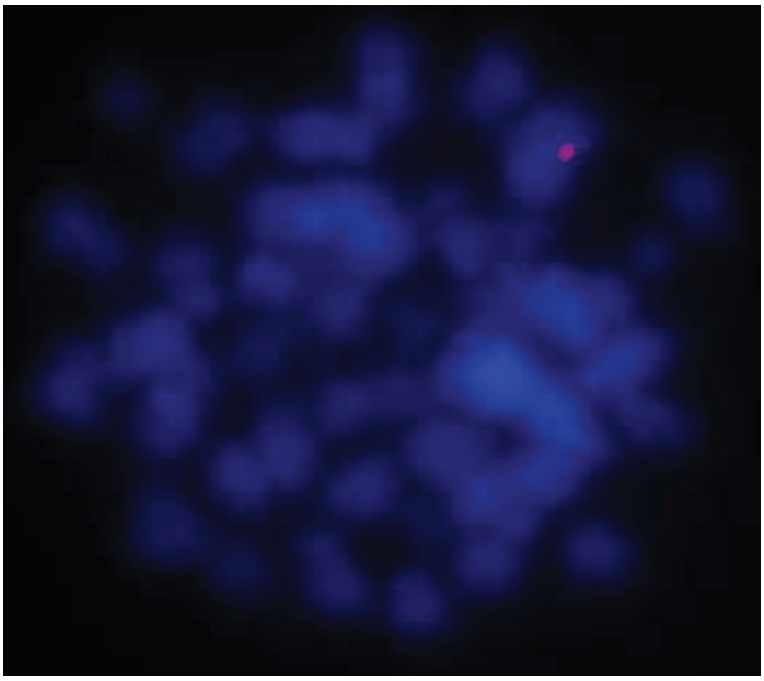

Figure 06. Patient R. P., 46 years, with deletion on the exon 1 of the AR

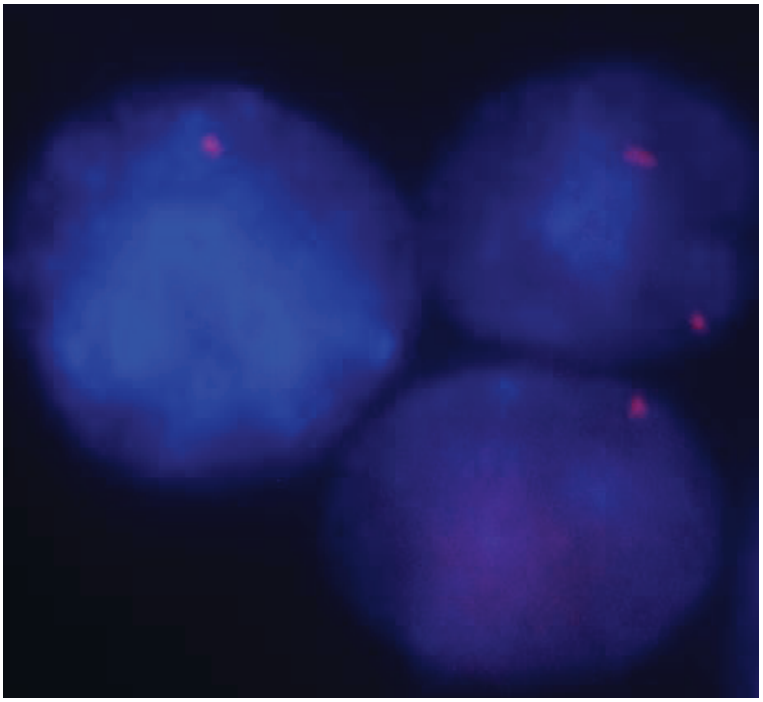

Figure 07. Patient A. G., 30 years old, with deletion on the exon 4 of the AR.

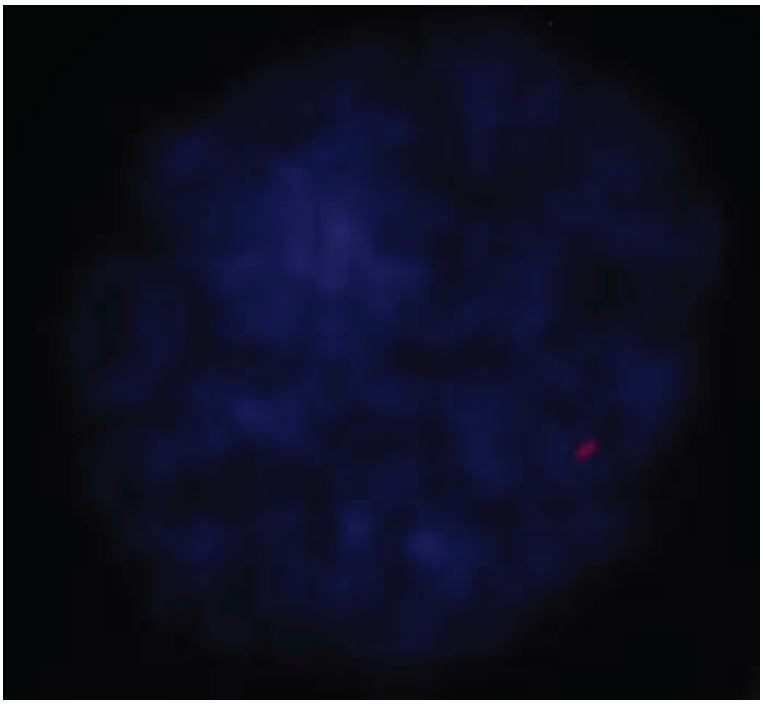

Figure 08. Patient M. A. C., 48 years old, with deletion on the exon 6 of the AR.

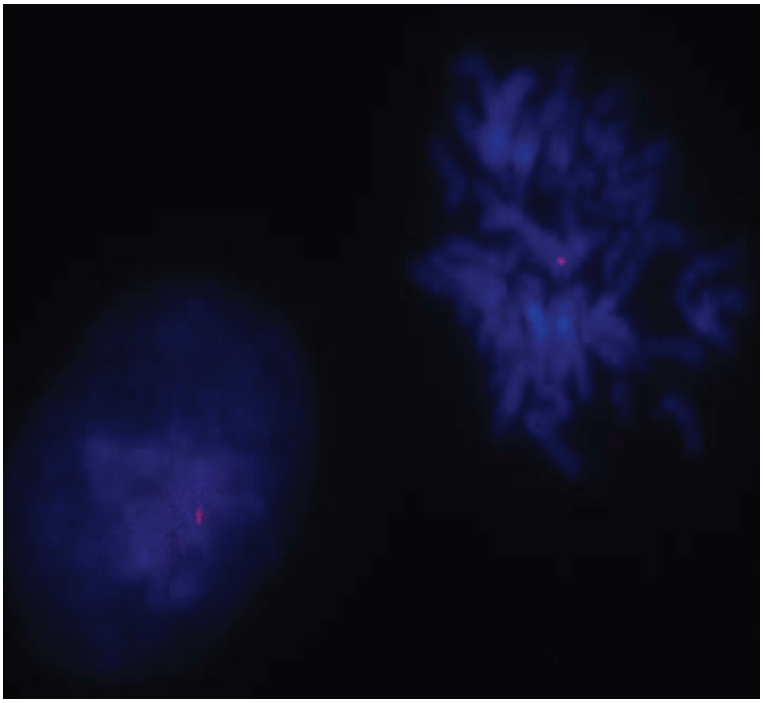

Figure 09. Patient E. S. O., 53 years old, with deletion on the exon 7 of the AR.

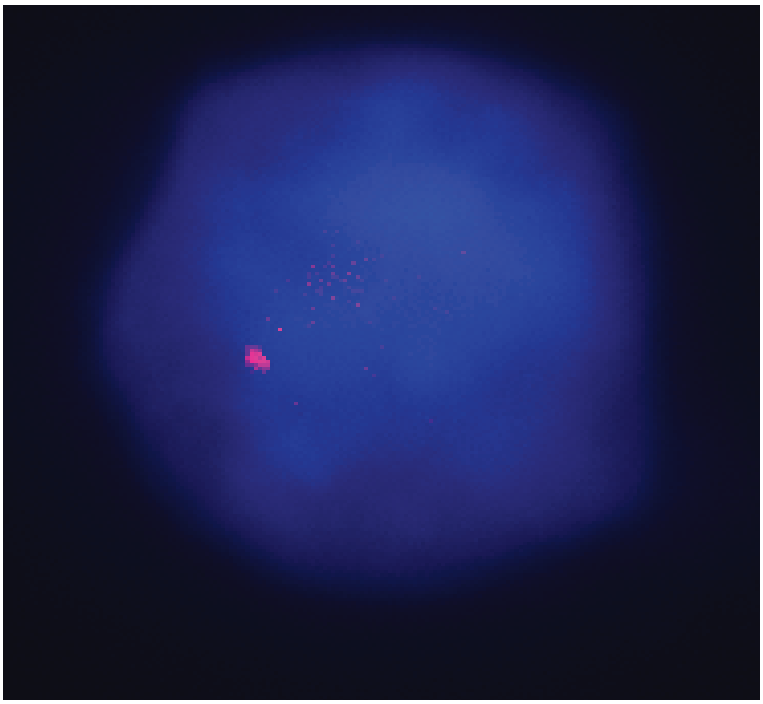

Figure 10. Patient O. S. T., 33 years old, with deletion in the exon 8 of AR.

DISCUSSION

In this study, we analyzed 62 normal men according to the results of semen analysis (20 X 10^6 sperm / mL) who were exposed to pesticides. Initially, it was verified the age of the patientes, with an averaged 40.7 years old (SD= ±11.53).

The data of molecular analysis showed that 67.8% of the subjects had mutation in at least one of the exons analyzed. Patients had a higher frequency of mutation in the exon 4 of the *AR* gene (32.25%). However, several studies showed a higher frequency of mutations on exon 1, as these genes have two polymorphic sites in such exon, characterized by different numbers of CAG and GGN repeats. The AR is highly polymorphic due to a repetition of glutamine encoded by (CAG) and a repeat NCAA glycine encoded by $(GGT)_3$ (GGG) $(GGT)_2$ $(GGC)_n$. Abnormal expansion of the CAG segment of 44 repetitions is known to reduce the role of RA both *in vivo* and *in vitro* (Ogata *et al*, 2001). A major expansion of the CAG segment, despite the variation within the normal range (approximately 10-30) was also previously described in 78 men with low masculinization, including hypospadias (Lim *et al*. 2000). These repetitions were also described in men with cryptorchidism, but no association between CAG

repeats and non-descent of the testes was found (Sasagawa *et al*., 2000, Lim *et al*., 2001). Despite the polymorphic GGN region of AR also have a function in the receptor, studies of this polymorphism related to hypospadias and cryptorchidism are unclear (Beilin *et al.* 2000; Radpour, 2007).

Under this condition, changes in any step of this process will induce changes in specific physiological function of AR. The leucine 707 residue that was mutated in the patient studied by Lumbroso *et al.* (1996), is conserved in all members of the subfamily of steroid receptor. This suggests a crucial role in the fundamental structure of the receiver. The substitution of leucine to arginine corresponds to a non-conservative change, ie, a change from neutral and hydrophobic residue by a heavier and highly hydrophilic amino acid. It is unclear whether the abnormal characteristics of binding of mutant Leu707Arg are caused by only one difference in charge or the conformation of the field in this region of AR is also changed. The amino acid change leads to a significant change in the hidropathicity profile by a decrease in hydrophobic character and this could explain the lack of ability to bind to androgens observed *in vitro*. In addition, previous studies have shown that androgen binding stabilizes the receptor. Thus, an AR mutant, with poor connection, it is probably degraded faster than the wild-type RA.

Another mutation identified by Ferlin *et al.* (2006) that may occur in exon 4 is the substitution of the amino acid alanine by aspartate (Ala645Asp) at codon 645 in men with low sperm concentration. According to Hiort *et al.* (2000), a possible mutation in exon 6 may be caused by the substitution of glutamine by glutamic acid at codon 798 (Gln798Glu) in men who have semen changed to the motility and sperm count.

There are reports showing that mutations found in infertile men were also found in fertile men. It is still not entirely clear what role these changes are and if they lead to infertility. According to studies by Dohle *et al*., (2003) and Vogt (2004), mutations in AR are able to lead to the formation of a defective protein, affecting its normal function. Mutations in AR can cause infertility in healthy men brcause of the effect exerted in the process of spermatogenesis.

In the present study we related the molecular analysis of the *AR* gene with the social habits of patients to verify if there was any relationship between them and we found that there was no significative results in such relationship. According to Künzle *et al*. (2003), who evaluated 839 smokers and 1266 nonsmokers to determine the effect of

smoking on sperm changes, it was concluded that smoking was associated with a significant reduction in sperm density (15.3% of cases), in total sperm count (17.5%) and in the total number of sperm motility (16.6%). The percentage of normal forms was significantly reduced in smokers, and vitality of sperm, ejaculate volume and concentration of fructose were not significantly affected. There was a relationship between cigarette smoking and reduced semen quality.

According to Eskenazi *et al.* (2003), exposure to chemical agents like alcohol and cigarettes can be harmful to sperm quality, and that damage is proportional to exposure time, ie, semen volume is greater in men who never smoked and never used alcohol and sperm quality was reduced in men who have these habits throughout life. Studies on the influence of alcohol, showed that the consumption of alcohol in the long term can result in erectile dysfunction, reduced libido and gynecomastia due to the reduction in serum testosterone caused by decreased testicular production and increased metabolism by the liver (Pasqualotto *et al* ., 2004).

According to a study conducted by Oliva *et al.* (2001), toxic effects to the testicles can result in various effects such as reduction in sperm production, the production of defective sperm production and impaired androgen. In this study, the exposure significantly reduces the seminal volume and reduced by approximately 50% sperm motility. Some chemicals have the ability to interfere with the functioning of the endocrine system, mechanisms of hormone action and are called endocrine disruptors (Brown, 2002). The main effects of exposure to endocrine disruptors on male fertility are a temporary reduction in sperm concentration and quality (Weiss *et al*., 2006) and high incidence of cryptorchidism and hypospadias (Queiroz, 2006).

The change in the international industrial and agricultural development in the 20th century involving the manipulation and exposure to various substances that are harmful to humans, some of which affect the male reproductive system, such as pesticides, metals, etc. (Pasqualotto *et al*., 2004). According to Jequier (2005), approximately 6% of men with reproductive age exhibit male infertility. The most frequent causes, accounting for 90% of the total, are associated with spermatogenesis. The other cases are related to changes in the transport of sperm and accessory glands of the male reproductive tract (6%), erectile disorder (2%), ejaculation disorder (1%) and changes in the functions of the sperm (1%).

There may be no sperm (azoospeermia), a decrease in the number (oligozoospermia), change in shape (teratozoospermia) and the capability of motility

(asthenozoospermic), or the vitality (necrospermia). Exposure to endocrine disruptors can alter the hormonal metabolism by altering the synthesis of testosterone, FSH, LH and other hormones. Most of the steroid metabolism occurs in the liver, the main target for some exogenous toxic substances (Gupta, 2004).

External causes include exogenous factors, these causes may occur consciously as alcohol or tobacco, or accidentally as infections or occupational or environmental contamination (Jequier, 2005). The various external causes that lead to infertility includes exposure of workers to substances related to work, contact with toxic substances such as pesticides (Pasqualloto *et al.* 2004; Joffe, 2001). Chia & Tay (2001) studied 640 men who were unable to conceive their wives, was asked if the men had been exposed to agents known to affect spermatogenesis, such as alcohol consumption, smoking and stress. They measured the total concentration of the seminal, density, motility, viability and morphology. Cigarette proved to be a risk factor and the electromagnetic field and high levels of stress can contribute to increased risk of infertility in work with these characteristics. Despite these findings, all these factors need further study to validate this hypothesis.

CONCLUSION

Infertility affects about 10% of couples and in half the cases are related to man. The genetics of fertility is complex and depends on several factors, such as aneuploidy, gene mutations, infectious diseases and exposure to pesticides. In this study, we analyzed agricultural aviators who were exposed to pesticides for at least three years and who had difficulty conceiving. We analyzed five exons of RA, they being the exons 1, 4, 6, 7 and 8. These patients were all normal semen analysis results in seconds. After the performance of the PCR technique and statistical analysis which compared the social habits of individuals with the results of molecular analysis, we observed that there was no statistically significant results from this relationship.

In particular, the effect of an organism exhibiting genetic heterogeneity within its tissues, and the possibility of an organisms genotype changing over its lifetime, are considered to have important implications for mutation databases in the future (Gottlieb et al., 2001).

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ANEXO I

PRONTUÁRIO

NOME: _____

IDADE: _____

FILHOS: ( ) SIM ( ) NÃO

QUANTOS: MENINOS (_____) MENINAS (_____)

DESTE CASAMENTO: ( ) SIM ( ) NÃO

TELEFONE: _____ TEL. CONTATO: _____

N° PRONTUÁRIO: _____

N° PRONT. ESPOSA: _____

1. FUMA: ( ) SIM ( ) NÃO

QUANTO TEMPO: ( ) MAIS 10 ANOS ( ) MENOS 10 ANOS

QUANTOS CIGARROS: 5-10/DIA ( ) 10-20/DIA( ) 20 OU MAIS ( )

2. BEBE ( ) SIM ( ) NÃO

TODO DIA ( ) DE VEZ EM QUANDO ( )

VINHO ( ) CERVEJA ( ) CACHAÇA( ) OUTROS_____

1 COPO( ) 2-3 COPOS( ) 3 OU + COPOS ( )

3. JÁ TRABALHOU COM:

AGRICULTURA: ( ) SIM ( ) NÃO TEMPO: _____

RAIO X: ( ) SIM ( ) NÃO TEMPO: _____

XEROX: ( ) SIM ( ) NÃO TEMPO: _____

MINERAÇÃO: ( ) SIM ( ) NÃO TEMPO: _____

OUTROS PRODUTOS PERIGOSOS: _____

4. DOENÇAS:

CAXUMBA: ( ) SIM ( ) NÃO_____

DIABETES: ( ) SIM ( ) NÃO

5. ACIDENTES EM ESPORTES: ( ) SIM ( ) NÃO
6. QUEDA DE CAVALO: ( ) SIM ( ) NÃO

HOUVE FERIMENTO NA ÁREA GENITAL: ( ) SIM ( ) NÃO

7. PACIENTE TEM OU TEVE:

VARICOCELE: ( ) SIM ( ) NÃO CRIPTORQUIDIA: ( ) SIM ( ) NÃO

8. OPEROU: ( ) SIM ( ) NÃO
9. ESPOSA FEZ:

LAQUEADURA: SIM ( ) NÃO ( )

TESTE PÓS-COITO: SIM ( ) NÃO ( )

10. INDICAÇÃO CLÍNICA: ICSI ( ) FIV ( )