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Relationship of *Androgen Receptor Gene* Polymorphism (rs2261634) in Male Infertility and Its Correlation Effect on some Hormones Levels in Samples of Iraqi who Utilize Anabolic Steroids

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Abstract: Polymorphisms that increase risk factor for male infertility. This study aimed to highlighted association between abuse anabolic steroids and male infertility by evaluate the genotyping and allele frequency of *androgen receptor* gene (*AR* gene) of (rs2261634), and found the relationship of this Single Nucleotide Polymorphism and some serum hormones levels. The samples for this study were obtained from the Kamal Al- Samara'ay IVF Hospital, Ministry of Health in Baghdad, Iraq. The study was conducted at the Institute of Genetic Engineering and Biotechnology, University of Baghdad. Genotyping of rs2261634 was done by High Resolution Melting technology. The Hormonal assay (Follicle-Stimulating-Hormone, Luteinizing Hormone, Testosterone, Estrogen and Prolactin) was performed by using cobas e 411 device. The results of genotypes and alleles frequencies at *AR* gene (rs2261634) SNP. in controls *versus* Iraqi Patients with abuse anabolic steroids showed that the percentage of wild-type TT genotype was significantly ($p \leq 0.01$) lower in Iraqi Patients with abuse anabolic steroids group than in controls group (16.6% *versus* 30%, *respectively*). The conclusion of this study found The TT genotype may represent a protective factor against the incidence of patients with abuse anabolic steroids in Iraqi patients. It was concluded that testosterone, prolactin, and estrogen levels statistically increased after using high dosages of anabolic steroids compared to control, but FSH and LH levels statistically decreased.

Keywords: Steroids-Estrogen,Testosterone, FSH-LH, Infertility- Polymorphisms.

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Introduction

Anabolic Androgenic Steroids (AAS) have been used by sportsmen since the early 1950s, with a massive increase in use since then. As time progressed, issues arose over the world as a result of the usage of these drug (1). Particularly in poorer nations, many young teenagers utilize Anabolic Androgenic Steroids to enhance their physical beauty and fitness. Also, It has been discovered that more athletes who play leisure and minor-league sports utilize AAS than those who compete at the highest levels (2). However, owing of their accessibility and low cost, AAS are one of the primary health-related issues in organized sports (3). The medical and scientific community doubt any real beneficial effects achieved from the use of steroids. This controversy is added to another point concerning the side effects of these drugs on athletes' health. AAS have significant impacts on the male endocrinological and reproductive

systems, including decreased male fertility, which has been shown to be reversible. Steroid misuse may also cause cases of long-term hypogonadotrophic hypogonadism (4). Prolonged misuse, on the other hand, may result in transitory testicular damage, manifested as decreased steroidogenesis in response to a normal gonadotrophin stimulation. Although there are recommendations that AAS are used by sportsmen in Iraq, the authors are unaware of any documentation. As a result, there is a real need to conduct a preliminary study to investigate the effects of these drug abuse on male reproductive health in order to pave the way for future studies to quantify the size of this problem in Iraq and to organize an effective educational program about the harmful effects these drugs may have on the health of users (4).

The formation and maintenance of the male phenotypic and spermatogenesis depend on androgens and a functional androgen receptor (AR). This is supported by the fact that variations in the *AR* gene result in a range of androgen insensitive abnormalities, from total feminization to phenotypically infertile males (5).

Materials and methods

The work was carried out at the University of Baghdad's Institute of Genetic Engineering and Biotechnology. The study's samples were taken at the Kamal Al- Samara'ay In Vitro Fertilization Hospital, Ministry of Health in Baghdad, Iraq. The time frame for this study has been extended from November 2022 to April 2023.

The study was designed as a case control study. Samples involved 100 semen samples as 50 abuse anabolic steroids of male infertility and 50 normal healthy controls. The samples were taken from men who had stopped from sexual activity for 3-5 days. Semen samples were collected in plastic, sterile containers and incubated at appropriate temperatures for enough liquefaction time before being exposed to Macroscopic and microscopic examinations in accordance with WHO guidelines (2010) (6).

The wizard genomic DNA purification kit (Promega) was used to extract genomic DNA from whole blood of infertile and fertile men. The collected DNA was then utilized for PCR amplification of selected fragments. After checking them with the Graphic software accessible on the NCBI website to evaluate both the specification and the size of the product, particular primers were utilized each gene. Alpha DNA Company provided all primers as lyophilized products in various picomole concentrations. The primer sequences are presented in (Table 1).

Table (1): Primer of polymorphism genotyping

Prime Name	Primer Sequence for *AR* rs2261634	Product bp	C
Common Reverse	TTCATGACATATCTTCAGACAATAAACAGC	30	60
T-FW	CTAGAAAATCACTTTATCCTTGACTTTACGA**T**	32	60
A-FW	TGCGAAAATCACTTTATCCTTGACTTTACGA**A**	32	60

The Real time -PCR conditions for SNPs fragment amplification were as follows: 35 cycles of denaturation at 95 C for 15 minutes, annealing at 60 C for 30 seconds, and extension at 72 C for 30 seconds.

Sperm production is a hormonal controlled process, hence it is necessary to evaluate the hormonal profile of this study participants, The Hormonal assay (FSH, LH, Testosterone, Estrogen and

Prolactin) was performed by using cobas e 411 device.

Genotyping assay of rs2261634 was done by High Resolution Melting technology.

The Statistical Analysis System-SAS (21) application was used to evaluate the impact of various variables on research parameters. In this investigation, the Chi-square test was utilized to compare percentages and odds ratio estimates (7).

Results and discussion

The present study included 100 subjects equally divided into two groups, Iraqi Patients with abuse anabolic steroids (AAS) of male infertility and normal healthy controls, semen sample were collected from both groups for seminal fluid analysis, and blood samples were collected from both groups for hormonal study. The age of patients and healthy control was ranged between 21 to 55 years.

Serum hormones

Sperm production is a hormonal controlled process, hence it is necessary to evaluate the hormonal profile of this study participants, the hormonal assay is clarified in (Table 2). LH concentration mean was (5.32 ±0.14 and 0.588 ±0.12) in control and patients *respectively*. FSH concentration mean was (5.27 ±0.23 and 1.276 ±0.12) in control and patients, *respectively*. Testosterone concentration was (5.95 ±0.13 and 13.02 ±0.92) in control and patients *respectively*. PRL concentration was (9.69 ±0.42 and 17.59 ±2.99) in control and patients, *respectively*. Estrogen concentration was (36.69 ±1.44 and 107.46 ±7.33) in control and patients *respectively*.

The results indicated that, the FSH and LH hormones level showed statistically decrease while testosterone, prolactin and estrogen hormones showed statistically increase after taking high doses of anabolic steroids compared with control.

Table (2): Comparison between patients and control groups in Hormones level

Group	Mean ± SE				
	FSH	LH	Testosterone	Estrogen	Prolactin
Patients	1.276 ±0.12	0.588 ±0.12	13.02 ±0.92	107.46 ±7.33	17.59 ±2.99
Control	5.27 ±0.23	5.32 ±0.14	5.95 ±0.13	36.69 ±1.44	9.69 ±0.42
T-test	0.509 **	0.382 **	1.844 **	14.822 **	5.996 **
P-value	0.0001	0.0001	0.0001	0.0001	0.010
** (P≤0.01).					

AAS usage disturbs the normal hypothalamic-pituitary gonadal axis in the same way as exogenous testosterone does, resulting in hypogonadotropic hypogonadism via reduction of pulsatile gonadotropin-releasing hormone (GnRH) production. This causes a drop in FSH and LH, which in turn causes a decrease in intratesticular testosterone (ITT) and total testosterone production. ITT is required for proper spermatogenesis, and its disruption can result in infertility (8). This might be related to a transitory impairment of AAS in pituitary function, which results in lower LH and FSH hormone levels, and lower testosterone levels (9,10). The use of exogenous hormone is hypothesized to suppress the synthesis of GnRH, which lowers the release of LH h and, as a consequence, decreases testosterone production (11,12). An animal model research carried out in India that demonstrated the damaging effects of intraperitoneal injection of AAS on the testes provided more support for it. These findings also looked at harmful modifications to the

seminiferous epithelium of the testes and a reduction in tubular diameter, which decreased pituitary gonadotropin (LH and FSH) and serum testosterone levels (13). Furthermore, large dosages of AAS compounds cause negative feedback on the hypothalamicpituitary axis, resulting in decreased LH and FSH production. The impact of intratesticular testosterone concentration and FSH on Sertoli cells is required for spermatogenesis. Only Sertoli cells have FSH and testosterone receptors within the seminiferous tubules. When FSH attaches to FSH receptors on these cells, it activates several signaling pathways and works synergistically with testosterone to boost the efficiency of spermatogenesis and fertility (14). AAS-related infertility usually appears as oligozoospermia and azoospermia with impaired sperm morphology and motility (15). In addition, altered regulation of serotonergic and oradregneric neurons, which in turn affect dopamine release, may have contributed to the hyperprolactinemia by increasing sensitivity to prolactin-releasing factor and prolactin inhibitory factor. Additionally, seizures cause neuronal discharges that activate the hypothalamus and boost the pituitary gland's production of prolactin (12).

Molecular study

Distribution genotype and allele frequency of androgen receptor gene (rs2261634) polymorphisms in patients and controls

The results of genotypes and alleles frequencies of rs2261634 SNP in controls versus Iraqi Patients with abuse anabolic steroids are presented in (Table 3).

Table (3): Genotype distribution and allele frequency of rs2261634 SNP

Genotype / rs2261634	Patients No. (%)	Control No. (%)	Chi-Square (χ^2)	P-value	O.R. (C.I.)	P-value
TT	8 (16.67%)	30 (60.00%)	12.736 **	0.0004	Ref. =1	---
TA	42 (84%)	20 (40.00%)	6.667 **	0.0098	7.5 (2.9 - 4.3)	0.0001
AA	0 (0.00%)	0 (0.00%)	0.00 NS	--	1.04(0.02-53.52)	0.98
Total	50	50				
Allele Frequency						
T	56 (0.58)	80 (0.80)	P-value = 0.0396 *			
A	40 (0.42)	20 (0.20)	P-value = 0.0098 **			
* (P≤0.05), ** (P≤0.01).						

The percentage of wild-type TT genotype was significantly (p≤0.01) lower in Iraqi Patients with abuse anabolic steroids group than in controls group (16.6% *versus* 60%, *respectively*). Then TT genotype may represent a protective factor against the incidence of patients with abuse anabolic steroids in Iraqi patients. Whereas, the percentage of heterozygous mutant TA genotype was significantly (p≤0.01) higher in patients with abuse anabolic steroids than in controls (84% *versus* 40%, *respectively*). Then TA genotype may represent a risk factor for the incidence of Iraqi patients with abuse anabolic steroids. This results agree with previous study show that this polymorphism is associated with idiopathic male infertility but result of genotyping of this study disagree with genotyping results of that study because it mentioned that cytosine allele is the risk allele due to its high frequency in infertile men diagnosed with while thymine allele is less frequent in patients and more frequent in fertile group differences in allele frequency between two studies referred to

variations between different communities (15,16).

Impact of polymorphism on semen parameters

The distribution genotype and allele frequency of *AR* gene (rs2261634) polymorphisms in patients and controls show that sperm death significantly associated (P≤0.05) with polymorphism genotype means of sperm death were gradually increasing from 74.37 (in homozygous TT) to 86.75 (in heterozygous TA) so means of sperm viability decreased by 12.38% in case of homozygous TT genotype in comparison with its value in case of homozygous TA genotype viability was higher significantly associated with polymorphism genotype.

The correlation between rs2261634 SNP and Seminal fluid are presented in (Table 4).

Table (4): Relationship between rs2261634 SNP and Seminal fluid of patients group

Genotype/ rs2261634	Mean ± SE							
	Count x 10^6	Motility %	Sluggish %	Dead %	Activity %	Normal %	Abnormal %	Volume
TT	2.75 ±0.49	4.62 ±0.80	75.00 ±10.85	74.37 ±10.71	7.75 ±1.16	8.25 ±1.22	78.62 ±11.26	2.12 ±0.24
TA	2.92 ±0.15	3.85 ±0.19	84.00 ±2.25	86.75 ±2.33	7.08 ±0.41	7.95 ±0.43	86.12 ±2.34	2.04 ±0.07
T-test	NS	NS	NS	*	NS	NS	NS	NS
P-value	0.494	0.391	0.134	0.050	0.746	0.925	0.299	0.864
* (P≤0.05), NS: Non-Significant.								

The results elucidate that sperm death significantly associated (P≤0.05) with polymorphism genotype means of sperm death were gradually increasing from 74.37 (in homozygous TT) to 86.75 (in heterozygous TA) so means of sperm viability decreased by 12.38% in case of homozygous TT genotype in comparison with its value in case of homozygous TA genotype viability was higher significantly associated with polymorphism genotype. There was no difference in means of sperms with no progressive motility in There were no significant differences in means of volume, immotile sperms and morphological abnormalities between various genotypes of polymorphism. This results showed that thymine allele is highly associated with sperm viability but it contradicts results of previous study about Chinese's population elucidate that cytosine allele is highly associated with poor count of sperms and decreased motility but it was agreeing with results of that study in absence of significant differences in other seminal fluid parameters like volume a morphological abnormalities and immobility (15,16).

***Androgen receptor gene* polymorphism and Hormones**

(Table 5) explained the relationship between rs2261634 SNP and Hormones level of patients group. The result shows significant negative correlation between (FSH-LH-Testosterone-Estrogen -Prolactin) hormones and *AR* gene (rs2261634) SNP, there are no effect on those hormones levels. Multiple types of infertility may result from mutations in the androgen receptor gene that disrupt the androgen receptor's normal function(17). Several studies were investigated the relationship of *AR* gene polymorphisms and hormones levels Although the number of CAG repeats of the *AR* are related to sex steroid levels and anthropometrics, but as our knowledge the current study is the first on which connect between *AR* gene (rs2261634) and hormones levels (18).

Table (5): Relationship between rs2261634 SNP and Hormones level of patients group

Genotype / rs2261634	Mean ± SE				
	FSH	LH	Testosterone	Estrogen	Prolactin
TT	1.391 ±0.32	0.742 ±0.39	11.90 ±2.73	95.20 ±17.12	12.36 ±2.77
TA	1.233 ±0.13	0.569 ±0.14	13.16 ±1.02	107.05 ±8.10	17.06 ±3.37
T-test	0.667 NS	0.713 NS	5.138 NS	39.178 NS	15.614 NS
P-value	0.735	0.623	0.586	0.512	0.506
NS: Non-Significant.					

Conclusion

It was concluded that hormones levels statistically increased after using high dosages of anabolic steroids compared to control

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