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Studies on the relationship between the pathophysiology of infertile Algerian couples and the I/D variant of the *ACE* gene

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The abstract

Objective : The prevalence of infertility is rising due to changes in genetics and enviremantals factors. This study was to investigate the pathophysiological and clinical characteristics of infertile Algerian couples. Also, this study aimed to assess the impact of the I/D variation of *ACE* gene in the patient population in question and determine the genotype-phenotype relationship for infertility.

Methods: The I/D variant of *ACE* gene were analyzed in 50 infertile Algerian couples and 55 healthy controls. Genotyping and frequency of this variant were determined using PCR amplification on genomic DNA extracted from blood leucocytes. IUI treatment is required for patients who are eligible to participate in the study.

Results: The frequencies of the I allele, D allele and the genotypic distributions (DD, ID and II) in the infertile patients and controls had significant difference (codominant models: DI vs. DD, P=0.0001; II vs. DD, P=0.39; dominant model: DI + II vs. DD, P=0.0007; allele model: I vs. D, OR=0.48, 95% CI 0.24-0.97, P=0.04). There were also significant an association between the I/D variant and PCOS for female infertility, OATS for male infertility, and obesity in both sexes. Only 10% of the fifty women who had IUI were able to successfully conceive.

Conclusion: This genetic investigation suggests that I/D variant of *ACE* gene have a significant effect on infertile couples in the Algerian community. Furthermore, different clinical characteristics of couples who undergo IUI can lead to a difference in rates of pregnancy.

Keywords: I/D variant, *ACE* gene, Infertility, couples, IUI

Introduction

Infertility is a disease of the reproductive system characterized by the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse. Infertility is recognized as a public health issue by the World Health Organization (WHO) and is prevalent around the globe. [1, 2]

Epidemiological data shows that the incidence of infertility differs between racial and ethnic groups. [3] Approximately 15% of couples are affected by infertility.[4] In 2016, the Algerian Fertility and Contraception Association (SAFEC) estimated that the rate of infertility in Algerian couples increased from 15 to 20%. For couples that basic fertility investigations do not identify a cause of infertility, intrauterine insemination (IUI) with controlled ovarian hyperstimulation is reported to be more effective.[5]

There are many different concepts attempting to explain the pathophysiology of infertility. Previous studies have assessed the potential genetic and environmental contributions to the etiology of infertility. Numerous potential genes were been examined thus far for their potential role in the pathophysiology of infertility.[6, 7, 8]

ACE (Angiotensin converting enzyme) gene is located on chromosome 17q23. The *ACE* gene encodes a protein of 1340 amino acids. There are two distinct isoformes of ACE, the somatic isoform (sACE) (170KDa) and the germinal or testicular isoform (tACE) (100KDa).[9]

In men reproductive tract, sACE is a soluble form of the enzyme found in seminal plasma and on the surface of epididymal epithelial and Leydig cells, while tACE is exclusively expressed in post meiotic spermatids and spermatozoa, the effects of ACE in the male reproductive tract have been attributed to tACE. In women, ACE regulates the angiogenesis of ovarian endothelium, steroidogenesis, resumption of meiosis and follicular growth.[10]

According to the National Centre for Biotechnological Information (NCBI), more than 160 genetic variants of *ACE* gene have been identified.[11] One of these variants predicated on the presence (insertion, I) or absence (deletion, D) of a 287 base pair ALU repeat sequence through intron 16; resulting in 3 genotypes: homozygous DD and homozygous II and heterozygous ID. Depending on the variant, different people have different plasma levels of the enzyme; those homozygous for the D allele have the greatest levels, while those homozygous for the insertion allele have the lowest levels.[12]

Previous research have analyzed the role of the D/I variant of *ACE* gene in men and women infertility factors, however, they have returned contradictory findings.[13, 14, 15]

The objective of the current study was to investigate the pathophysiological and clinical features of infertile Algerian couples and utilize IUI treatment for them. Furthermore, this study aimed to assess the impact of I/D variation of ACE gene in the patient population in question and determine the genotype-phenotype relationship for infertility.

Materials and Methods

Study population

Fifty infertile Algerian couples were selected from the obstetrics, gynecology, and medically assisted procreation services at the Mother and Child Public Hospital, Constantine, Algeria, as candidates for IUI. The control group in this study consisted of 22 women and 33 men who were fertile.

Each couple completed an extensive questionnaire including demographic and clinical characteristics regarding age, body mass index (BMI), blood type, smoking habits, type of infertility, familial infertility and the reproductive history. All participating subjects gave written, informed consent before the molecular analyses.

Intra-uterine insemination (IUI)

IUI is often considered first-line treatment for couples with unexplained or mild male-factor infertility because it is less invasive and less costly.

+ Semen analysis and preparation

A total of 50 men were included in this study. On the day of ovulation, semen samples were taken on the spot by masturbating following 48 to 72 hours of sexual abstinence. The samples were placed in sterile containers and liquefied for 30 minutes at 37°C. In accordance with the 2010 World Health Organization (WHO) guidelines, semen samples were examined for each couple at least once. Then, we used centrifugation to gather the sperm from the seminal plasma sperm preparation methods for IUI, using swim-up or density gradient techniques. This processing technique provides better sperm quality regarding concentration and motility, which may lead to higher success rates after IUI.

+ **Ovarian stimulation (OS)**

IUI can be performed in a natural ovulatory cycle or it can be combined with OS, which is intended to induce multiple follicular developments to improve the chances of pregnancy.

On cycle day three of a spontaneous or progesterone-induced menstrual bleed, recombinant follicle-stimulating hormone (rFSH) was initiated. Serum estradiol (E2) concentration and serial ultrasound evaluation were used to observe the gonadotropin response, and dose modifications were performed as clinically needed. Recombinant human chorionic

gonadotropin (rhCG) was used to induce ovulation when at least one follicle measuring 16 mm or more has been observed. 35–36 hours following the ovulation trigger, an IUI was carried out. Approximately 16 days following the event, if no spontaneous menses had started, a quantitative serum β-hCG was obtained; a positive pregnancy test resulted from a serum β-hCG concentration of greater than 6 mIU/ml.[11]

Extraction and genotyping of the ACE

DNA was isolated from peripheral blood leucocytes of every participant using common salting-out techniques.[16]

The polymerase chain reaction (PCR) experiment was used to genotype the ACE gene's I/D variant under the following conditions: first denaturation at 95°C for 5min, followed by 37 cycles (denaturation at 95°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 30s) and a final elongation at 72°C for 4min.

Reaction PCR mixture containing 20 pmol/I of each primer forward primer 5′-GAT GTG GCC ATC ACA TTC GTC AGAT- 3′and reverse primer 5′-CTG GAG ACC ACT CCC ATC CTT TCT- 3′, dNTPs (2mM) , Taq polymerase enzyme (5U/ µl Biomatik), $MgCl_2$ (1.5 mM). The PCR products were separated by electrophoresis on 2% agarose gel.

The PCR amplification would result in 190 bp products if the “D” allele was present. On the other hand, 490 bp of DNA would show up if the “I” allele was present. When a person has a heterozygous genotype, both fragments will show up, giving two bands on electrophoresis.

Statistical analysis

The association between genotype and phenotypic characteristics of infertile couples was estimated by the odds ratios (OR) with 95% confidence intervals (CI). The genotype frequencies observed in patients and controls were compared with Hardy-Weinberg expectations by using the $\chi 2$ statistics. P-values <0.05% were considered to be significant. Analysis was carried out using the Statistical Package for Social Sciences software (SPSS, Windows version release 22; SPSS Inc.; Chicago, IL).

Results

Clinico-pathological features

The average age of men included in this study was 39.3±6.8 years. The women partners had a mean age of 33.4±5.67 years.

A total of 100 patients with infertility have included, 39 couples have primary infertility, 10 couples have primary-secondary infertility, and only one couple has secondary infertility (78%, 20% and 2%, respectively).

In all, 50 men were been recruited in our study. The demographic and clinical data are depicted in Table I.

Table I: Clinico-pathological features in men group

Characteristics	Total N (%)
BMI	
18.5 Kg/m²>BMI<2.5 Kg/m²	15(30%)
25 Kg/m²>BMI<30 Kg/m²	25(50%)
BMI≥30 Kg/m²	10(20%)
ABO blood grouping	
A	15(30%)
B	4(8%)
O	31(62%)
AB	0(0%)
Cigarette smoking	
Never	22(44%)
Past	4(8%)
Current	24(48%)
Varicocele antecedents	
Yes	5(10%)
No	45(90%)
Family history	
Yes	14(28%)
No	36(72%)
Spermatic anomalies (28 cases)	
Azoospermia	2(7,14%)
Oligospermia	4(14.29%)
Asthenospermia	9(32.14%)
Oligoasthenospermia	7(25%)
Oligoasthenoteratospermia	4(14.29%)
Crypto-astheno-teratospermia	1(3.57%)
Hypospermia	1(3.57%)

The analysis of the clinical data of infertile women showed an average age of puberty of 12.8 years and ranged between 9 and 16 years. We note that the majority of patients are between 11 and 14 years of puberty age. The collection of data is represented in Table II.

Table II: Clinico-pathological features in women group

Characteristics	Total N (%)
BMI	
18.5 Kg/m²>BMI<25 Kg/m²	15(30%)
25 Kg/m²>BMI<30 Kg/m²	21(42%)
BMI≥30 Kg/m²	14(28%)
ABO blood grouping	
A	27(54%)
B	8(16%)
O	15(30%)
AB	0(0%)
Menstruel cycle regularity	
Regular	33(66 %)
Irregular	13(26%)
Not determined	4(8%)
Obstetrical history (abortion)	
Yes	10(20%)
No	40(80%)
Family history	
Yes	9(18%)
No	41(82%)
Medical history	
Diabetes	2(4%)
Dysthyroidism	2(4%)
Fibroma	5(10%)
OPK	7(14%)
Endometrial polyps	1(2%)
Ovulation disorders	2(4%)
Tubal infertility	1(2%)
Post-partum infection	1(2%)
No priors	29(58%)

Results of IUI

Controlled ovarian hyperstimulation, semen preparation, and insemination regimens were done according to hospital-specific protocols. The study protocol recommended the use of follicle-stimulation hormone for controlled ovarian hyperstimulation. In our study, five (10%) of the fifty women who had IUI successfully conceived.

ACE Genotypes

The results of the molecular analysis of I/D variant of the *ACE* gene were been found in only 45 patients (22 women and 23 men). The genotyped population was been divided into two groups: 45 patient's in-group 1 (men-women) and 55 fertile individual's in-group 2 (men-women).

The study's findings demonstrated that each cohort of infertile patients and controls had different percentages for each of the three potential genotypes (Table III).

Table III: Genotype and allele frequencies of I/D variant of *ACE* gene in Algerian infertile patients and controls

	Patients n %	**Controls n %**	**OR**	***P-value***
DD	36 80%	25 45.45%	/	/
DI	3 6.67%	28 50.91%	13.44 3.67-49.08	**0.0001**
II	6 13.33%	2 3.64%	0.48 0.856-2.57	0.3918
DD Vs II+DI	9 20%	30 54.55%	4.80 (1.94-11.84)	**0.0007**
II Vs DD+DI	39 86.67%	53 96.36%	1.66 (0.78-21.28)	0.0956
Allele D	75 83.33%	78 70.9%	/	/
Allele I	15 16.67%	32 29.1%	0.48 0.24-0.97	**0.0414**

The significant difference was found in the ID variant of *ACE* gene between infertile cases and controls (codominant models: DI vs. DD, OR=13.44, 95% CI 3.67-49.08, P=**0.0001**; II vs. DD, OR=0.48, 95% CI 0.856-2.57, P=0.39; dominant model: DI + II vs. DD, OR=4.80, 95% CI 1.94-11.84, P=**0.0007**; recessive model: II vs. DI + DD, OR=1.66, 95% CI 0.78-21.28, P=0.09; allele model: I vs. D, OR=0.48, 95% CI 0.24-0.97, P=**0.04**).

Genotype-Phenotype Correlations

Our infertile couples underwent a thorough genotype/phenotype correlation study. Percentages of I/D variant patients have been associated with clinico-pathological data of infertile couples (Table IV), with medical and obstetrical women history data (Table V) and with men's sperm defects (Table VI).

Table IV: Genotype-Phenotype correlations in infertile couples

	DD	**DI**	**II**	**OR**	***P value***	**I allele**	**D allele**	**OR**	***P value***
Age									
<35 ans (n=22)	19	1	2	-	0.58	9	35	1.71(0.49-6.12)	0.34
>35 ans (n=23)	17	2	4			6	40		
BMI									
<30 kg/m² (n=29)	26	0	3	-	**0.031**	6	52	0.29(0.08-1.05)	**0.030**
>30 kg/m² (n=16)	10	3	3			9	23		
Tobacco									
Past+current (n=14)	11	2	1	-	0.48	3	23	1.04(0.12-10.29)	0.96
Never (n=9)	8	0	1			2	16		

Obesity is observed in 16 patients six patients carry the allele I. thus, obesity had a significant association with the I/D variant in the *ACE* gene among infertile patients

Table V: Genotype-Phenotype correlations in women

	DD	**DI**	**II**	**Allele I**
Fibroma (n=5)	5	0	0	0
OR(95%IC)	/	0(0-71.28)	0(0-6.12)	0(0-1.82)
P-value	0.16	0.57	0.23	0.06
PCOS (n=7)	3	0	4	8
OR(95%IC)	0.05(0-0.89)	0(0-42.25)	/	3,87(0,62-25.07)
P-value	**0.008**	0.48	**0.001**	0.08
Dysthyroidism (n=2)	2	0	0	0
OR(95%IC)	/	0(0-311.62)	0(0-25.8)	0(0-6.63)
P-value	0.42	0.74	0.48	0.28
Abortion (n=8)	7	1	0	1
OR(95%IC)	2.8(0.2-81.62)	/	0(0-2.7)	0.19(0.01-1.82)

P-value	0.38	0.17	0.09	0.10

There is a significant correlation between PCOS and the I/D variant of the *ACE* gene ($p<0.05$).

Table VI: Genotype-Phenotype correlations in men infertility

	DD	**DI**	**II**	I Allele
Asthenospermia (n=6) OR(95%IC) ***P-value***	6 0.92(0.05-31.74) 0.95	0 2.5(0-113.76) 0.52	1 0(0-14.08) 0.37	1 0.42(0.02-4.45) 0.43
Azoospermia (n=2) OR(95%IC) ***P-value***	2 - 0.49	0 0(0-94.69) 0.64	0 0(0-94.69) 0.64	0 0(0-12.56) 0.41
Oligospermia (n=3) OR(95%IC) ***P-value***	3 - 0.39	0 0(0-44.68) 0.56	0 0(0-44.68) 0.56	0 0(0-7.16) 0.30
Oligoasthenospermia (n=7) OR(95%IC) ***P-value***	6 1.38(0.09-42.68) 0.79	0 0(0-10.91) 0.32	1 2.5(0-113.76) 0.52	2 1.17(0.13-9.17) 0.86
OATS (n=2) OR(95%IC) ***P-value***	0 0(0-0.76) **0.001**	1 20(0-4946.33) **0.03**	1 20(0-4946.33) **0.03**	3 - **0.0000037**
Hypospermia (n=1) OR(95%IC) ***P-value***	1 - 0.63	0 0(0-327.29) 0.75	0 0(0-327.29) 0.75	0 0(0-33.95) 0.57

The result of genotyping of the *ACE* gene reviled that DD, II and ID genotype frequency was significantly higher in infertility men with Oligoasthenoteratospermia (OATS) ($p = 0.001$, $p = 0.03$ and $p = 0.03$, respectively).

Discussion

Infertility research has increased dramatically throughout the past 20 years. An infertile couple's capacity to procreate could been severely impacted by a variety of health problems, such as genetic abnormalities, bacterial illnesses, lifestyle decisions, and environmental variables.[17]

No previous or ongoing research has been done, as far as we are aware, involving infertile couples and the I/D variant of the *ACE* gene in any racial or ethnic group. The purpose of the study was to clarify the significance of the ACE variation in Algerian infertile couples undergoing IUI.

Clinico-pathological features

Age was significantly associated with infertility. Several studies have shown that women over 35 are at a higher risk of becoming infertile. Furthermore, research clearly shows a correlation between older men and declining testicular function and sperm quality.[18, 19, 20, 21]

In comparison with Europeans surveys of infertile women, the prevalence of both primary infertility and secondary infertility were different. Only 2.1% of couples had secondary sterility, according to Salazar Mederos, while 84.2% of couples had initial sterility. Compared to the opposite scenario (4.2%), the combination of primary sterility in women and secondary sterility in men occurs more frequently (9.5%). This could been explained by the social background and the fact that childless couples tend to seek infertility treatment more frequently than other couples do.[22, 23]

Studies have shown that extremes of body weight and lifestyle factors can promote excess free radical production, which could affect fertility.[24, 25, 26]

There is sufficient evidence to conclude that smokers have lower sperm parameters and sperm function tests than non-smokers and that the effects are dose-dependent. However, smoking has not proven to have a detrimental effect on male fertility yet.[27] There is good evidence that smoking in the females is associated with impaired fecundity and accelerated the loss of reproductive function and may advance the time of menopause by 1-4 years.[28]

In some developed countries, overweight and obese men and women are more prevalent than 50%. Obesity has a direct impact on multiple reproductive outcomes, including anovulation, subfertility, and infertility.[29]

ABO blood type was been related to a number of infertility processes, but it is unclear whether and how ABO blood type affects ovarian reserve. Liangshan et al (2016) explored that blood type O is a risk factor for diminished ovarian reserve while the B antigen (blood type B or AB) is a protective factor for ovarian reserve in Chinese women with subfertility.[30] Xingyu Sun confirmed this association from the same geographical area (China).[31]

Varicoceles, or abnormally dilated scrotal veins, were been found in about 40% of men who present with infertility.[32] In our study, just 10% of infertile men has varicoceles.

In previous research, 86.5% of the participants had aberrant spermogram parameters, primarily in cases of 44.8% of oligoasthenoteratozoospermia. The most frequent abnormality, accounting for 90.9% of cases, was asthenozoospermia. Other prevalent abnormalities included oligozoospermia (87.4% of cases), teratozoospermia (66.9% of cases), necrozoospermia (55.6% of cases), and azoospermia (10.4% of cases).[33] Kalfando Mwamba et al., however, noted various frequencies.[34]

PCOS is a heterogeneous and very frequent endocrinological disease associated with reproductive alterations. The presence of ovulatory dysfunction is a key factor in the pathogenesis of PCOS, resulting in reduced fertility in women with PCOS.[35, 36]

Results of IUI

IUI is frequently used as the first-line of treatment for many types of infertility. Successfully treating recommended couples with IUI can reduce the number of unneeded in-vitro fertilization treatments. There is little proof of pregnancy after IUI treatment, despite its widespread use.[37] Different clinical characteristics of couples who undergo IUI can lead to a difference in pregnancy prevalence.

Rates of pregnancy among women receiving IUI were similar to those seen in previous research conducted on populations in Nepal (10.22%) and India (11.29%) and.[37, 38] As well, in a similar study conducted in southeast of Nigeria, the prevalence as confirmed by ultrasound was 10.4%.[39] Azantee et al. found that the pregnancy rate per cycle was 14.5% in the same geographic area. Comparably, Siam evaluated the conception rate for men with idiopathic infertility (12-14%) and for those with infertility (6-8%).[40, 41] However, Ahmed et al. observed that Sultan Qabos Hospital had a high success rate of IUI (21.58%).[42]

ACE Genotypes

This study shows that the genotypic and allelic distribution of the I/D variant varied significantly between the controls and the patients. ($p = 0.0001$). Several studies reporting the association between I/D variant of *ACE* gene and the risk of men infertility. The findings were contradictory.

Infertile groups had a higher frequency of the DD genotype, according to a study by Zalata et al., and a greater proportion of II healthy fertile men in the Egyptian community.[9] Among the populations of Turkish, Saoudienne, and Indienne. There may be a high risk of infertility with the DD genotype.[43, 44, 45] Furthermore, compared to men with normal fertility, Kucera et al. found that men with a pathological sperm count had a different allele frequency of the I/D variant of the *ACE* gene variation. This was been explained by variations in the variant's

distribution and disease prevalence across regional and ethnic lines.[46] However, Liao and Roy (2002) demonstrated that the pathophysiology of men infertility in the Singapore community was not likely to be caused by ACE variants.[47]

Genotype-Phenotype Correlations

Obesity has a complicated effect on fecundity. Several cohort studies have found that anovulatory infertility is more than twice as frequent in obese women as in normal BMI controls.[48- 49]

Obesity can also cause endometrial changes. Obese women have altered endometrial gene expression during the implantation window of their natural cycles. There is a trend toward an insignificant relationship between BMI and the *ACE* gene I/D variant, which may account for the various BMI cut-off points used by researchers to define overweight and obesity.[50]

In the other hand, Kioka et al. with Greek population and Jia et al. with Caucasian population, found a statistically significant association between ACE variant and PCOS in the Caucasian population but not in the Asian population. Additionally, it shows that those with the D allele have a higher risk of developing PCOS than people with the I allele.[51,52]

In Pakistani PCOS patients, the I/D variant was significantly associated with an atypical LH: FSH ratio. As a consequence, the presence of the I/D variant is thought to affect the steroidogenesis process, which can lead to the developement of PCOS in women of reproductive age. Although the I/D variant is not the primary cause of PCOS, it is thought to play a role in its pathogenesis.[53]

Additionally, In Iranian female population, the findings of this study clearly show that the ID variant was prevalent in infertile groups with or without PCOS, with a statistically significant difference compared to the control group, highlighting its potential as a PCOS and infertile biomarker.[54]

Moreover, the I/D variants were not associated with the pathogenesis of PCOS in the Chinese population. The variants, on the other hand, were linked to steroidogenesis in the ovary. The findings indicated that I/D variant s were not the primary etiological factor, but rather may be associated with aggravated clinical manifestations of PCOS.[55]

Kucera et al, (2001) detected different allele frequencies of ACE I/D in men with a pathological sperm count compared with men with normal fertility.[46]

In the Singapore Chinese population, *ACE* gene is unlikely to play a role in the pathogenesis of male infertility.[47]

Conclusion

We are the first to analyze the genotype-phenotype correlation and the frequency of *ACE* genetic changes in infertile Algerian couples who treated with IUI. This genetic analysis shows that infertile couples in the Algerian community have been significantly affected by the *ACE* variation. Furthermore, our data validate a relationship between the *ACE* gene's I/D variant genotype and PCOS for infertility in women, OATS for infertility in men, and obesity in both sexes. For subjects with primary or secondary infertility, more research is required to fully examine this link. In addition, the clinical traits of couples who undergo IUI can have an impact on the rates of conception.

Ultimately, the results demonstrate the need for more research and more extensive studies to identify more genes.

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