

The Role of G22 A Adenosine Deaminase 1 Gene Polymorphism and the Activities of ADA Isoenzymes in Fertile and Infertile Men



Amir Fattahi, Iraj Khodadadi, Iraj Amiri, Zeinab Latifi, Marzieh Ghorbani, and Heidar Tavilani

OBJECTIVE	To evaluate frequency distribution of adenosine deaminase 1 (ADA1) G22 A alleles and genotypes in fertile and infertile men.
METHODS	In this study we evaluate frequency distribution of ADA1 G22 A alleles and genotypes in 200 fertile and 200 infertile men. The polymerase chain reaction-restriction fragment length polymorphism technique was used for determining ADA1 G22 A variants. In addition, ADA isoenzymes activities (ADA1 and ADA2) were measured using colorimetric method.
RESULTS	The frequency of GG genotype was significantly higher and GA genotype was lower in infertile males compared with fertile men ($P = .048$ and $P = .045$, respectively). However, there was not any noticeable difference in allele distribution between groups ($P > .05$). Based on logistic regression analysis, the GA genotype has a protective role and can decrease the risk of male infertility 1.7 times ($P = .046$). There were significantly higher activities of ADAT and its isoenzymes in infertile males compared with fertile men ($P < .05$). Also, the ADA1 activity with GG genotype was higher than GA carriers in all population ($P = .001$).
CONCLUSION	Our results revealed that the activity of ADA isoenzymes and distribution of ADA1 G22 A genotypes were different among fertile and infertile men and more likely the GA genotype, which had lower ADA1 activity and was higher in fertile men is a protective factor against infertility. UROLOGY 86: 730–734, 2015. © 2015 Elsevier Inc.

Adenosine deaminase (ADA: EC 3.5.4.4) participates in deamination of adenosine or deoxy-adenosine and produces inosine or deoxy-inosine, respectively; thereby it regulates adenosine and deoxy-adenosine concentration in different tissues.¹ This enzyme has two isoenzymes ADA1 and ADA2, in which ADA1 is widely distributed in various tissues but ADA2 is more abundant in serum and may be originated from monocyte-macrophage system.¹

Some studies have examined the importance of this enzyme in male and female reproductive system.^{2–9} ADA activity has been detected in whole semen, seminal plasma, and sperm cells.¹⁰ It has been reported that

inhibition of ADA1 modulates sperm fertilizing ability.¹¹ Further studies have led to the discovery of ADA1 on mammalian spermatozoa surface (ecto-ADA) and also confirmed the presence of both dipeptidyl peptidase IV (CD26) and adenosine A1 receptor (A1R) as ecto-ADA complexing proteins on spermatozoa.^{8,12} It has been demonstrated that addition of ADA to sperm suspension changes adenylatecyclase activity by decreasing adenosine concentration,¹³ so extracellular adenosine concentration has to be regulated by ecto-ADA bounded to the cell surface. Previously, Rostampour et al⁶ has shown a higher activity of plasma ADA in infertile men compared with fertile subjects. In addition, researches have revealed nonenzymatic roles for ecto-ADA such as interaction and/or fusion between prostasomes and spermatozoa.⁷

ADA1 encoded by gene located on long arm of chromosome 20 (20q 13.11) consists of 12 exons and 11 introns. The amino acid sequence of ADA1 is highly conserved among species.¹⁴ Many single nucleotide polymorphism have been observed in ADA1 gene, but the most important polymorphism that affects enzyme function is G22 A in exon 1, resulting from substitution of asparagine (Asn) instead of aspartic acid (Asp) at the

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From the Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; the Department of Biochemistry, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran; the Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran; the Department of Clinical Biochemistry, School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran; and the Urology & Nephrology Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

Address correspondence to: Heidar Tavilani, Ph.D., Hamadan University of Medical Sciences, Shaheed Fahmideh Ave., Hamadan, Iran. E-mail: tavilani@umsha.ac.ir

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eighth codon.¹⁵ Studies have shown that the activity of this enzyme is related to the G22 A genotypes.¹⁶ Bottini et al¹⁷ have reported that there is a lower frequency of GA genotype among infertile couples and in women who have recurrent miscarriages. However, most studies about relation of ADA with fertility were conducted on women.^{9,18-20}

On the basis of role of ADA in regulating adenosine concentration, which may impact sperm capacitation, motility, and function, any changes in the structure and activity of this enzyme can affect male fertility.⁷ To our knowledge, there is no reported study addressing the impact of ADA polymorphism in infertile men. Thus, in this study we aimed to evaluate the frequency distribution of ADA1 G22 A alleles and genotypes in fertile and infertile men. In addition, the correlation of different G22 A genotypes and activity of ADA isoenzymes were investigated in this population.

METHODS

Subjects, Sample Collection, and Analysis

This study was performed with participation of 200 fertile (who had at least one child) and 200 infertile males, who have been referred to Fatemeh Fertility Clinic of Hamadan University of Medical Sciences. Patients were selected according to the World Health Organization (WHO) metrics. Patients with abnormal semen analysis were enrolled in this study. According to [Supplementary Table 1](#), most of infertile men were categorized as asthenozoospermic group. All patients who had certain infertility causes such as abnormal karyotype, inflammation and infectious diseases, and varicocele were excluded from the study population. There was not any significant difference between mean ages and age ranges (29-40) of two groups. All participants completed and signed informed consent, according to the criteria of the Ethical Committee of Hamadan University of Medical Sciences.

Ten milliliters of peripheral blood were collected from each subject in heparin containing tubes. After centrifugation, plasma was separated and stored at -70°C until biochemical measurements. Cell containing part (pellet) was used for DNA extraction and genotype analysis using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Semen samples were collected from infertile males after 3 days of sexual abstinence. After liquefaction, semen-related parameters including sperm concentration, morphology, and motility grades (a: rapid progressive; b: slow progressive; c: nonprogressive; and d: immotile) were assessed, according to the WHO procedures.²¹

Genotype Analysis of ADA1

Genomic DNA was extracted from peripheral blood using the phenol-chloroform method, and its quality was determined as a ratio of A260:A280 absorbance. Forward 5'-GCCCCGCC CGTTAAGAAGAGC-3' and reverse 5'-GGTCAAGTCAG GGCAGAAGCAGA-3' primers were used to amplify a 397 bp PCR product containing polymorphism site using premix PCR kit (Bioneer, Korea) as previously described by Napolioni et al.²² After confirming the size of PCR products by agarose gel electrophoresis, PCR products were digested by TaqI restriction enzyme (Fermentas, USA) for 90 min at 65°C and then were

electrophoresed on 2% agarose gel. The AA genotype was cleaved into 245 and 152 bp fragments, while the GA genotype yielded three 397, 245, and 152 bp fragments. In contrast, the GG genotype was identified by a solo 397 bp fragment (see [Supplementary Fig. 1](#)).

Determination of ADA Isoenzymes Activity

Giusti and Galanti colorimetric method was used for assessment of total ADA (ADAT) activity.²³ To evaluate ADA2 activity, plasma ADA activity was determined after the addition of 0.1 mM of Erythro-9 (2-hydroxy-3-nonyl) adenine, which specifically inhibits ADA1 activity. The ADA1 activity was calculated by the subtraction of ADA2 activity from ADAT.

Statistical Analysis

SPSS 16.0 (IBM, USA) program was used for statistical analysis of data. One-sample Kolmogorov-Smirnov test was applied to determine the normal distribution of data. To obtain the frequencies of ADA1 genotypes and alleles in fertile and infertile men, χ^2 -test was used. In addition, logistic regression test was applied to estimate the relative risk of ADA1 genotypes. For comparison of the ADA activity between groups *t*-test was used, and one-way analysis of variance was used to compare ADA activity and sperm parameters among different genotypes. The results were expressed as mean $\pm$ standard deviation (SD), and $P < .05$ was considered as statistically significant level.

RESULTS

The semen profiles of infertile group are presented in [Supplementary Table 1](#) and the ADA1 genotype and/or allele frequencies in all subjects are described in [Table 1](#). As it is shown, the frequency of GG genotype was significantly higher in infertile males compared with fertile men (86% vs 78.5%), whereas the frequency of GA genotype was statistically higher in fertile males in comparison to infertile men (20.5% vs 13%). However, there was not any noticeable difference in allele distribution between groups. Based on logistic regression analysis, the GA genotype has a protective role and can decrease the risk of male infertility 1.7 (1/0.579) times, but a similar protective role for carrying AA genotype has not been observed ([Table 1](#)).

No significant differences were found in semen parameters among three ADA1 genotypes in infertile group ([Table 2](#)). Our results revealed that there were significantly higher activities of ADAT and its isoenzymes in infertile males compared with fertile men. In addition, the mean activity of ADA2 isoenzyme was higher than ADA1 in all of subjects ([Table 3](#)).

Unlike ADA2 activity, the activity of ADA1 was statistically different among different genotypes in both groups as shown in [Table 4](#). The activity of ADA1 with GG genotype was higher than GA carriers in all population.

CONCLUSION

Numerous studies have confirmed the presence of ADA enzyme in seminal plasma, sperm, and testes.^{10,11} In addition, it was found that this enzyme had important

Table 1. The frequency of ADA1 (G22 A) genotypes, alleles, and odds ratios in fertile (n = 200) and infertile (n = 200) males using χ^2 -test and regression logistics analysis

	Frequency		<i>P</i> -Value (χ^2 , df)	Odds Ratio, 95% CI (Lower-Upper, <i>P</i>)
	Fertile n (%)	Infertile n (%)		
ADA1 genotypes				
GG	157 (78.5)	172 (86)	0.048 ($\chi^2 = 3.853$, df = 1)	Reference group
GA	41 (20.5)	26 (13)	0.045 ($\chi^2 = 4.034$, df = 1)	0.579 (0.338-0.990, <i>P</i> = .46)
AA	2 (1)	2 (1)	1 ($\chi^2 = 0$, df = 1)	0.955 (0.356-2.561, <i>P</i> = .928)
GA+AA	43 (21.5)	28 (14)	0.048 ($\chi^2 = 3.853$, df = 1)	0.594 (0.352-1.003, <i>P</i> = .049)
ADA1 alleles				
G	355 (88.8)	370 (92.5)	0.069 ($\chi^2 = 3.310$, df = 1)	Reference group
A	45 (11.2)	30 (7.5)		0.800 (0.628-1.019, <i>P</i> = .071)

AA, adenine-adenine; ADA, adenosine deaminase; CI, confidence interval; df, degrees of freedom; GA, guanine-adenine; GG, guanine-guanine.

Table 2. Semen parameters according to the various genotypes of ADA1 in infertile group

Genotypes	Sperm Motility (%)				Sperm Concentration (Million/mL)	Sperm Normal Morphology (%)
	Grade a*	Grade b*	Grade c*	Grade d*		
GG	4.20 $\pm$ 4.74	13.67 $\pm$ 8.55	20.83 $\pm$ 12.54	62.13 $\pm$ 19.62	60.28 $\pm$ 39.62	23.16 $\pm$ 12.79
GA	4 $\pm$ 4.73	12.90 $\pm$ 9.41	19.45 $\pm$ 11.24	59.79 $\pm$ 22.30	60.15 $\pm$ 38.44	25.19 $\pm$ 15.19
AA	4.10 $\pm$ 5.79	12.20 $\pm$ 10.18	8.60 $\pm$ 5.09	75.10 $\pm$ 21.07	67.50 $\pm$ 3.53	15 $\pm$ 7.07
P-value	.979	.892	.340	.555	.967	.507

Results are presented as mean $\pm$ SD. Grade a: rapid progressive, b: slow progressive, c: nonprogressive, and d: immotile.

* Grade of sperm movement according to WHO [1999] criteria.

Table 3. ADA_{Total} and its isoenzymes activities in plasma of fertile and infertile men

	Fertile n = 200	Infertile n = 200	P-value
ADA _{Total} (U/L)	28.87 $\pm$ 10.02	40.13 $\pm$ 11.72	<.001
ADA ₁ (U/L)	9.13 $\pm$ 4.47	10.77 $\pm$ 6.48	.003
ADA ₂ (U/L)	19.73 $\pm$ 9.04	29.07 $\pm$ 9.49	<.001

Abbreviation as in Table 1.

Results are presented as mean $\pm$ SD.

roles in sperm maturation, motility, capacitation, and acrosomal reaction through adjusting adenosine concentration.⁷ Moreover, it was well reported that ecto-ADA acts as a bridge for the interaction between sperm and prostasome, which is essential for regulation of sperm function.⁷

In the present study, we have investigated the ADA1 G22 A gene polymorphism and plasma activities of ADAT, ADA1, and ADA2 in fertile and infertile men. Our results showed significant different distribution of genotypes between fertile and infertile groups, as the genotype frequencies of GG and GA were significantly higher and lower, respectively, in infertile men compared with fertile males. According to our knowledge, there is one study relatively similar to ours, which reported higher frequency of GG genotype in infertile couples than fertile group.¹⁷

The activity of ADA1 in carriers of GG genotype was higher than that of subjects carrying GA genotype. Previously, studies have shown that the activity of this enzyme in subjects with genotype GA is 20-30% lesser than that of those carrying GG genotype and, carriers of A allele have higher level of intra- and extracellular adenosine.¹⁶ Therefore, these observations may explain that higher frequency of GG genotype in infertile patients, which is

associated with increased enzyme activity, leads to reduced adenosine concentration in male reproductive system, thereby affecting its function and male fertility. Adenosine plays important roles in sperm motility, maturation, capacitation, and fertilizing ability.⁷ Therefore, decreasing adenosine concentration around sperm leads to lesser activation of adenosine receptors on sperm surface and alterations in sperm function, although we did not observe significant differences in sperm motility, concentration, and morphology between different genotypes.

According to statistical analysis, the GA genotype has a protective role and reduces the risk of male infertility over 1.7-fold. It can be concluded that the catalytic activity of ADA1 in GA genotype carriers, which is lower than that of observed in GG genotype, leaves higher levels of adenosine around the sperm and enhances sperm fertility. It is known that the G22 A polymorphism results in substitution of noncharged polar asparagine (Asn) instead of negatively charged aspartic acid (Asp) at the eighth position located on the surface of protein.¹⁵ It is therefore likely that such a change affects interaction between ecto-ADA and its binding proteins (CD26 and A1AR) and thus impairs sperm binding to prostasome. Since the binding of sperm to prostasomes regulates sperm functions, any changes in this interaction will be effective on fertility.⁷ In a study on women with recurrent spontaneous abortions, frequency of GA genotype has been reported lower than that of women with normal pregnancies, concluding that G22 A polymorphism may influence on connection between maternal and fetal cells via ecto-ADA.¹⁹ However, the amino acid substitution (N to D) is not in binding site of ADA with CD26 or A1R,²⁴ but so far the exact impact of this variation on the attachment is not known.

Table 4. ADA_{Total} and its isoenzymes activities (U/L) according to the various genotypes of ADA1 in studied population

	Genotype			P-value
	GG	GA	AA	
All population (n = 400)				
ADA _{Total}	34.96 ± 12.29	32.8 ± 12.02	25.48 ± 9.77	.142
ADA ₁	10.43 ± 5.86	7.72 ± 3.67	7.51 ± 1.98	.001
ADA ₂	24.52 ± 10.34	24.25 ± 10.59	16.67 ± 8.14	.320
Fertile (n = 200)				
ADA _{Total}	29.43 ± 10.14	27.23 ± 9.33	18.69 ± 9.14	.162
ADA ₁	9.56 ± 4.42	7.53 ± 4.40	7.59 ± 3.33	.030
ADA ₂	19.86 ± 9.23	19.70 ± 8.31	9.98 ± 4.23	.310
Infertile (n = 200)				
ADA _{Total}	40 ± 11.94	41.59 ± 10.54	32.28 ± 4.28	.519
ADA ₁	11.22 ± 6.84	8.01 ± 2.10	7.44 ± 1.78	.047
ADA ₂	28.78 ± 9.44	31.41 ± 9.92	23.36 ± 1.41	.292

Abbreviation as in Table 1.

Results are presented as mean ± SD.

We showed that the activities of ADAT, ADA1, and ADA2 are significantly higher in infertile men than in fertile males. High level of ADA2 activity in serum of infertile men might be due to monocyte-macrophage system activation, however, we did not survey this system. Moreover, we did not observe significant difference in activity of ADA2 among various genotypes because the polymorphism was related to ADA1. In his previous study, which was performed on 50 fertile and 50 infertile men, Rostampour et al⁶ reported higher level of ADAT and ADA2 activities in infertile men compared with fertile subjects, whereas no significant difference was observed in ADA1 activity between two groups maybe because of small sample size. However, in the present study high level of ADA1 activity in infertile men perhaps may be related to genotype frequency as we found high frequency of GG genotype in this group and high level of ADA1 activity in subjects with GG genotypes in comparison with other genotypes (GA and AA). We speculate that ADA polymorphism could be considered as a parameter in genetic counseling for male fertility. So evaluating ADA1 polymorphism could also be considered for improving reproductive success and embryo health.

The ADA activity might be a potentially important marker as an additional diagnostic biochemical tool for evaluating male infertility. However, determining the ADA activity in seminal fluid is also necessary for powerful interpretation. Moreover, we found high activity of ADA1 in infertile males and it may decrease adenosine concentration around spermatozoa. So, selective adding of adenosine to sperm environment in infertile men with high ADA activity might improve fertilization and pregnancy rates. As previous studies have shown significant improvements can be found in sperm motility, number of recoverable sperms, fertilization rate, number of replaceable embryos, and pregnancy rate following sperm incubation in analog of adenosine.²⁵⁻²⁸

Considering our study results and lack of appropriate research about this polymorphism and male fertility, study of ADA isoenzymes activity in seminal fluid with evaluation of G22 A polymorphism in fertile and infertile men seems necessary.

Furthermore, there is a need to assess impact of this polymorphism on binding of ecto-ADA to its cell surface receptors.

In conclusion, the results of our study showed that distribution of G22 A genotypes was different among fertile and infertile men and more likely the GA genotype, which was higher in fertile men is a protective factor against infertility. Moreover, the activities of ADA_{Total}, ADA1, and ADA2 enzymes were significantly higher in infertile men compared with fertile subjects, which indicates the importance of this enzyme in male fertility.

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References

- Gakis C. Adenosine deaminase (ADA) isoenzymes ADA1 and ADA2: diagnostic and biological role. *Eur Respir J*. 1996;9:632-633.
- Napolioni V. ADA (22G A) polymorphism: a possible genetic marker for predictive medicine of human reproduction? *Metabolism*. 2010;59:e9-e10.
- Mokhtari M, Hashemi M, Yaghmaei M, et al. Serum adenosine deaminase activity in gestational diabetes mellitus and normal pregnancy. *Arch Gynecol Obstet*. 2010;281:623-626.
- Lee SJ, Hwang HS, Kim BN, et al. Changes in serum adenosine deaminase activity during normal pregnancy. *J Korean Med Sci*. 2007;22:718-721.
- Wen X, Perrett D, Jones N, et al. High follicular fluid adenosine levels may be pivotal in the metabolism and recycling of adenosine nucleotides in the human follicle. *Metabolism*. 2010;59:1145-1155.
- Rostampour F, Biglari M, Vaisi-Raygani A, et al. Adenosine deaminase activity in fertile and infertile men. *Andrologia*. 2012;44: 586-589.
- Allegrucci C, Liguori L, Mezzasoma I, Minelli A. A1 adenosine receptor in human spermatozoa: its role in the fertilization process. *Mol Genet Metab*. 2000;71:381-386.
- Minelli A, Allegrucci C, Piomboni P, et al. Immunolocalization of A1 adenosine receptors in mammalian spermatozoa. *J Histochem Cytochem*. 2000;48:1163-1171.
- Setarehbad R, Hosseinipana SM, Vatannejad A, et al. Adenosine deaminase activity during menses, follicular and luteal phases of the menstrual cycle. *Eur J Obstet Gynecol Reprod Biol*. 2011;155: 233-234.
- Crabbe M. The development of a qualitative assay for male infertility from a study of enzymes in human semen. *J Reprod Fertil*. 1977; 51:73-76.

11. Monks NJ, Fraser LR. Inhibition of adenosine-metabolizing enzymes modulates mouse sperm fertilizing ability: a changing role for endogenously generated adenosine during capacitation. *Gamete Res.* 1988;21:267-276.
12. Mironov S, Langohr K, Richter D. A1 adenosine receptors modulate respiratory activity of the neonatal mouse via the cAMP-mediated signaling pathway. *J Neurophysiol.* 1999;81:247-255.
13. Stein D, Fraser L, Monks N. Adenosine and Gpp (NH) p modulate mouse sperm adenylate cyclase. *Gamete Res.* 1986;13:151-158.
14. Hershfield MS. Genotype is an important determinant of phenotype in adenosine deaminase deficiency. *Curr Opin Immunol.* 2003;15:571-577.
15. Hirschhorn R, Yang D, Israni A. An Asp8Asn substitution results in the adenosine deaminase (ADA) genetic polymorphism (ADA 2 allozyme): occurrence on different chromosomal backgrounds and apparent intragenic crossover. *Ann Hum Genet.* 1994;58:1-9.
16. Battistuzzi G, Iudicone P, Santolamazza P, Petrucci R. Activity of adenosine deaminase allelic forms in intact erythrocytes and in lymphocytes. *Ann Hum Genet.* 1981;45:15-19.
17. Bottini E, Carapella E, Cataldi L, et al. Adenosine deaminase polymorphism. Associations at clinical level suggest a role in cell functions and immune reactions. *J Med Genet.* 1981;18:331-334.
18. Nunes DP, Spegiorin LC, Mattos CC, et al. The ADA* 2 allele of the adenosine deaminase gene (20q13. 11) and recurrent spontaneous abortions: an age-dependent association. *Clinics (Sao Paulo).* 2011;66:1929-1933.
19. Nicotra M, Bottini N, Grasso M, et al. Adenosine deaminase and human reproduction: a comparative study of fertile women and women with recurrent spontaneous abortion. *Am J Reprod Immunol.* 1998;39:266-270.
20. Jadhav AA, Jain A. Adenosine deaminase activity in normal pregnancy and pregnancy associated disorders. *Arch Physiol Biochem.* 2013;119:88-91.
21. Organization WH. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction.* Cambridge: Cambridge University Press; 1999.
22. Napolioni V, Lucarini N. Gender-specific association of ADA genetic polymorphism with human longevity. *Biogerontology.* 2010;11:457-462.
23. Giusti G, Galanti B. Colorimetric method. In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis.* 3rd ed. Weinheim, Germany: Verlag Chemie; 1984:315-323.
24. Fan H, Tansi FL, Weihofen WA, et al. Molecular mechanism and structural basis of interactions of dipeptidyl peptidase IV with adenosine deaminase and human immunodeficiency virus type-1 transcription transactivator. *Eur J Cell Biol.* 2012;91:265-273.
25. Imoedemhe D, Sigue A, Pacpaco E, Olazo A. Successful use of the sperm motility enhancer 2-deoxyadenosine in previously failed human in vitro fertilization. *J Assist Reprod Genet.* 1992;9:53-56.
26. Imoedemhe D, Sigue A, Pacpaco E. Pregnancy following sperm incubation in 2-deoxyadenosine (2-DXA) prior to in vitro fertilization (IVF). *J Assisted Reprod Genet.* 1991;8:59-61.
27. Edirisinghe WR, Junk S, Yovich JM, et al. Sperm stimulants can improve fertilization rates in male-factor cases undergoing IVF to the same extent as micromanipulation by partial zona dissection (PZD) or subzonal sperm insemination (SUZI): a randomized controlled study. *J Assist Reprod Genet.* 1995;12:312-318.
28. Shen MR, Linden J, Chiang PH, et al. Adenosine stimulates human sperm motility via A2 receptors. *J Pharm Pharmacol.* 1993;45:650-653.

APPENDIX

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.urology.2015.06.034>.