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Original Article

PROTAMINE1 and *PROTAMINE2* genes expression in the sperms of oligoasthenospermic individuals and intrauterine insemination candidates couples: Is there any significant differences?

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Abstract

Background: Male infertility refers to a male's inability to cause pregnancy in a fertile female. It seems the large portion of this category of infertility, has roots in genetic factors. *PROTAMINE* family is one of the most important genes which are involved in male factor infertility. Hence, the aim of this study is to evaluate *PROTAMINE1* and *PROTAMINE2* (P1 and P2) genes expression in oligoasthenospermic individuals and intrauterine insemination (IUI) candidate couples' sperms.

Materials and Methods: Samples were gathered from the patients referred to the Isfahan Infertility Center of Shahid Beheshti, 80 semen samples were in IUI candidates groups and 16 semen samples were in oligoasthenospermia group was collected. The outcome of IUI procedure was followed up after 14 days. Through these samples, 16 couples achieved pregnancy (IUI$^+$) and from the top of the list, 16 semen samples with negative β-HCG were obtained (IUI$^-$). After RNA extraction from sperms, *PROTAMINE* genes family expression was evaluated in our three groups by real time-reverse transcription polymerase chain reaction.

Results: Our study revealed that P1 gene expression has no significant differences between IUI$^-$, IUI$^+$, and oligoasthenospermia groups, whereas P2 gene expression showed significant differences between oligoasthenospermia with two IUI groups. Main sperm parameters have no significant differences between IUI groups.

Conclusion: This study reveals P1 and P2 genes expression value have no significant differences between IUI$^-$ and IUI$^+$. On the other hand, P2 gene expression value has significant differences between oligoasthenospermia with two IUI groups.

Key Words: Intrauterine insemination, oligoasthenospermia, *PROTAMINE* genes, sperm

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INTRODUCTION

Reproduction is one of the main natural desires of human and one of the most important key factors of the community survival. In addition, infertility has long been a source of concern for all human societies.[1,2]

According to the World Health Organization (WHO), infertility is "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse." Global estimates say that approximately 15% of couples worldwide are infertile. In the Middle East, it is estimated about 10–15%.[3,4]

Male infertility problems make up the major cause of infertility in the world.[5] Infertility in couples has many reasons such as male problems (35%), problems with fallopian tubes and pelvis in women (35%), ovulatory disorders (15%), unexplained infertility (15%), and unusual problems (5%).[6,7] Male infertility refers to a male's inability to cause pregnancy in a fertile female.[5] Many studies have mentioned that social progress in developing countries, adverse weather conditions, job stress, and noise pollution from industrial plants and so on; reduce the level of male fertility.[7-9] Factors involved in male infertility in so many researches have examined. The most important of these factors can be mentioned the following: Reduction of produced spermatozoa, production of sperm with poor quality, sperm outflow obstruction, production of anti-sperm antibodies, sexual problems such as premature ejaculation, alcohol and cigarette consumption, environmental factors such as X-ray and genetic factors.[10-15]

Despite significant advances in the diagnosis and treatment of infertility, 50% of male infertility cases, remain as idiopathic infertility.[15,16] Although it seems the large proportion of this category of infertility has roots in genetic disorders while thousands of genes involved in spermatogenesis phenomenon, only a small fraction of them have been identified and even the much smaller, have been under review and analysis. Genetic damage has been detected in infertile men in all groups and increasingly in many studies they mentioned as a key factor in male infertility.[17,18] There is a positive relationship between genetic damage and defects in sperm parameters. Many studies have shown that the integrity and accuracy of sperm DNA are more and more critical factors in its performance against parameters such as the number and mobility of sperm. Hence, this is the truth that the conventional semen parameters, not evaluated as well as their biological characteristics.[19-21]

So far, several genes associated with sperm parameters analyzed, *PROTAMINE* family is one of the most important of this genes. Protamines are the main proteins involved in sperm DNA density. They are small nuclear proteins, rich in arginine. In the late phase of spermatogenesis, histones are replaced by protamines that are necessary for condensation of sperm head.[22] It is reported that abnormalities in histone transition and protamine expression in human have been found to be associated with male infertility.[23] In mammals, two types of protamine, PROTAMINE1 and PROTAMINE2 (P1 and P2) are studied. Both of them are situated on short arm of chromosome 16. In all mammals, P1 packs the sperm DNA while P2 is only exist in primates, many rodents and some of the placental mammals.[22] Defects of these proteins associated with sperm ultra-structural chromatin defects. In addition, one of the causes that result in reproductive failure, is a defect in Protamine proteins.[24]

According to Nasr *et al*. (2009), sperm parameters in infertile men with protamine deficiency were lower than the parameters defined by the WHO reported.[25,26] In several studies it has noted that the protamine deficiency is associated with male infertility. Nasr *et al*., Oliva, Nasr *et al*., Tavallaee *et al*., Hammoud *et al*., and Shokri *et al*., and in so many other studies, relationship between protamine deficiency and semen parameters or male infertility have been studied.[27-32]

Hence, the aim of this study is P1 and P2 genes expression in oligoasthenospermic individuals and intrauterine insemination (IUI) candidates' couple's sperms and to evaluate if there is any significant differences between these two groups.

MATERIALS AND METHODS

Sample collection and parametric analysis of semen

Semen samples from 96 patients referred to the Isfahan Infertility Center of Shahid Beheshti was collected. Totally, 80 semen samples were in IUI candidate groups. Two weeks after performing IUI procedures, 16 cases became pregnant (IUI$^+$ group). We selected 16 semen samples from the top of the list as our IUI$^-$ groups. Moreover, 16 semen samples were gathered as oligoasthenospermia group. Therefore, we have three groups for sperm evaluation (IUI$^+$ group, IUI$^-$ group, oligoasthenospermia group). Gathering the samples was performed after obtaining consent according to Isfahan University of Medical Science's Guidelines of Ethical Committee.

According to the WHO, semen volume should be more than 1/5 ml per ejaculation. Sperm concentration

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should be 15 × 10^6/ml at least. About 40%–50% of sperm should be moving forward and the sperm with normal morphology in each ejaculation should be more than 4% totally. All of these semen samples were analyzed under the computer-assisted sperm analysis. IUI candidate samples were in normal sperm parameters range according to the WHO manual laboratory guideline (2010) and 16 samples with low quality of parameters, were chosen as oligoasthenospermia group. In the recent group, the progressive forward moving of sperms reported <10% and sperm concentration was <15 mil/ml.

Sperm preparation and RNA extraction

All of the samples were taken from the patients after 5–3 days of abstinence from sexual intercourse. After that, they were washed by using two methods: Swim up and Simple washing. Swim up used for washing the normal quality samples (IUI candidates), according to the following protocol: Semen is washed with Ham's F10 and then samples placed in centrifuge, about 5 min. Finally, it is washed with combination of Ham's F10 and Albumin and placed in incubator at 37°C about 30 min. After the aforementioned time, sperms placed at the top of the solution were isolated. Simple washing method which we used for washing low quality semen samples (oligoasthenospermia), has no significant difference with previous method. In this way, sperms were washed with the solution of Ham's F10 and Albumin, deposited at the bottom of the container and isolated from the solution.

After washing, the IUI semen samples were frozen at −70°C. 21 days later, patients were contacted and asked about the result of the IUI procedure. In this way, samples were divided into two groups: IUI^+ (successful pregnancy) and IUI^- (failure in pregnancy). From oligoasthenospermia immediately and from IUI candidates after the mentioned time and defreezing the samples, RNA extraction was performed. In this method, using RNase plus universal minikit (Qiagen Company, Cat. No 73404), according to the protocol RNAs of semen samples were extracted and to verify quality of the extracted RNAs, agarose gel electrophoresis was performed. The concentration of the RNAs was determined by spectrophotometry. Then, cDNA strands synthesized by using ReverAid First strand cDNA synthesis kit of Fermentas Company from RNAs with oligo-dT primers. To evaluate the expression of P1 and P2, real time polymerase chain reaction (PCR) and ΔCT method was performed. The real time PCR was performed with gene specific primers and the SYBER Green PCR Master mix (Qiagen) using a thermal cycler rotor-gene 6000 (Qiagen). GAPDH as a housekeeping gene was used. Finally, these gene expressions were examined in the case groups compared with control group according to relative quantitation method.

Quantitative real time-PCR was performed using specific exon junction primers for P1 and P2 mRNAs [Table 1], with the Beacon Designer 8.01 (Premier Biosoft Company, Palo Alto, California, USA).

Statistical analysis

To statistically analyze the data, Student's *t*-test and ANOVA were used and also to analyze and comparison the data between two groups, Duncan's tests were done. The SPSS software (Statistical Package for the Social Sciences version 22, SPSS Incorporated, Chicago, Illinois, USA) was utilized for statistical analyses, and differences were considered significant if $P < 0.05$.

RESULTS

Relative expression of *PROTAMINEs* gene was determined respect to the expression of GAPDH as a housekeeping gene. According to Table 2, the mean expression of the both P1 and P2 gene in oligoasthenospermia group is lesser than the two other groups. Moreover, the standard deviation in IUI^- is higher than the two other groups. The *P* value of ANOVA test of P1 gene expression is >0.05, so the mean of P1 gene expression has no significant difference between these three groups [Figure 1]. The *P* value of ANOVA test of P2 means expression is <0.05, so the mean expression of P2 has significant difference between our groups [Figure 2] by using Duncan's *post hoc* test, the mean expression of P2 gene were calculated in pairwise form between the

Table 1: Sequences of the real time-polymerase chain reaction primer sets

Gene	Primer sequence	Size of amplicon
PROTAMINE1	F: 5'-AGGAGAGCCATGAGGTGCT-3' R: 5'-CAGGAGTTTGGTGGATGTGCTATT-3'	89 bp
PROTAMINE2	F: 5'-CGGAGGAGGCATCGCAGAGG-3' R: 5'-GGTGTTTCTTGGGCAGGTGACTTT-3'	198 bp
GAPDH	F: 5'-ACCACAGTCCATGCCATCAC- 3 R: 5'-TCCACCACCCTGTTGCTGTA-3	452 bp

Table 2: Means and standard deviation of PROTAMINE1 and PROTAMINE2

Sample group	Mean±SD	
	PROTAMINE1	PROTAMINE2
IUI^+	0.03444±0.017048	0.06410±0.032449
IUI^-	0.04457±0.035879	0.05197±0.038840
Oligoasthenospermia	0.02950±0.019718	0.01022±0.006683
P	0.248	0.000

SD: Standard deviation, IUI: Intrauterine insemination

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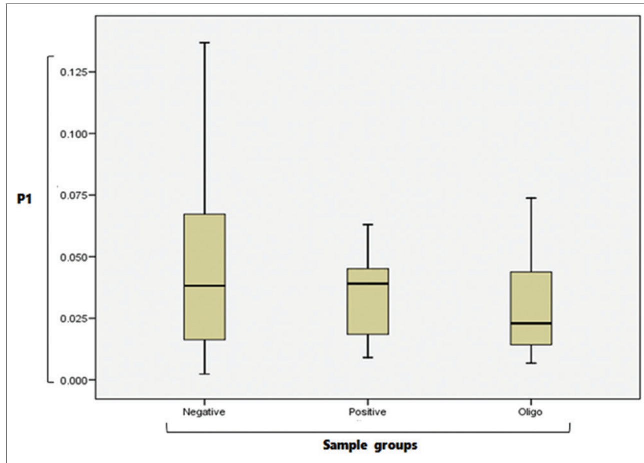

Figure 1: Relative expression of *PROTAMINE1* in three samples groups. There is no significant difference

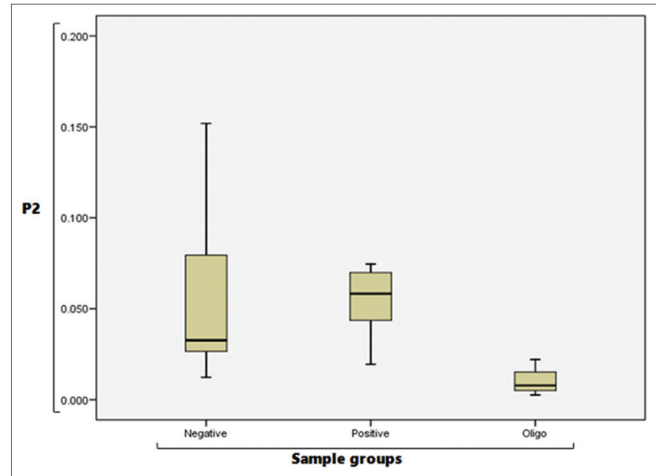

Figure 2: Relative expression of *PROTAMINE2* in three samples groups. There is a significant difference between oligoasthenospermia group and IUI+ and IUI−. The expression of *PROTAMINE2* in these two groups is significantly higher than oligoasthenospermia

groups. The result showed that the mean of this gene expression in oligoasthenospermia has a significant difference with IUI+ and IUI− groups and this group has the least expression of P2 gene compared to the two. The mean of this gene expression has no significant difference between IUI+ and IUI− [Table 2]. Again, as Table 2 reveals the gene expression ratio PI/PII in IUI+, IUI−, and oligoasthenospermia groups were 0.5372, 0.8576, and 2.8864, respectively. Table 3 illustrates men semen parameters have no significant differences between two IUI groups.

Table 3: Means and standard deviation of mail semen parameters

Parameters	Mean±SD		*P*
	IUI+	IUI−	
Concentration	112.75±51.99	106.62±61.99	0.755
Normal morphology	8.06±5.07	6.75±2.40	0.376
Fast motility	13.31±6.80	11.06±5.47	0.379
Slow motility	22.93±13.21	32.06±9.35	0.152

SD: Standard deviation

DISCUSSION

In this, differences between IUI+ candidates as fertile group and oligoasthenospermia as infertile group were evaluated by *PROTAMINE* genes expression. Based on our findings, P1 gene expression has no significant differences between IUI candidates and oligoasthenospermia groups, whereas P2 gene expression has significant differences between sperms in oligoasthenospermia and our IUI candidate couples.

During sperm production, the spermatogonia A and B were undergoing profound changes. Obviously, this phenomenon is performed in three phases; spermatogenesis phase, meiotic phase and spermiogenesis phase. Through the *PROTAMINE* family, P1 presents in almost all mammalian species and *PROTAMIN*2 is observed in mice, hamster, horse, and human.[33-35] In some species the P2 gene is present but no protein production occurs. Any changes in process of substitution of protamine directly effects on fertilization rate and embryo quality and pregnancy outcome.[36]

In mammals, two types of protamine P1 and P2 are studied. Protamines are essential for density of sperm genetic material during spermatogenesis phenomena and probably have other important functions such as down regulation of the DNA transcription in sperm. Unfortunately, many of their functions still remain unknown. So far, three main functions of protamines were detected: (1) Condensation of sperm nucleus (2) protection of paternal genome from nucleases and free radicals (3) paternal genome imprinting.[28,37-42]

Nasr-Esfahani *et al*. was using CAM3 staining along with semen parameters in *in vitro* fertilization (IVF) candidates couples. They declared that insufficient protamines contents could affect the outcome of IVF procedure.[25] In present study, the comparison of the P1 and P2 gene expression in three groups: IUI candidates with positive and negative outcomes and one group with oligoasthenospermia. We compared the protamine content in nuclear region of sperms in oligoasthenospermia with IUI+ and IUI− candidates' sperms. No significant differences in main sperms parameters and *PROTAMINE1* gene expression observed between IUI+ and IUI− candidates and oligoasthenospermic individuals. However, expression of P2 genes reveals significant differences in oligoasthenospermia with IUI candidate's couples.

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In our study, IUI^+ outcome was taken as control groups in which main semen parameters are in normal range and fertilization test is positive. On the other hand, IUI^- outcomes illustrate on situation in which main semen parameters are in normal range but no fertility occurs.

Considering the finding of this study emphasize on; standard semen parameters which were developed by the WHO guideline (2010), still is the unique way to evaluate male fertility status. Because of, here we have no significant differences in main semen parameters between IUI^+ and IUI^- groups as well as no significant differences between *PROTAMIN1* and P2 genes expression between mentioned to IUI candidates groups and these genes expression could not be a valuable marker to obtain the prediction of IUI outcome.

CONCLUSION

This study reveals P1 and P2 genes expression value have no significant differences between IUI^- and IUI^+ and should not use these parameters to predict of outcome of IUI. On the other hand, P2 gene expression value has significant differences between oligoasthenospermia with two IUI groups.

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Conflicts of interest

There are no conflicts of interest.

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