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Evaluation the expression of insulin and insulin receptor-beta (IR-β) in sperm of infertile male with failed intracytoplasmic sperm injection (ICSI)

Fateme Alizadeh[1] , Masoud Hemadi[2*] , Ghasem Saki[3] , Akram Gavahi[2] , Farideh Moramezi[2] , Armita Valizadeh[4]

[1]Department of Anatomy, School of Medicine, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran
[2]Fertility, Infertility and Perinatology Research Center, School of Medicine, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran
[3]Physiology Research Center School of Medicine, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran
[4]Department of Bone Marrow Transplantation, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran

***Correspondence to**
Masoud Hemadi, Ph.D,
Email: hemadi-m@ajums.ac.ir

Abstract

Introduction: Most infertility treatment centers are currently using semen analysis to differentiate between fertile and infertile individuals. Recent studies have evaluated the expression and secretion of independent insulin and related beta receptor (IR-β) in mammals' ejaculated spermatozoa such as humans and pigs, and its effect on motility parameters capacitation and acrosomal reaction.
Objectives: This study aimed to investigate the expression of insulin, IR-β and the parameters of sperm and DNA fragmentation index (DFI) in men with intracytoplasmic sperm injections (ICSI) failed.
Patients and Methods: This study was conducted on 15 men with unknown infertility with a history of unsuccessful ICSI and 10 fertile men. After collecting samples, sperm parameters (concentration and motility), sperm DNA fragmentation and expression of insulin and IR-β genes were investigated. To assess DFI from SDFA kit and to review the expression, the genes were analyzed via RT-PCR method. Data were analyzed statistically by t-test and covariance tests.
Results: Based on the findings of this research, the concentration and percentage of motility in infertile men with a history of unsuccessful ICSI was significantly lower than fertile individuals, moreover, the amount of DNA fragmentation significantly increased in infertile men with a history of unsuccessful ICSI compared to fertile men ($P < 0.05$). Insulin expression in the sperm of infertile men with a failed ICSI history was significantly lower than fertile men ($P < 0.05$); however, the IR-β expression rate was not significantly different between the two groups.
Conclusion: Evaluation of insulin gene expression can be helpful as a good marker for identifying infertility with an unknown cause.

Introduction

Insulin (Ins) is secreted from B-cells of the endocrine pancreas in adult mammals, and also from the thymus in young animals (1). During embryonic development, the yolk sac (2) and brain (3) of rodents contain the extra-pancreatic insulin gene. Ins first bind to and activates membrane protein receptors to initiate its effects on the target cell. What causes the effect is the activated receptor, not Ins (4). A heterotetrametric transmembrane insulin receptor beta (IR-β) is located on somatic cells, consisting of two A and B subunits that are linked together using disulphide bonds (5). The A subunit is extracellular component and the B-subunit is found in the plasma membrane prominently in the cell cytoplasm. The insulin is linked to the a-subunits and subsequently causes activation of intrinsic tyrosine-specific kinase of the B-subunits

Key point
- Assessment of insulin gene expression in ejaculated semen can be a good marker for evaluation of sperm quality and predicting male fertility.
- Lower expression of the insulin gene may be a helpful marker for identifying infertility with an unknown cause.

within the cell to phosphorylate tyrosine residues and intracellular phosphorylate substrates, playing a central role in the signal transduction to trigger different functions of the insulin such as the regulation of gonadal hormones (6). The males with insulin-dependent diabetes show severe defects in sperm structure, obviously poor motility, lower count, abnormal morphology (7, 8) and weak penetration into hamster eggs (9). In addition, the spermatozoa of patients

with type 1 diabetes have high level of oxidative DNA modification, mitochondrial DNA deletion and nuclear DNA fragmentation (10).

Objectives
Recent studies have shown the expression and independent insulin secretion and related IR-β receptor in mammals' ejaculated spermatozoa such as humans and pigs and its effect on motility parameters, capacitation, and acrosomal reaction (11). Therefore, the aim of this study is to evaluate the expression of insulin and IR-β in human ejaculated spermatozoa, as well as the biofunction of insulin for obtaining the ability to fertilize and metabolize sperm.

Materials and Methods
Chemicals
The total RNA isolation system kit was purchased from Qiagen, Germany, c-DNA synthesis kit, Syber Green & DNase1 purchased from Thermo Fisher Scientific company, USA, primers from Macrogen, South Korea. SDFA kit was purchased from Dianbioassay, Iran.

Sample selection and routine inspection of sperm
The present study included 15 unexplained infertile men with history of unsuccessful intracytoplasmic sperm injections (ICSI) and also 10 healthy fertile men having children (control group), aged 25 to 44 years. Sampling was done at Imam Khomeini Hospital in Ahvaz. The patients were instructed to avoid any sexual activity for three to five days prior to transferring semen sampling through masturbation into a sterile plastic container. Completely liquefied semen volume (mL), sperm motility (percentage) and sperm count (10/mL) were determined. Furthermore, the semen sample of 0.5-1 ml was separated for evaluation of DNA fragmentation.

Detection of DNA fragmentation in sperm samples
The DNA fragmentation in the sperm samples was detected by the sperm DNA fragmentation assay kit (SDFA kit, Dianbioassay, Iran). Thus, the fresh semen was washed and then diluted by PBS to the desired level (15-20 ×10/mL), and the DNA fragmentation was detected in accordance with the kit instruction.

RNA isolation and RT-PCR
First, for preparation of cell pellet, semen sample was washed twice with phosphate buffered saline (PBS), then by using RNeasy mini kit (Qiagen, Germany) and as directed, total RNA isolated from ejaculated spermatozoa. The remaining DNA was removed by digestion with DNase I, and the RNA concentration was determined by a NanoDrop device. For the construction of cDNA, 1μg of DNAase-treated RNA samples were transcribed reversely by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) on a thermocycler. After synthesis of cDNA to quantify the expression of Insulin and IR-B genes, RT-PCR was performed. The primers for Ins and IR-B genes, as well as the primer for the GAPDH (as an internal control) were designed using the NCBI site and the Oligo7 software whose specification is given in Table 1.

Materials needed for RT-PCR included: primer (1 μL), cDNA (1 μL), Syber green/Rox qpcr master mix (5.5 μL) and RNase-free water (3.5 μL). Cycling conditions were the initialization at 95°C for 4 minutes, and then 40 cycles including denaturation at 95C for 5 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds. The normalization of target gene level was performed to the GAPDH expression. The negative control, with no reverse transcription, was considered for all genes. The comparative formula of Ct method $2^{-\Delta\Delta ct}$ was used to analyze the PCR findings.

Ethical issues
The Declaration of Helsinki was observed to conduct the present study at Ahvaz Jundishapur University of Medical Sciences. Accordingly, written informed consent was taken from all participants before any intervention. This study was extracted from the Master of Science thesis of Fateme Alizadeh at this university (Thesis ethical code# IR.AJUMS.REC.1397.457).

Data analysis by statistical tests
All data are reported as mean ± standard deviation (SD). Data normal distribution was checked by Kolmogorov-Smirnov test. Independent sample *t* test and analysis of covariance (ANCOVA) were used to analyze the results using SPSS statistics version 17.01 (SPSS Inc., Chicago, USA) at a significance level of $P<0.05$.

Results
Semen analysis parameters
The analysis of semen parameters showed no statistically

Table 1. The primers used in this study

Company	Sequence name	Primer sequence	Primer length
Macrogen	GAPDH	5-CGGTGAGTACGTCGTGGAGTC-3 F 5-GCAGGAGGCATTGCTGATGA-3 R	21 mer 20 mer
Macrogen	Insulin	5-TGAACAAAGATGACAACGAGG-3 F 5-GTGTGACTTACAGATGGTCGG-3 R	21 mer 21 mer
Macrogen	IR-β	5- ACAACCAGAGTGAGTATGAGG-3 F 5-TGAAGAGGTTTATCTGGGGAC-3 R	21 mer 21 mer

significant difference in the mean semen volume in the study groups ($P = 0.180$). Assessment of sperm count and motility showed a significant difference in sperm count ($P = 0.001$) and sperm motility ($P < 0.001$) between the two groups (Table 2).

Analysis on sperm DNA fragmentation:

In this study, there were 15 infertile males (unsuccessful ICSI) and 10 fertile males having children. The analysis showed higher sperm DNA fragmentation in the infertile males when comparing with the controls ($P = 0.003$), the data of which are presented in Table 1. Analyzing the correlation of sperm DNA fragmentation and routine semen parameters negatively correlated sperm DNA fragmentation with semen volume, count and motility (Table 2, Figure 1).

Evaluation of insulin and IR-β expression

To compare Ins and IR-B gene Expression in sperm between two groups, RT-PCR was conducted. Since the two groups showed a significant difference in the mean sperm concentration, motility and DNA fragmentation index (DFI), Co-variance analysis was used to adjust these variables and compare the gene expression between the two groups. The result showed that insulin gene expression was significantly lower in infertile men with a history of unsuccessful ICSI than in fertile men ($P = 0.033$), but there was no significant difference in IR-B gene expression ($P = 0.102$) between the two groups (Tables 3-5; Figures 2 and 3).
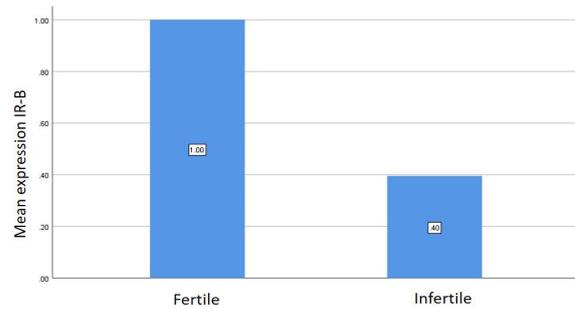

Figure 1. Evaluation of DFI to check integrity DNA sperm. A Fertile group; - B; Infertile with unsuccessful ICSI group.

Discussion

The ejaculated spermatozoa in human beings has been reportedly shown to express the insulin autonomously capable of controlling their metabolically energy balance, independent of systemic regulation (12). According to documents, spermatozoa transmit the paternal haploid genome, and activate the signals and sperm centrosomes to the oocyte. Moreover, the spermatozoa are responsible for providing unique paternal mRNAs. It is not residual to spermatogenesis regarding sperm RNA functions, including de-novo degraded protein translational replacement, chromatin repackaging, and post-fertilization transfer of multiple needed RNAs to the oocyte (13). Physiological functions of insulin and IR-B have been previously evaluated on either capacitation or acrosome processes. The mammalian spermatozoa must

Table 2. Comparison of sperm parameters between two groups

Variable	Count	Mean	Standard Deviation	Minimum	Maximum	P value
DFI	25	39.88	22.23	15	95	0.001*
Motility%	25	43.40	9.10	30	60	0.000*
Concentration	25	32.76	10.54	18	62	0.001*

Table 3. Comparison of genes expression

Variable	Groups	Count	Mean	Standard Deviation	Minimum	Maximum	P value*
Ins expression	Fertile	10	1.00	1.49	0.00	4.38	0.154
	Infertile	15	0.24	0.57	0.00	2.19	
IR-B expression	Fertile	10	1.00	1.00	0.01	3.32	0.068
	Infertile	15	0.40	0.59	0.00	1.66	

*Results from independent *t* test.

Table 4. Comparison of Insulin expression

Parameter	B	Standard Error	T	P value
Fertile/infertile	1.497	0.651	2.298	0.033*
DFI%	0.026	0.014	1.833	0.082
Motility%	-0.021	0.035	-0.616	0.545
Concentration	0.014	0.033	0.421	0.678

*Results from independent ANCOVA analysis.

Table 5. Comparison of IR-β expression

Parameter	B	Standard Error	T	P value
Fertile/infertile	0.906	0.529	1.713	0.102
DFI%	0.003	0.011	0.288	0.776
Motility%	-0.017	0.028	-0.607	0.551
Concentration	0.000	0.026	-0.007	0.995

*Results from independent ANCOVA analysis.

be maturated for oocyte fertilization during capacitation and acrosome processes (14), so that the capacitation prepares sperm from a specialized exocytosis process as the acrosome reaction (15), enabling the penetration of the zona pellucida of the ovum.

According to previous studies that have revealed Ins and its IR-β mRNA expressed in the spermatozoa of pig, in this study for the first time, we investigated the expression of mRNA for Ins and IR-β in sperm of infertile men and their effect on obtain the ability to fertilization. We demonstrated that the expression of insulin in the infertile male sperm with history of unsuccessful ICSI is significantly lower when comparing with fertile males. However, IR-β expression between two groups did not show significant differences. Given that the quality of sperm and the integrity of sperm DNA are lower in

infertile patients with history of unsuccessful ICSI, it can be concluded that the quality and function of sperm cells and the ability to fertilization can be altered by decreasing insulin gene expression.

Conclusion
The current study for the first time demonstrated that the expression of insulin mRNA in infertile men with history of unsuccessful ICSI is lower than fertile men. Therefore, assessment of insulin gene expression in human ejaculated sperm can be used as a good marker for evaluation of sperm quality and predicting male fertility.

Limitations of the study
This study has an insufficient sample size for statistical measurements, which is due to problems in sampling.

Conflicts of interest
The authors of this study declare no conflicts of interest.

Authors' contribution
FA, AG, and AV carried out the experiments and contributions to the study design. FA and FM prepared the primary draft. MH and GS analyzed the obtained data. MH was responsible for the final draft edition and final manuscript approval. The paper article was written and signed by all authors.

Ethical considerations
The authors observed completely the ethical considerations, such as redundancy, double publication or submission, falsification, data fabrication, misconduct and plagiarism.

Figure 2. Comparing the expression of insulin in the sperm of fertile males and infertile patients with unsuccessful ICSI.

Figure 2. Comparing the expression of insulin in the sperm of fertile males and infertile patients with unsuccessful ICSI.

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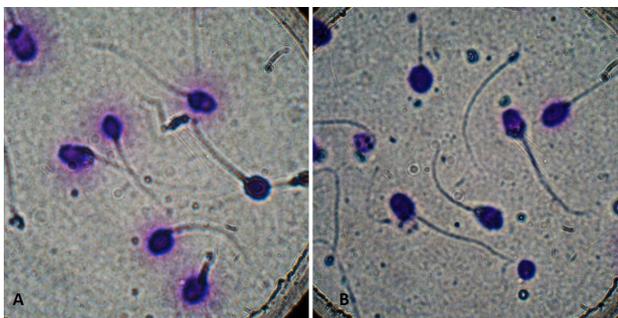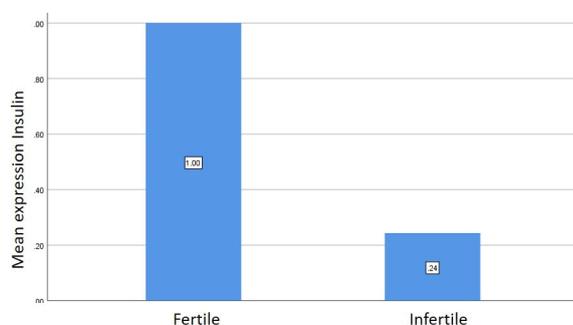3. Alpert S, Hanahan D, Teitelman G. Hybrid insulin genes reveal

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