Abstract

Estrogen receptors (ERs) mediate estrogen action in regulating at all levels of the hypothalamic-pituitary-testis axis in male. It has an essential role in male reproductive tract and spermatogenesis. In male mice estrogen receptor alpha (ERα) knock-out (ER koα), these mice were infertile and severe impairment in spermatogenesis, sperm motility and in vitro fertilizing potential was observed. Recently it has been reported that microRNA (miRNA) mir-99a and mir-196b were predicted to target ERα gene. miRNAs are small, endogenous, single stranded RNA molecules that regulate gene expression and have been implicated in various disease states. It has been proved that some miRNAs expression is tissue-specific and disease-specific, giving potential for identifying miRNAs as a diagnostic tool. About 60-75% of male infertility cases is idiopathic and their molecular mechanisms are unknown. Semen analysis and diagnostic tests are not accurate in this case. Therefore, taking the proper approach in treatment is difficult. In this study, changing in the expression levels of mir-99a, mir-196b and ERα expression levels were evaluated in oligospermic infertile patients (n=43) compared to control fertile subjects (n=43). After washing and separating sperms, total RNA was isolated and then cDNA was synthesized. The expression levels of mir-99a and mir-196b and ERα were evaluated by real time PCR method. mir-99a, mir-196b levels were significantly higher than those in normal controls(p< 0.0001, p=0.004 respectively). Also, we have found that, ERα level significantly decreased in comparison with normal group (p< 0.0001). The increases of mir-99a, mir-196b and decreases of ERα expression in spermatozoa cells in oligospermic patients may be associated with the susceptibility and progression of infertility. The results of this study indicate that miRNA can be have a key role in spermatogenesis and might have a diagnostic and prognostic value in men infertility.

Key words: Has-mir-99a, hsa-mir-196b, microRNA, Male Infertility, ERalpha.

Introduction

Infertility is defined as a failure to conceive after more than one years of regular intercourse without contraception (1). Approximately 15% of the couples are infertile, about 45% of which is related to male factors. Oligospermia and asthenospermia are common causes of infertility in males, however, the molecular mechanisms causing these effect is not entirely clear. The causes are known in less than 35% of these cases, out of which is genetic disease with unknown molecular mechanisms. In oligospermia the number of spermatozoa is reduced and in asthenospermia abnormality in sperm motility is seen(2). About 60-75% of infertility cases are idiopathic with or without abnormal semen analysis(3). It was seen that in bulls, spermatoza with normal motility and morphology, the fertility of some bulls was reduced which may be due to molecular defects in the sperm cells (4).

More than hundreds of genes are involved in spermatogenesis. Molecular and cellular integrity of sperm cells is important for fertilization any deletion or mutation in the sequence of genes and inappropriate gene expression causes disorder in spermatogenesis and fertility (5, 6). Estrogen has a positive impact on function of sperm by stimulating of capacitating and fertilizing ability (7), as well as it has a key role in modulating of the male
reproductive tract. Cellular signaling of estrogen is mediated through the estrogen receptors (ER) that are present throughout the male reproductive tract and spermatozoa. ERα mediates estrogen action in regulating at all levels of the hypothalamic-pituitary-testis axis in male. ERα has an essential role in male fertility, it was proved that ER knockout (Era/-/) mice become infertile.

ERα gene expression is regulated by small noncoding RNAs (microRNAs). Previous studies showed that mir-99a and mir-196b were predicted to target ERα gene. MicroRNAs (miRNAs) were first detected in human spermatozoa by Ostermeier et al. They are abundant in spermatozoa but their function in spermatogenesis and fertilization is unknown. miRNAs are small (18-25 nucleotides) noncoding regulatory RNAs which negatively regulate gene expression. They participate in designation of cell fate, embryonic development, and control of growth, differentiation, and death of cells. Number of miRNA is expressed in mouse male germ cells. miRNAs are involved in regulating of gene expression during mitotic, meiotic, and post-meiotic stages of spermatogenesis. Impaired biogenesis of miRNAs disrupts spermatogenesis and causes infertility in male mice.

In the present study we investigated expression levels of mir-99a, mir-196b and their common target gene (ERα) and their correlation in oligospermic infertile and normospermic fertile men.

Material and methods

Study design

From infertile men (n=723) referred to the Tabriz Alzahra Infertility Center (mean age 27.5 years), which despite of continuous intercourse they had a background of infertility for more than two years, 43 oligospermic infertile patients were selected. The written consent of the subjects was done according to the rules of medical ethics. Control samples (n=43), selected from normal volunteers, had a baby in the last two years and their semen analysis was normal. Two months before sampling, none of the control subjects nor patients treated with the drug, as well as they didn’t have intercourse 3-5 days before sampling. This research was approved by the Ethics Committees of Tabriz medical University.

Exclusion criteria

The volunteers with infertile partner, infection in the genital tract, autoimmune disorders, reproductive tract abnormality, smoking, alcohol and drug consumption were excluded from the study.

Hormone detection

The blood was placed at 37 °C for 10 min to clot formation. The clot from the wall of the test tube was gently removed. Then supernatant was centrifuged at 1000×g for 10 min at 4 °C. Samples were assayed for follicle stimulating hormone (FSH), luteinizing hormone (LH), 17β-estradiol (estrogen) and testosterone using an enzyme-linked immunosorbent assay (ELISA) by commercial ELISA kit (AccuBind ELISA, Monobind, USA) according to the manufacturer’s instructions.

Isolation of spermatozoa from seminal fluid

Semen samples were collected in a sterile container and incubated at 37 °C for 30 minutes to get the fluid then according to WHO guidelines, semen analysis was performed. Sperms were purified by Goodrich methods. In brief, the samples were washed two times in 1×PBS buffer solution, then somatic cells were absent in SCLB solution (0.1% SDS, 0.5% TX-100 in DEPC water). The cells were counted, if somatic cells were, present the process was repeated. Finally, the solution was frozen at –80 °C.

RNA isolation

Total RNA was isolated using exiqon miRCURY RNA isolation kit (Exiqon, Denmark) according to the manufacturer’s instructions. Quantity and quality of the isolated RNA was measured by NanoDrop1000 (NanoDropND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA). Total RNAs were reversed to cDNA using LNA universal RT miRNA PCR kit (Exiqon, Denmark). Briefly, 20ng of total RNA was reverse transcribed. cDNA Synthesis was performed by thermal cycler (Eppendorf, Germany) with the following parameter value, 60 minutes at 42 °C, 5 minutes at 95 °C and immediately cool to 4 °C until use.

Real-time PCR analysis

Quantitative real-time reverse transcriptase-PCR was carried out by using the Corbett Rotor-Gene 6000 Real-Time PCR system (Qiagen,
Germany). miRNAs quantification was performed using MiRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Denmark). Mir-16 was used as the endogenous control miRNA. The relative expression level of ERα was measured by qPCR with primers (ERα: 5′-CCACATCAGTCACATGAGTAA-3′ and 5′-GGTCCATCAGCATCTACAG-3′) using the Quantitect SYBR Green PCR Kit (Qiagen, Germany). The expression levels were normalized to β-actin as housekeeping gene with the following primers (5′-TGGACTTCCGAGCAGAGATG-3′ and 5′-GAAGGAAGGCTGGAAGAGTG-3′). The reactions were performed in triplicate.

Statistical analysis
Statistical analysis was performed using SPSS software (version 18). The results were expressed as mean ± SD. Relative expression level of genes were calculated by using the 2^{-ΔΔCt} model (18). Unpaired Student’s t-test was used to analyze the differences in gene expression between oligospermic and control group. Correlation analysis was performed using the spearman rank correlation test. In all analyzes P-value < 0.05 was considered as significant.

Results
Expression level of mir-99a, mir-196b and ERα in oligospermic and control group
We determined the expression levels of mir-99a, mir-196b and ERα in oligospermic and control group. By real-time quantitative RT-PCR analysis, we found that expression levels of mir-99a and mir-196b were much higher in oligospermic than control group (p<0.0001 and p=0.004, respectively; figure 1). Inversely, expression level of ERα was significantly lower in oligospermic than control group (p<0.0001; figure 1).

Correlation between expression levels of ERα and seminal plasma parameters
Correlation between expression levels of ERα and semen were analyzed using spearman’s rank correlation test (table 1). Expression levels of ERα were strongly and positively correlated with those of sperm count, quick progressive, slow progressive, non-progressive, immotile, normal morphology and pH, was performed using Spearman’s rank correlation test (table 1). Expression levels of mir-196b were strongly and positively correlated with pH (spearman’s correlation coefficient; 0.7295).

Correlation between hormones and ERα, mir-99a, mir-196b
Expression levels of miRNAs were not significantly correlated with hormones. Expression levels of ERα were strongly and positively correlated with estrogen(spearman’s correlation coefficient; 0.7818) (table 2)

Discussion
Spermatogenesis is an intricate process of germ cell development that many genes are involved. Any defect in genes expression or their regulation disrupt spermatogenesis and cause infertility (19). miRNAs regulate gene expression by modification of special mRNA translation. Few studies have been conducted about miRNAs function in spermatogenesis and male fertility. In a study conducted on non-obstructive azoospermic infertile patients, significant change in miRNAs expression was seen compared to fertile control men (20). In that study Lian et al. showed that miRNAs have a regula-
A role in spermatogenesis. In this study we investigated mir-99a, mir-196b and their common target ERα gene expression in oligospermic infertile patients compared with normospermic fertile control by real-time PCR methods. Our result showed that mir-99a in oligospermic was significantly over expressed compared to control group. We also demonstrated that high mir-99a expression was associated with significant decreases in ERα gene expression level in oligospermic group. It was proved that mir-99a was predicted to target ERα, which has been implicated in ERα regulation(10, 21). In the present study we have shown that expression level of mir-196b was increased unlike ERα expression, which was decreased. Guarducciet al. showed that ERα promoter polymorphism were inversely associated with sperm count (22). As our results indicated inhibition of germ cell proliferation by reducing the expression of ERα gene by mir-196b is possible. In the male reproductive tract, there are higher levels of ERα in the efferent ductules (region of the male tract) than female reproductive system, it occupy one third of epididymis. It shows the importance of ERα in the male reproductive system and fertility. ERα has a key role in the regulation of fluid reabsorption, and in the epididymis the receptor is re-

### Table 1. Correlation analysis between ERα, mir-99a, mir-196b and seminal plasma parameters

<table>
<thead>
<tr>
<th>variable</th>
<th>mir-99a</th>
<th>mir-196b</th>
<th>ERα</th>
</tr>
</thead>
<tbody>
<tr>
<td>volume</td>
<td>0.06791a</td>
<td>0.4770</td>
<td>-0.092</td>
</tr>
<tr>
<td></td>
<td>0.8521b</td>
<td>0.1666</td>
<td>0.799</td>
</tr>
<tr>
<td>Sperm count</td>
<td>0.01824</td>
<td>0.1220</td>
<td>0.863*</td>
</tr>
<tr>
<td></td>
<td>0.9601</td>
<td>0.7372</td>
<td>0.001</td>
</tr>
<tr>
<td>Quick progressive</td>
<td>0.4975</td>
<td>0.2138</td>
<td>0.723*</td>
</tr>
<tr>
<td></td>
<td>0.1435</td>
<td>0.5530</td>
<td>0.018</td>
</tr>
<tr>
<td>Slow progressive</td>
<td>0.2601</td>
<td>0.02174</td>
<td>0.875*</td>
</tr>
<tr>
<td></td>
<td>0.4679</td>
<td>0.9525</td>
<td>0.0009</td>
</tr>
<tr>
<td>Non-progressive</td>
<td>0.3525</td>
<td>0.3472</td>
<td>0.551</td>
</tr>
<tr>
<td></td>
<td>0.3179</td>
<td>0.3256</td>
<td>0.098</td>
</tr>
<tr>
<td>Immotile</td>
<td>-0.4390</td>
<td>-0.1835</td>
<td>-0.691*</td>
</tr>
<tr>
<td></td>
<td>0.2043</td>
<td>0.6119</td>
<td>0.026</td>
</tr>
<tr>
<td>Normal morphology</td>
<td>0.4280</td>
<td>0.4640</td>
<td>0.642*</td>
</tr>
<tr>
<td></td>
<td>0.2173</td>
<td>0.1767</td>
<td>0.045</td>
</tr>
<tr>
<td>pH</td>
<td>0.2848</td>
<td>0.7295*</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>0.4250</td>
<td>0.0166</td>
<td>0.328</td>
</tr>
</tbody>
</table>

*a* Spearman correlation coefficient  
*b* P, spearman’s rank correlation test  
*P<0.05 is consider significant

### Table 2. Correlation analysis between ERα, mir-99a, mir-196b and hormones

<table>
<thead>
<tr>
<th>variable</th>
<th>mir-99a</th>
<th>mir-196b</th>
<th>ERα</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>-0.5152a</td>
<td>0.1033</td>
<td>0.2364</td>
</tr>
<tr>
<td></td>
<td>0.1276b</td>
<td>0.7763</td>
<td>0.5109</td>
</tr>
<tr>
<td>LH</td>
<td>-0.3697</td>
<td>0.1398</td>
<td>0.3455</td>
</tr>
<tr>
<td></td>
<td>0.2931</td>
<td>0.7001</td>
<td>0.3282</td>
</tr>
<tr>
<td>E2</td>
<td>-0.2970</td>
<td>-0.3587</td>
<td>0.7818*</td>
</tr>
<tr>
<td></td>
<td>0.4047</td>
<td>0.3088</td>
<td>0.0075</td>
</tr>
<tr>
<td>test</td>
<td>0.2000</td>
<td>0.2918</td>
<td>-0.1879</td>
</tr>
<tr>
<td></td>
<td>0.5796</td>
<td>0.4133</td>
<td>0.6032</td>
</tr>
</tbody>
</table>

*a* Spearman correlation coefficient  
*b* P, spearman’s rank correlation test  
*P<0.05 is consider significant
sponsible for keeping fluid osmolality and pH (23). In our study reduced ERα expression was associated with little change in semen pH. Gunawan et al. showed that a polymorphism in the coding region of ERα in exon 1 was related to sperm motility (24). Recent findings are consistent with our results. We proved that expression level of ERα in oligospermic significantly was down regulated compared to control group, as well as motility and pH in seminal fluid of oligospermic was lower than those in control. Our data showed that Spermatozoa with normal morphology decreased in oligospermic group compared to control group as well as our results showed that significant positive correlation with the expression of ERα and morphology. This is consistent with recent findings of Josepha and colleagues. They proved that in ERαKO mice sperm maturation and capacity to fertilize was destroyed that contributes to infertility (25). ERα play an important role in modulation of sperm metabolism (26), disruption in its performance, reduced values for sperm density, sperm motility, and percentage of sperm with normal a unique property of GS (germline stem cell) morphology (27). ERα in the acrosome of the spermatozoa is more than in other sections, acrosome contains lytic which Puncture the outer coat of the egg and allow the infiltration of sperm (28). Acrosome dysfunction impairs oocyte fertilization and cause of male infertility (29). Oligospermic patients have a high frequency of defective sperm–zona pellucida (outer coat of the egg) interaction (30, 31) according to our finding decrease in ERα may cause acrosome dysfunction.

It is possible that miRNA interfere in spermatogenesis through other genes. Possible target of mir-99a are SLC2A and DICER1. SLC2A (solute carrier family 2) is belong to glucose transporter (GLUT) family. It is expressed in the testes and sperm. It was showed that altered SLC2A expression may be responsible for the decreased spermatogenesis, sperm maturation, and fertilization in the male mice. (32, 33). DICER1 (RNAse III endonuclease) is essential for the biogenesis of microRNAs (miRNAs). DICER1 is essential for differentiation of the male germ line and spermatogenesis. (34, 35). AKT1 is a target for mir-196b. AKT1 is a serin/threonin kinases enzyme that it has been proved to be the mediator of cellular growth, proliferation, survival, and metabolism in various cell types (36). Kim et al. showed that in Akt1-/- male mice apoptotic sperm in null mice were more than wild-type mice, and sperm motility and concentration were significantly lower in the null sperm (32).

In conclusion, we have defined efficacy of mir-99a, mir-196b and ERα in oligospermic infertile patients. Our results obtain more information about the molecular mechanism of infertility, and their possible regulatory role in spermatogenesis and fertilization.

Acknowledgment

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