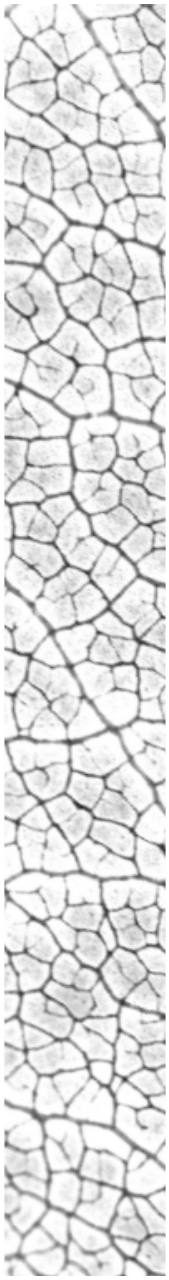

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# The relationship between expression of Mir30 and Let7 genes in infertile males and non-obstructive azoospermia

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**Abstract**

Any defect in the process of spermatogenesis can lead to a type of male infertility disorder called Non-Obstructive Azoospermia. Investigating factors involved in spermatogenesis, including genes, can help to understand the mechanism of infertility in men. On the other hand, a number of miRRNAs communicate with spermatogenesis and regulate it. The current paper aims at determining the let7 and mir30 genes expressions in testicular tissue of Azoospermic patients. This case-control study was performed on 40 infertile males with Non-Obstructive Azoospermia and 40 healthy fertile males. Genomic RNA from the testicular tissue samples as well as semen of the fertile individuals was extracted and converted to CDNA. Using Real-time PCR technique, using the specific primer of the examined genes and primer U6 as internal control, the expression rate in samples and controls was evaluated, and the amount of ΔCT was calculated, and finally, the relative number of multiplied mRNA copies of formula $2^{-\Delta\Delta CT}$ was used. Data were then analyzed using Excel software, and compared using SPSS software and t-test and ANOVA. Based on the quantitative results obtained from the examination of the let7 and mir30 genes expression, the results showed that the let7 and mir30 gene expression in Azoospermic individuals was significantly different from that of fertile male sperm samples. The mean of let7 expression in Azoospermic individuals was more than 18 times higher than normal samples. (P-value = 0.04). There was a significant difference in expression of let7 in fertile and Azoospermic male samples by t-test. (P-value = 0.03). There was no significant difference in the expression of mir30 in fertile and infertile males with Azoospermia. (P-value = 0.27). There is a significant difference in the expression of let7 gene in Azoospermia samples compared to fertile individuals, and it can be concluded that this gene may play a role in the spermatogenesis process. By examining larger populations and proving this change of expression, the miRNA expression patterns were used to diagnose and treat Non-obstructive Azoospermic individuals.

**Keywords:** azoospermia, Let7, Mir30, male infertility

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## INTRODUCTION

Infertility is one of the most common and rising problems in the world (Hamada et al. 2012). Non-reproductive fertility is called infertility after one year of sexual intercourse without interruption (Krausz and Forti 2000). The number of infertile couples is increasing in many ways in today's world (Ikuerowo et al. 2010, Moncada et al. 1991, Thonneau et al. 1991). Several factors have a negative effect on the number, shape, maturity and sperm motility. Azoospermia is one of the causes of male infertility. The prevalence of this mark has been reported in over 2% of the population and 10-25% of the infertile population (Rowe et al. 2000).

In non-obstructive Azoospermia, sperm is not made and it is believed that this Azoospermia is due to a defective process of spermatogenesis and is associated with the absence of mature sperm (Ezeh 2000).

Spermatogenesis is a complex process of differentiation and cellular changes derived from a diploid stem cell (spermatogonium), ultimately results in a haplotypes male gamma that is regulated by the development and maturation of germ cells by 2300 genes (Yu and Tan 2012).

It seems that the disruption and mutation of each of the genes that are effective in spermatogenesis can lead to male infertility (Turner 2003). Various genetic reasons have been investigated in this regard, for example, examination of the types of deletions in chromosomes (Y 10), MLH3 mutations in gonadal tissue (Ferrás et al. 2007), mutations in the methylthematetrahydrofolate reductase gene (MTHFR) (Nuti and Krausz 2008), epigenetic changes (Hajkova et al. 2002), and ..... A change in the expression of the short 22 RNA expression nucleotides regulating the expression of

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genes called miRNA is one of the recent highly regarded causes of many disorders including infertility (Stark et al. 2008).

In addition to mRNA or coding RNAs, other transcripts called microRNAs have also been identified in the sperm that are not protein coding and by binding to the mRNA inhibit the translation process and regulate the gene expression at the post-transcription level. miRNAs are present at different stages of spermatogenesis and have the highest expression in spermatocin pectin cells as well as spermatids (Hackstein et al. 2000, Mardani et al. 2014, Sutovsky and Schatten 1999).

The study of the role of miRNAs in male reproductive organs is essential as mRNAs play an important role in biological processes and cellular disorders. Expression of the specific miRNA concentration is associated with certain male reproductive disorders. Therefore, the evaluation of several miRNA gene expressions may be useful as a suitable molecular marker for detecting these male infertility disorders.

Accordingly, the importance of miRNAs in the differentiation and proliferation of sex cells is more and more evident.

On the other hand, according to studies conducted over the years, many miRNAs have been introduced as effective factors in spermatogenesis, which requires further studies to use them in the treatment and diagnosis of infertility disorders; for example, Barad et al. (2004) determined miRNA expression in the testicle. The technique used by this group was microarray technique (Barad et al. 2004). Lian et al., also conducted a series of Semen tests on infertile and fertile males in 2009, and were able to identify different 52miRNA experessions (Lian et al. 2009).

Kotaja et al. (2006) managed to detect chromatic aberrations as a control center for intracellular miRNA functional pathways in a sexually transmitted cell, that Let7 is also located at this center (Kotaja et al. 2006).

Locating some of the miRNA regulatory components in Excel 2008 was carried out by Marcon et al., using FISH techniques, microarrays and qPCR (Marcon et al. 2008).

Mclver et al. (2013) identified miRNAs 7 that were important in differentiating the egg cell after fertilization (20,33).

The authors of the current paper (2011) reported the use of seminal fluid molecular markers as a non-invasive diagnostic method to evaluate the spermatogenesis in Non-obstructive Azoospermic patients (Aslani et al. 2011). In this study, the expression of specific genes AKAP4 and PRM2, DAZ of sex cells was performed by RT-PCR, then it was concluded that the presence of the transcripts of DAZ and protamine 2 genes in seminal fluid of Non-obstructive Azoospermic individuals can be used as a noninvasive molecular marker for prediction of the presence or absence of mature spermatozoa. Recent studies have used miRNAs in seminal fluid to study spermatogenesis in Non-obstructive Azoospermic males and to evaluate the profile of these transcripts in infertile males, as well. For example, Wie Wu et al. (2013) investigated the miRNA profile in seminal fluid of Azoospermic males with the aim of finding noninvasive methods to find out the cause of Non-Obstructive Azoospermia. Thus, they examined the expression of miRNA miR-141, miR-429 and miR-7-1-3p in 100 patient samples versus 100 controls. The method used in this study was RT-PCR and the results showed that the expression of all three is significantly increased, and by examining larger populations and proving this change of expression, it may be possible to examine the pattern of miRNAs in diagnosis and treatment of Non-Obstructive Azoospermia (Magro 2015, Wu et al. 2013).

The current paper aims at evaluating the expression of two miRNAs, titled Let7 and Mir30, in patients with Non-Obstructive Azoospermia.

## MATERIALS AND METHODS

Testicular biopsy specimens were obtained from 40 infertile men who were referred to the Najmieh Infertility Center of Kerman in order to treat infertility. The inclusion criteria for azoospermia were based on the analysis of semen and the absence of sperm extraction from epidiam. Approximate sample size was mm 2 * 2 * 2 and stored at -70 ° C. Semen specimens of a sample of 40 fertile individuals with normal sperm parameters (according to WHO standards) was collected after diagnostic spermogram.

The study was approved by the Ethics Committee and written consent was received from both the case and control groups for participation in the research project. Moving sperms with normal morphology (Nidacon, sweden) Pure sperm were isolated from pure semen, following concentration slope steps. Therefore, 40% and 80% Pure sperm were prepared by adding Sigma, Usa and Ham's F-10 in a concentration of 40% and 80% Pure sperm. Then, in a centrifuge tube, ml2 was added at 80% concentration and Zn 2 ml of 40% Pure sperm concentration. The addition of the layers was done in such a way that the two layers were not mixed together, then the 2 ml semen was added to and centrifuged for about 20 min at 300 g, then the sperms deposited from the 80% layer were carefully separated without mixing with other layers. Having washed the sperms with the culture medium, they added about 0.7 ml of solution (RNA-Bee (tel-tes Inct, USA) and transferred to the molecular laboratory for RNA extraction in liquid nitrogen (196 ℃). Sperm miRNA extraction and testicular tissue using an Exigon ™ Mercury ™ RNA Isolation Kit kit of Biofluids, Inc., according to the company's instructions, the extracted RNA was converted to cDNA using the Exigon CDNA

**Table 1.** Primer sequence used for let7 and mir30 and U6 genes

| Gene | Primer sequence |
|---|---|
| Mir30a | GGCGTGTAAACATCCTCGACTG |
| Let7b | TGAGGTAGTTAGGTTGTATAGTT |
| U6 | CTCGCTTCGGCAGCACA |

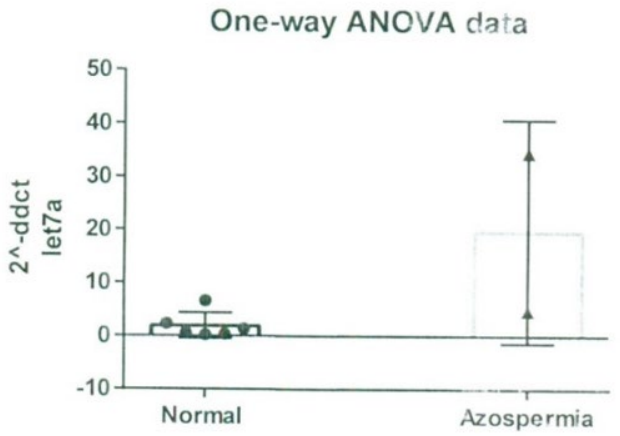


**Fig. 1.** Mean expression of mir-let7 in Azoospermic individuals compared to fertile individuals (ANOVA)

builder. Having produced the cDNA, the cDNA was prepared to prepare the components of the qPCR reaction, first cDNA was diluted and prepared for qPCR reaction. The prepared product was placed in the Real Time step one plus made of ABI Company and then contracted using the specific primers of let7 and mir30 genes and the primer U6 as a control of specimen's expression and $\Delta CT$ Value was calculated. In the end, $2^{\wedge-\Delta\Delta CT}$ was used for relative calculation of the number of copies of mRNA replicated.

**Table 1** shows sequences and primers used in this paper.

### Statistical Analysis

Data were categorized using Excel software and analyzed using t-test, ANOVA and SPSS software and comparing expression using ΔΔCT. P <0.05 was considered as significance level.

## FINDINGS

Detection of mir-let7a expression in semen samples from fertile and infertile males was performed using quantitative PCR method and computational ΔΔCT method. In this study, the UC6 reference gene was used to calculate ΔCT, as well as all ΔΔCT calculations. The ΔCT of each sample was subtracted from the mean ΔCT of normal samples. The results showed that mir-let7 expression in infertile samples was significantly different from that of fertile male sperm samples, with the mean expression of mir-let7 in infertile Azoospermia was 18 times higher than that of normal subjects (P- value = 0/04).

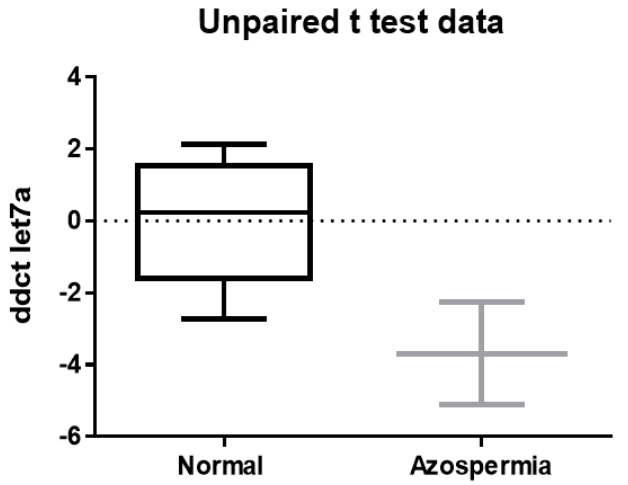


**Fig. 2.** Mean expression of mir-let7 in Azoospermic samples compared to fertile individuals (t-test)

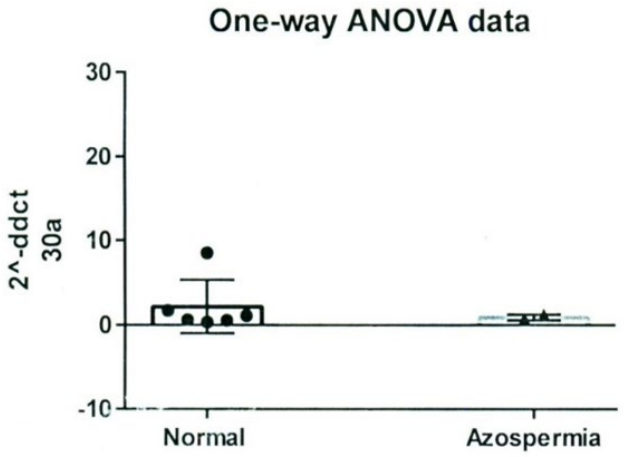


**Fig. 3.** Mean expression of mir-30a in Azoospermic samples compared to normal subjects (ANOVA)

Measurement of mir-let7 expression in fertile and Azoospermia male samples was statistically significant by t-test. P value = 0.03.

The mir-30a expression in infertile samples did not show any significant difference with fertile male sperm samples. Mean expression of mir-30a in infertile Azoospermic samples was about 1 times higher, but the difference in expression in fertile and infertile samples was not significant.

There was no significant difference in the expression of mir-30a in fertile and Azoospermic male samples by t-test. P value = 0.039.

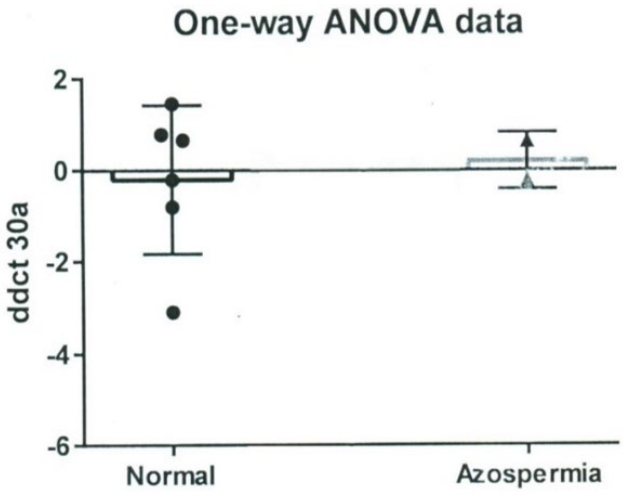


**Fig. 4.** Mean expression of mir-30a in Azoospermic samples compared to normal subjects (t-test)

## DISCUSSION AND CONCLUSION

The current paper shows that the expression of let7 gene in Azoospermic individuals was significantly different with fertile individuals, and the expression of mir30 gene was not significantly different with fertile individuals. The following studies also show the effects of these genes on spermatogenesis and fertility in men.

Mir-let7 is present in chromatid bodies and is responsible for controlling the expression of its downstream genes in the pre-transcription phase and is more involved in the differentiation of sex cells, while mir30 has a role in controlling the performance and production of sperm after expression (Kotaja et al. 2006).

Finding miRNA target genes affecting infertility can also be effective in understanding this process. Brehm et al. (2007) showed that the target gene is the let-7a, Fndc3a, which binds spermatids to mitotic ducts, and that the mice that mutated artificially in the gene were infertile (Brehm et al. 2007). Therefore, probably the imbalance in expressing this miRNA can cause infertility in men. There are many studies that show the importance of this mir-30, in fertility, for example:

Weimin et al. (2016), investigated the effect of some miRNAs on embryo implantation, among which there was mir-30, which was a major factor in the implantation of the fetus (Liu et al. 2016).

A. Salas-Huetos (2014) examined the expression of 227 micro-RNAs in healthy and infertile people, which was also included in the profile of the Mir-30 family. 46 micro-RNA was present only in fertile individuals, and 67 of them were not present in any fertile or infertile samples. Micro-RNAs with high specific expression in the infertile group included miR-375, miR-19b, miR-200c, miR-132 and miR-30c. It should be noted that mir-30 was also found in the healthy group, but its expression in the infertile group was far more than that of the fertile group (Salas-Huetos et al. 2014).

Mercedita et al. (2011) examined the expression of 7 micro RNA miR-338, miR-222, miR-18, miR-30, miR-10, miR-196 and miR-365 in fertile and infertile samples. The team showed that the target genes of these micro-RNAs are all but the zinc finger and Hox family, and all of these micro-RNAs are involved in the spermatogenesis process. They showed that Mir-30 was involved in both spermatogenesis in both mice and humans (Akoz et al. 2018, Koudehi et al. 2014, Köseoğlu et al. 2018, Madison-Villar and Michalak 2011).

Therefore, it can be concluded that these genes may play a role in the spermatogenesis process. By studying larger populations and proving this change of expression, it may be possible to study patterns of miRNA expression in the diagnosis and treatment of Non-Obstructive Azoospermia.

## REFERENCES

Akoz A, Yildiz V, Orun S, Turkdogan KA, Duman A (2018) Management of Poisonous Snake Bites: Analysis of 29 Cases. J Clin Exp Invest., 9(4): 140-4. https://doi.org/10.5799/jcei/3998

Aslani F, Modarresi MH, Soltanghoraee H, Akhondi MM, Shabani A, Lakpour N, Sadeghi MR (2011) Seminal molecular markers as a non-invasive diagnostic tool for the evaluation of spermatogenesis in non-obstructive azoospermia. Systems biology in reproductive medicine, 57(4): 190-6. https://doi.org/10.3109/19396368.2011.569906

Barad O, Meiri E, Avniel A, Aharonov R, Barzilai A, Bentwich I, Einav U, Gilad S, Hurban P, Karov Y, Lobenhofer EK, Sharon E, Shiboleth YM, Shtutman M, Bentwich Z, Einat P (2004) MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. Genome Res., 14: 2486–94. https://doi.org/10.1101/gr.2845604

Brehm R, Zeiler M, Ruttinger C, Herde K, Kibschull M, Winterhager E, Willecke K, Guillou F, Lecureuil C, Steger K, et al. (2007) A sertoli cell-specific knockout of connexin43 prevents initiation of spermatogenesis. Am J Pathol, 171: 19-31. https://doi.org/10.2353/ajpath.2007.061171

Ezeh UIZO (2000) Beyond The clinical Classification of azoospermia. Hum Reprod., 15: 2356-9. https://doi.org/10.1093/humrep/15.11.2356

Ferrás C, Zhou XL, Sousa M, Lindblom A, Barros A (2007) DNA mismatch repair gene hMLH3 variants in meiotic arrest. Fertil Steril, 88(6): 1681-4. https://doi.org/10.1016/j.fertnstert.2007.01.063

Hackstein JHP, Hochstenbach R, Pearson PL (2000) Towards an understanding of the genetics of human male infertility: lesson from files. Trends in Genetics, 16(12):565-72. https://doi.org/10.1016/S0168-9525(00)02140-5

Hajkova P, Erhardt S, Lane N, Haaf T, ElMaarri O, Reik W, et al. (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev, 117(1-2): 15-23. https://doi.org/10.1016/S0925-4773(02)00181-8

Hamada A, Esteves SC, Nizza M, Agawal A (2012) Unexplained male infertility; diagnosis and management. Int braz J urol, 38(5): 576-94. https://doi.org/10.1590/S1677-55382012000500002

Ikuerowo SG, Izeybu MC, Benebo AS, Fadeyibi IO, Omodele FO (2010) Testicular Biopseis of Azoospermic Men at the Lagos State University Teaching Hospital. African journal of Urology, 16(3): 69-72. https://doi.org/10.1007/s12301-010-0016-5

Kilitci A, Kaya Z, Acar EM, Elmas ÖF (2018) Scrotal Calcinosis: Analysis of 5 Cases. J Clin Exp Invest., 9(4): 150-3. https://doi.org/10.5799/jcei/4002

Köseoğlu D, Karcı AÇ, Acar A, Tuna EE, Berker D (2018) Progressive severe dyspnea and hypoxia due to primary thyroid lymphoma: steroid administration may be life-saving. Electronic Journal of General Medicine, 15(5): em77. https://doi.org/10.29333/ejgm/94011

Kotaja N, Bhattacharyya SN, Jaskiewicz L, Kimmins S, Parvinen M, Filipowicz W, Sassone-Corsi P (2006) The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. Proc Natl Acad Sci U S A., 103: 2647–52. https://doi.org/10.1073/pnas.0509333103

Koudehi FA, Farazmand R, Mirzayian S, Mohamad S (2014) Customer Value Assessment Methodology Using DM Approach. UCT Journal of Research in Science, Engineering and Technology, 2(3).

Krausz C, Forti G (2000) Clinical aspects of male infertility. Result probl cell Diffcr, 28: 1-21. https://doi.org/10.1007/978-3-540-48461-5_1

Lian J, Zhang X, Tian H, Liang N, Wang Y, Liang C, et al. (2009) Altered microRNA expression in patients with non-obstructive azoospermia. Reprod Biol Endocrinol, 7: 13. https://doi.org/10.1186/1477-7827-7-13

Liu W, Niu Z, Li Q, Pang RT, Chiu PC, Yeung WS (2016) MicroRNA and embryo implantation. American Journal of Reproductive Immunology, 75(3): 263-71. https://doi.org/10.1111/aji.12470

Madison-Villar MJ, Michalak P (2011) Misexpression of testicular microRNA in sterile Xenopus hybrids points to tetrapod-specific microRNAs associated with male fertility. Journal of molecular evolution, 73(5-6): 316-24. https://doi.org/10.1007/s00239-011-9478-8

Magro VM (2015) An Unclear Anemia in an Elderly Subject with Aortic Stenosis. European Journal of General Medicine, 12(2): 170-3. https://doi.org/10.15197/sabad.1.12.35

Marcon E, Babak T, Chua G, Hughes T, Moens PB (2008) miRNA and piRNA localization in the male mammalian meiotic nucleus. Chromosome Research, 16(2): 243-60. https://doi.org/10.1007/s10577-007-1190-6

Mardani M, Lavasani SM, Omidvari M (2014) An investigation into DOW and MOND indices with fuzzy logic based on fire and explosion risk assessment in Iran oil refinery. UCT Journal of Research in Science, Engineering and Technology, 2(3): 126-37.

McIver SC, Stanger SJ, Santarelli DM, Roman SD, Nixon B, McLaughlin EA (2013) A unique combination of male germ cell miRNAs coordinates gonocyte differentiation. PloS one, 7(4): e35553. https://doi.org/10.1371/journal.pone.0035553

Moncada S, Palmer RM, Higgs EA (1991) Nitric oxide: Physiology, Pathophysiology and Pharmacology, Pharm. Rew., 43(2): 109-142.

Nuti F, Krausz C (2008) Gene polymorphisms/mutations relevant to abnormal spermatogenesis. Reprod Biomed Online, 16(4): 504-13. https://doi.org/10.1016/S1472-6483(10)60457-9

Rowe PJ, Comhaire FH, Hargreave TB, Mahmoud AMA (2000) WHO manual for standardized investigation, diagnosis and management of the infertile male. Cambridge: Cambridge University Press: 33.

Salas-Huetos A, Blanco J, Vidal F, Mercader JM, Garrido N, Anton E (2014) New insights into the expression profile and function of micro-ribonucleic acid in human spermatozoa. Fertility and sterility, 102(1): 213-22. https://doi.org/10.1016/j.fertnstert.2014.03.040

Stark A, Bushati N, Jan CH, Kheradpour P, Hodges E, Brennecke J, et al. (2008) A single Hox locus in Drosophila produces functional microRNAs from opposite DNA strands. Genes Dev, 22(1): 8-13. https://doi.org/10.1101/gad.1613108

Sutovsky P, Schatten G (1999) Paternal contributions to the mammalian Zygote: fertilization after sperm-egg fusion International review of cytology, 195: 1-65. https://doi.org/10.1016/S0074-7696(08)62703-5

Thonneau P, Marchand S, Tallec A, Ferial ML, Ducot B, Lansac J, et al. (19991) Incidence and main causes of infertility in a resident Population of three French regions (1988-1989), Hum. Reprod., 6(6): 811-6. https://doi.org/10.1093/oxfordjournals.humrep.a137433

Turner RM (2003) Tales from the tail: what do we reably know about sperm motility? J Androl., 24(6): 790-803. https://doi.org/10.1002/j.1939-4640.2003.tb03123.x

Wu W, Qin Y, Li Z, Dong J, Dai J, Lu C, Guo X, Zhao Y, Zhu Y, Zhang W, Hang B (2013) Genome-wide microRNA expression profiling in idiopathic non-obstructive azoospermia: significant up-regulation of miR-141, miR-429 and miR-7-1-3p. Human Reproduction, 28(7): 1827-36. https://doi.org/10.1093/humrep/det099

Yu JT, Tan L (2012) The role of clusterin in Alzheimer's discase; Pathway, Pathogesis, and therapy. Mol Neurobial, 45(2): 314-26. https://doi.org/10.1007/s12035-012-8237-1