EXOSOMAL MIR-449C AND MIR-135A EXPRESSION IS ASSOCIATED WITH MALE INFERTILITY AND EFFICACY OF ASSISTED REPRODUCTIVE TECHNOLOGIES

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Abstract. Infertility is a major public health problem worldwide and its prevalence is as high as 17, 5% depending on the population studied The research is aimed to study the possibility of using miR-20a, miR-135a, miR-34b, miR-449v, miR-449c as markers of male infertility and to evaluate the dependence of the effectiveness of ART programs on the level of expression of exosomal microRNAs. The experimental group included patients entering the program of ART (assisted reproductive technologies) with the diagnosis of idiopathic male infertility (n = 30), the control group included couples with female infertility of tubal origin (n = 19). The isolation of exosomal microRNA from ejaculate was performed using the exoRNeasy Midi regent kit. The miRCURY LNA miRNA SYBR® Green PCR System was used to evaluate the expression of the following exosomal microRNAs: miR-20a, miR-135a, miR-34b, miR-449c, miR-449c and control miR-16. The expression of exosomal miR-449c and miR-135a was significantly different in the experimental group ($p = 0.03$ and $p = 0.04$, respectively). There was also a tendency to decrease the expression for such microRNAs as: miR-20a, miR-34b and miR-449c. Moreover, the expression level of miR-34b, miR-449c, and miR-449c and miR-135a has a direct correlation with the effectiveness of ART programs ($p < 0.05$ for miR-135a). The strength of correlation relationship of the above relationships using Cheddock scale was moderate. MicroRNA molecules selected for the study not only demonstrated their potential ability to be used as a diagnostic marker of male infertility, but also showed the ability to reflect the efficiency of fertilization and embryo formation processes.

Keywords: exosomal microRNA, male infertility, microRNA expression levels.

List of Abbreviations
ART – Assisted reproductive technologies
IVF – In vitro fertilization
NOA – Non-Obstructive Azoospermia

Introduction

Infertility is a major public health problem worldwide and its prevalence is as high as 17, 5% depending on the population studied (Cox *et al*., 2022). According to a recent meta-analysis by the Levine H. et al. group, global trends in the decline of total sperm count in the ejaculate are of serious concern: for example, between 1973 and 2018, it decreased by 62.3% at a rate of minus 4.70 million/year, while the percentage of decline in sperm concentration per year doubled, increasing from 1.16% after 1972 to 2.64% after 2000 (Levine *et al*., 2023).

The causes of male infertility are highly diverse and, in most cases, poorly understood, such that idiopathic genesis accounts for up to 30-40% of all identified cases (Jungwirth *et al.*, 2019; Agarwal *et al*., 2021). Although various diagnostic tests exist, their interpretation is inaccurate and often subjective (Esteves *et al*., 2020).

The current gold standard for the diagnosis of male infertility has a number of drawbacks: (1) normal spermogram results do not guarantee conception, (2) there is a significant overlap between semen parameters of fertile and infertile men, (3) semen analysis does not provide information about the fertilizing ability of the sperm (Daneshmandpour *et al.*, 2020).

Spermatogenesis is a complex process that requires precise and highly organized regulation of gene expression at multiple levels, including transcriptional, post-transcriptional and epigenetic mechanisms. A wide variety of investigations have found out more than thousands of protein coding genes responsible for the regulation of spermatogenesis (Mateo & Sassone-Corsi, 2014). Spermatozoa are also rich in small non-coding RNAs (Khadhim & Manshd, 2023), which could have played an essential role in the process of spermatogenesis or may have critical roles in the post-fertilization development (Alves *et al*., 2020).

Recent scientific studies have suggested that small non-coding RNA molecules known as microRNAs (miRNAs), single-stranded RNA molecules of 22 to 24 nucleotides in length, whose main function is to regulate gene expression by forming semi-complementary structures in the 3' untranslated region (3'UTR) of their target matrix RNA (mRNA) may be involved in the pathogenesis of male infertility (Daneshmandpour *et al*., 2020).

Regulation of gene expression by miRNA is one of the most important levels of post-transcriptional regulation levels. miRNA expression varies at different stages of spermatogenesis, so there are specific types of miRNAs at each stage of sperm production and maturation and miRNA alterations can result in molecular etiology of male infertility and dysregulated expression of miRNA can be inherited by next generations (Hua *et al*., 2019). The presence of microRNAs in testes, epididymis, spermatozoa, seminal plasma, and extracellular vesicles (i.e., exosomes and microvesicles) and the ability of these molecules to modulate different steps of spermatogenesis by upregulating or downregulating genes has been shown to suggest to many researchers that microRNAs may be of interest as a potential screening method of investigation (Salas-Huetos *et al*., 2020; Barbu *et al*., 2021). Altered levels of their expression in men have been found to be associated with reduced sperm count (oligozoospermia), low sperm motility (asthenozoospermia) and abnormal sperm morphology (teratozoospermia) (Zhou *et al*., 2019).

Nixon et al. found that spermatozoa are loaded with different miRNAs during epididymal maturation, suggesting their role in post-fertilisation development (Nixon *et al.*, 2015). Sperm from Drosha and Dicer germline conditional knockout mice showed reduced embryo developmental potential, which was rescued by injecting sncRNAs from wild-type sperm into the embryos, suggesting that sncRNAs are critical for pre-implantation embryo development (Yuan *et al*., 2016). Sperm-borne miRNAs, such as miR-34c, have been shown to be critical for the first cleavage in the zygote (Liu *et al*., 2012). Hua et al. (2019) identified five miRNAs (miR-132-3p, miR-191-3p, miR-520a-5p, miR-101-3p and miR-29a-3p) that could serve as potential markers for sperm quality assessment in IVF (Hua *et al.*, 2019). It has also been reported high miR-191-5p expression level in sperm had a higher fertilisation rate (FR), effective embryo rate (EER) and high quality embryo rate (HQER), suggesting that miR-191-5p could be used as a potential biomarker to detect high quality sperm for IVF (Xu *et al*., 2020). Abu-Halima et al. selected five miRNAs (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-122 and hsa-miR-429) and suggested their potential to discriminate the subfertile group from the fertile group (Abu-Halima *et al*., 2014). Later in 2019 Corral-Vazquez et al selected the best miRNA pairs from their previously reported data and found out that the hsa-miR-942-5p/hsa-miR-1208 pair were the best markers for the diagnosis of infertile men with seminal changes, while the hsa-miR-34b-3p/hsa-miR-93-3p pair showed the potential for the diagnosis of infertile men with unexplained male infertility or when seminal parameters were close to the threshold values (Corral-Vazquez *et al*., 2019). Several studies on fertile and infertile sperm samples have identified hundreds of differentially expressed miRNAs (Abhari *et al*., 2014; Salas-Huetos *et al*., 2015; Muñoz *et al*., 2015; Salas-Huetos *et al*., 2016; Tian *et al*., 2018; Mokánszki *et al*., 2020). In the study by Ahmet Tektemur et al. (2018) it was shown that changes in testicular and/or spermatozoal ion channels and miRNA expressions due to verapamil treatment may affect male fertility. They

found downregulated levels of miR-20a, miR-92a, miR-132, miR-320 and miR-760 expressions in spermatozoa. These altered gene expressions were found to be associated with decreased sperm concentration (Tektemur *et al*., 2022).

Moreover Gianmartin Cito et al. (2020) showed that miR-20a-5p could represent a novel non-invasive diagnostic biomarker of male infertility. They detected that median blood plasma miR-20a-5p was significantly higher in patients affected by NOA (0.16 2-ΔCt, range: 0.05-0.79 2-ΔCt) than in fertile controls (0.06 2-ΔCt , range: 0.04-0.10 2-ΔCt), P < .001. MiR-20a-5p was positively correlated with follicle-stimulating hormone (FSH) (rrho = -0.490, P = .015) and luteinizing hormone (LH) (rrho = -0.462, P = .023), and negatively correlated with serum total testosterone (TT) (rrho = -0.534, P = .007) and right and left testicular size (rrho = -0.473, P = .020 and rrho = -0.471, P = .020, respectively) (Cito *et al*., 2020). Li et al. (2021) aimed to determine the molecular mechanism by which JAK2 mRNA and miR-135a-5p affect asthenozoospermia. The authors showed that MiR-135a-5p overexpression suppressed the expression of JAK2 mRNA and protein by targeting the 3'UTR of JAK2 mRNA. Correlation analysis revealed that miR-135a-5p level was significantly negatively correlated with sperm progressive motility, whereas JAK2 mRNA level was significantly positively correlated with sperm progressive motility. They concluded that low JAK2 mRNA expression and high miR-135a-5p expression were associated with asthenozoospermia and male infertility (Li *et al*., 2021).

Stefano Comazzetto identified two miR-34 family miRNA loci (miR-34b/c and miR-449) that are specifically and highly expressed in postmitotic male germ cells. Reduced expression of several miRNAs, including miR-34b/c in sperm has been associated with reduced fertility in humans. The scientists found that deletion of the miR34b/c and miR-449 loci results in oligoasthenoteratozoospermia in mice. Deficiency of miR-34bc/449 impairs both meiosis and the final stages of sperm maturation. Analysis of miR-34bc-/-;449-/- pachytene spermatocytes revealed a small cohort of dysregulated genes that were highly enriched in miR-34 family target genes. Thus, the miR-34 family has been suggested to be important for spermatogenesis, and its dysregulation may lead to oligoasthenoteratozoospermia and infertility (Comazzetto *et al*., 2014). Despite numerous studies on the role of microRNAs in the development of male infertility, the data obtained are contradictory and poorly reproduced in subsequent studies, but the search for a new diagnostic marker of the resulting condition is one of the urgent tasks of modern medicine (Li *et al*., 2021). Taking into account the conflicting results of different authors on the expression of miR-20a, miR-135a, miR-34b and miR-449, we aimed to study the possibility of using them as markers of male infertility and to evaluate the dependence of the effectiveness of ART programs on the level of expression of exosomal microRNAs.

Materials and Methods

The selection of patients for participation in the study was carried out from married couples who entered the programs with the use of assisted reproductive technologies (ART), on the basis of the Family Medical Center. The experimental group included 30 couples diagnosed with idiopathic male infertility, abnormal spermogram parameters, absence of female factor and sufficient response to ovarian stimulation (more than 8 oocytes obtained after transvaginal follicle puncture). Patients with female tubal factor infertility, with normal ejaculate parameters and proven fertility in the male partner were allocated to the control group. The criteria for exclusion from the study were the age of the male partner over 50 years old and the age of the female partner over 35. In addition to age, patients without pronounced somatic pathology were selected for the study. In accordance with the inclusion and exclusion criteria, 30 married couples were selected for the experimental group and 19 for the control group. The study was conformed to the standards set by the latest revision of the Declaration of Helsinki. All study participants signed informed voluntary

consent. Local ethics committee of Bashkir State Medical University approved the project and all the procedures used in the investigation (protocol#10-24, 2024).

After two days of abstinence, patients from the experimental and control groups donated ejaculate by masturbation. Part of the material was used for repeated extended evaluation of ejaculate morphokinetic parameters, the remaining material, with a volume of at least 1 ml, was transported to the Research Institute of Urology and Clinical Oncology after preliminary freezing to -90 o for the main stage of the study.

During further investigation, the expression of the following exosomal microRNAs was characterized: miR-20a, miR-135a, miR-34b, miR-449c, miR-449c and 1 control miR-16. Subsequently, the information obtained was used to assess correlations with the efficacy of ART programs (assessed by relying on embryological results).

Prior to analysis, the ejaculate was centrifuged after thawing at 1600 g for 10 minutes and then at 16000g for another 10 minutes to remove cells and cell fragments. The exoRNeasy Midi regent kit was used to isolate and isolate exosomal microRNA contained in extracellular vesicles. The method was based on the ability of special membranes to bind and isolate exosomes and other extracellular vesicles from biological fluids previously purified from cells. At the same time, particles other than vesicles, such as protein complexes, were removed in the washing step. QIAzol Lysis Reagent containing phenol and guanidine thiocyanate was used for lysis of exosomes and elution of microRNAs contained in them. The reagent facilitated almost complete removal of proteins and residual DNA from the solution by organic extraction. After adding chloroform to the lysate, and separating the resulting solution into two phases, the upper aqueous phase containing all the RNA was withdrawn. The resulting material was then mixed with 96% alcohol in a 1:2 ratio (phase:alcohol) to give the RNA the desired properties and poured into RNeasy MinElute spin columns, where the microRNA was bound to the membrane. Finally, microRNA of high concentration and quality was eluted into a small tube by adding 14 μl of RNA-purified water.

The miRCURY LNA miRNA SYBR® Green PCR System characterized by high specificity to microRNAs and the ability to form strong thermostable complementary bonds between RNA strands - LNA (Locked nucleic acids) technology - was used to assess the expression of the microRNAs under study. The method is based on universal reverse transcription followed by amplification by real-time PCR.

Reverse transcription was performed on a BIO-RAD Real-time CFX96 Touch amplifier. The reaction was performed using 5x miRCURY RT SYBR® Green Reaction Buffer, 10x miRCURY RT Enzyme Mix reverse transcriptase (containing Poly(A) polymerase), RNA purified water and microRNA isolated during the previous step of the study. Reverse transcription was performed at 42 ºC for one hour, followed by enzyme inactivation by rapid warming to 95 ºC for a 5 minute period. Then, immediately, the resulting solution was cooled to a temperature of 4 °C. The above process allowed to obtain single-stranded cDNA matrix for all types of PCR analysis allowed by the kit.

The cDNA obtained in the previous step was amplified in real time on a Rotor Gene Q 6 plex machine. The reaction used 2x miRCURY SYBR® Green PCR Master Mix solution containing both buffer - 2x miRCURY SYBR® Green PCR Buffer and DNA polymerase QuantiNova DNA Polymerase enzyme together with SYBR® Green I fluorescent dye. SYBR® Green I dye is able to bind to double-stranded DNA and produce fluorescent staining at wavelengths between 494 nm and 521 nm. The QuantiNova polymerase used in the reaction was in an inactive state due to tight affinity binding to the QuantiNova Antibody. To activate the enzyme, it was necessary to increase the temperature to 95 ºC, which resulted in denaturation of the protective antibody and release of the polymerase. Real-time amplification was performed for 50 cycles at 60 -95 ºC. Simultaneously, fluorescence detection at 494 nm was performed for the final determination

of microRNA expression. In total, the expression of 6 microRNAs was determined: miR-20a, miR-135a, miR-34b, miR-449c, miR-449c and control miR-16.

The bioinformatic analysis 2^-ΔΔCt was used to interpret the obtained results. The method is based on the assumption that the difference in the value of the "threshold cycle" (ΔΔCt) - the cycle at which detectable fluorescence was formed - between the gene of interest and the control gene is proportional to the relative expression level of the gene of interest. Spearman's rank correlation was used to assess the effect of microRNA expression on the efficacy of HRT programs, using the Cheddock scale to characterize the strength of correlations. The results were visualized using the capabilities of Statistica 10 package (Tibco, USA) and Microsoft Excel 2019.

Results

According to the results of this study, it was found that the expression of exosomal miR-449c and miR-135a was significantly lower in the experimental group (p = 0.03 and p = 0.04, respectively) (Fig. 1, 2). There was also a trend

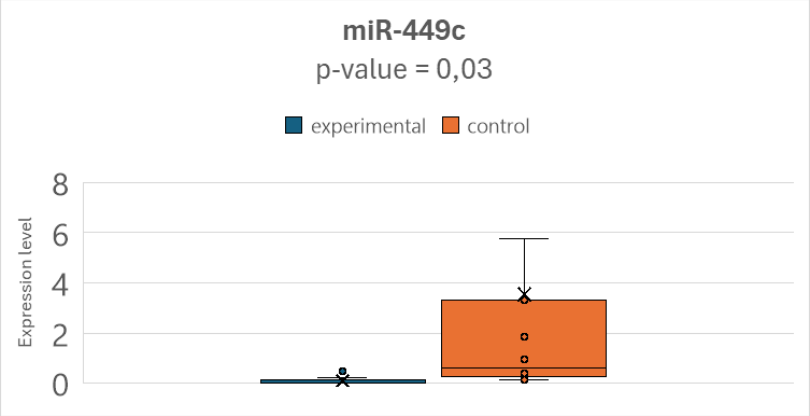

Fig. 1. Expression level of exosomal microRNA-449c in experimental and control groups

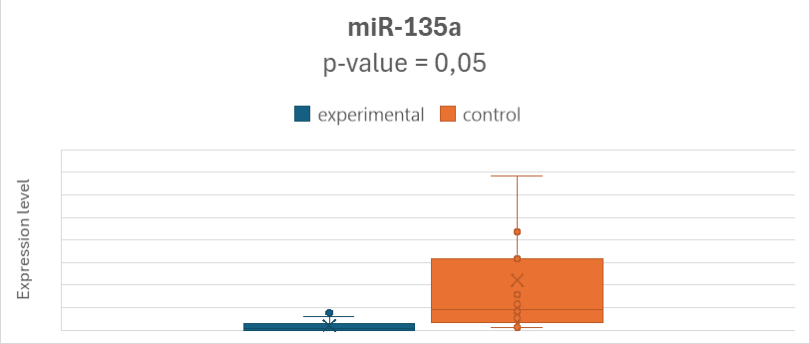

Fig. 2. Expression level of exosomal microRNA-135a in experimental and control groups

towards lower expression for miRNAs such as: miR-20a, miR-34b and miR-449c (p = 0.08, p = 0.15 and p = 0.18, respectively) (Fig. 3, 4, 5).

The insufficient reliability of the obtained correlation interactions can be explained by both the small number of study participants and the exosomal localization of the microRNAs under study.When comparing the results of the embryological stage of ART programs, it was found that with high reliability the expression level of miR-34b, miR-449c, and miR-449c has a direct correlation with the frequency of obtaining embryos of day 5 of good quality (p < 0.01). Simultaneously, the strength of the correlation relationship of the above relationships according to the Cheddock scale corre-

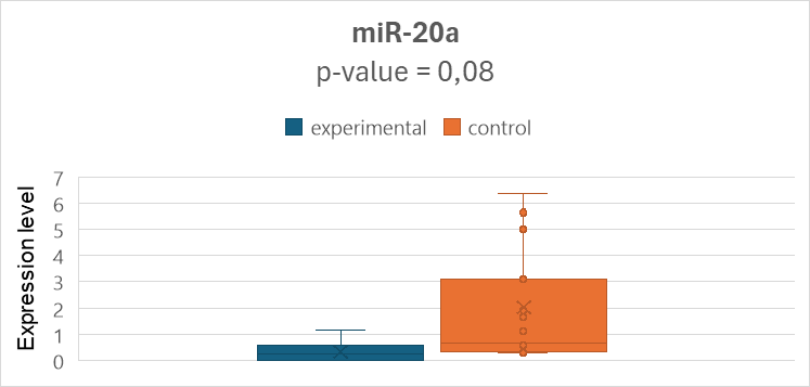

Fig. 3. Expression level of exosomal microRNA-20a in experimental and control groups

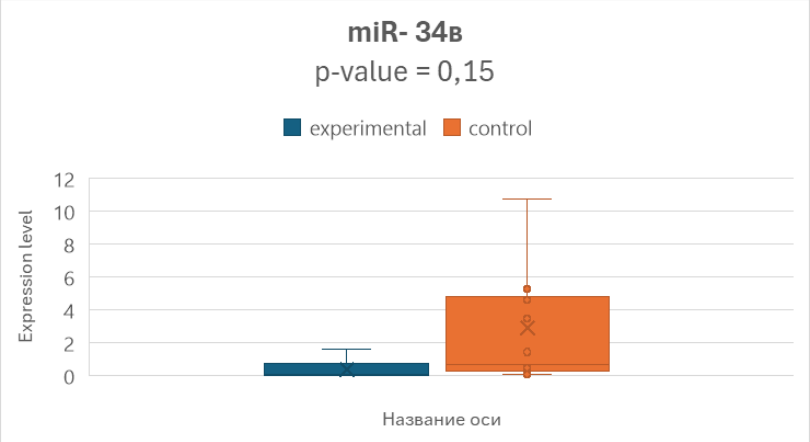

Fig. 4. Expression level of exosomal microRNA-34b in experimental and control groups

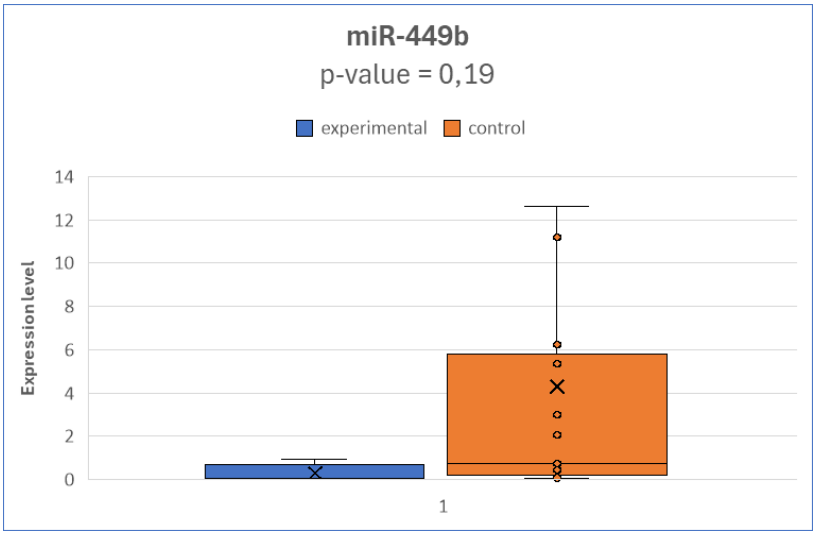

Fig. 5. Expression level of exosomal microRNA-449b in experimental and control groups

sponds to moderate, with the highest value obtained for miR-449c (r = 0.48762), then miR 34b and the final mir-449b (r = 0.44375 and r = 0.37661, respectively) (Fig. 6a, 6b, 6c). The expression of miR-135a was also characterized by a correlation with the frequency of obtaining high quality blastocysts, but with a lower degree of certainty ($p < 0.05$). The strength of the

correlation of this relationship was moderate (r = 0.33333), and with the lowest significant value obtained (Fig. 6d). For miR-20a, no associations were found with the effectiveness of ART programs (p > 0.05, r = 0,059912) (Fig. 6e).

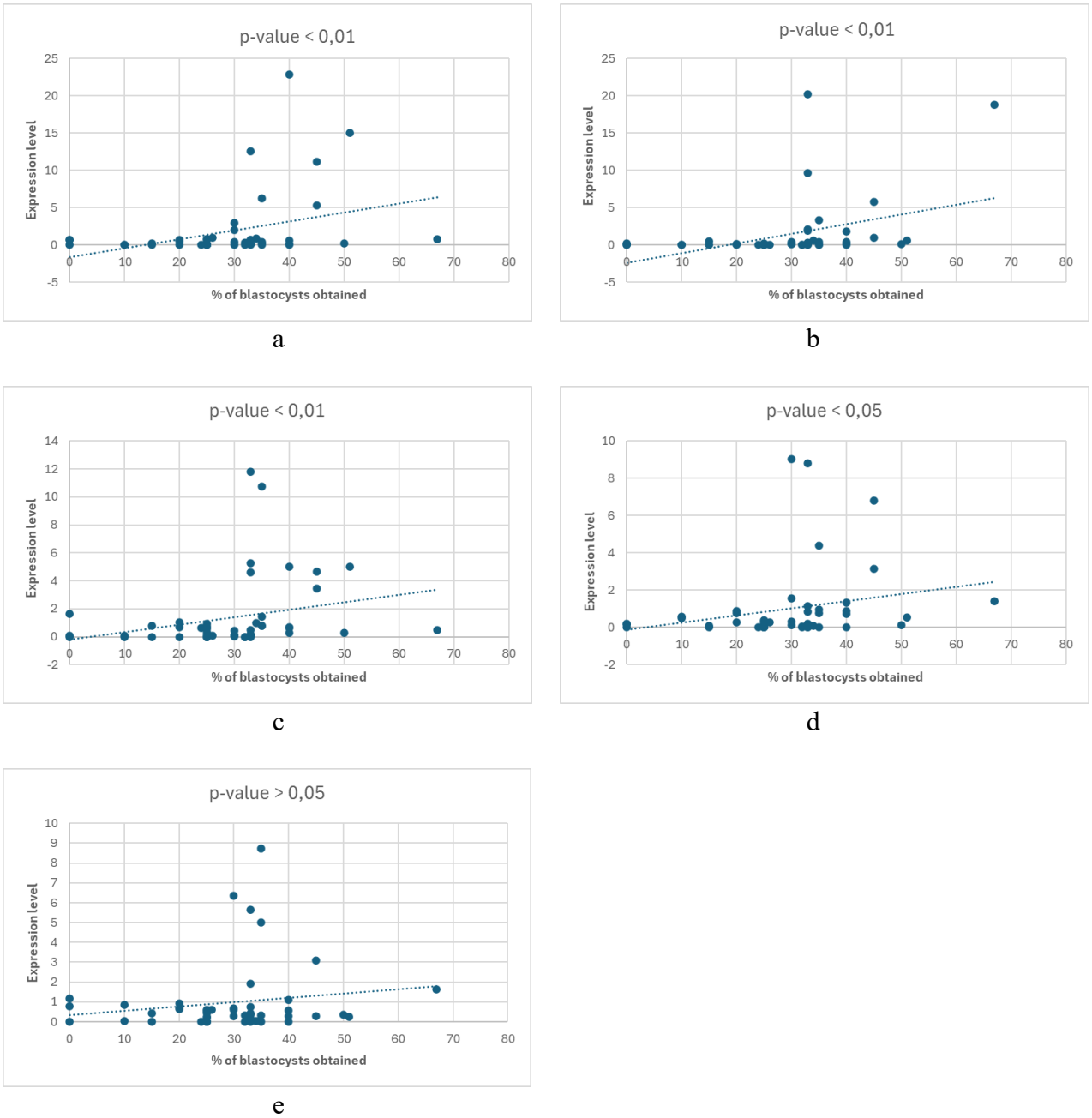

Fig. 6. a) Correlation of microRNA-449b expression with the results of the embryological stage of ART programs. b) Correlatin of microRNA-449c expression with the results of the embryological stage of ART programs. c) Correlation of microRNA-34b expression with the results of the embryological stage of ART programs. d) Correlation of microRNA-135a expression with the results of the embryological stage of ART programs. e) Correlation of microRNA-20a expression with the results of the embryological stage of ART programs

Discussion

The choice of microRNAs evaluated in our study was determined by the existing original and basic scientific works devoted to the evaluation of the role of small RNA forms as biomarkers of male infertility. Thus the attention of researchers has been attracted for several years by the miR-34 family, which consists of six members (miR-34a, miR-34b, miR-34c, miR-449a, miR-449b, miR-449c) located on three different chromosomes 1p36.22, 11q23.1 and 5q11.2 (Momeni *et al.*, 2020; Najafipour *et al.*, 2021; Eikmans *et al.*, 2020; Khadhim *et al.*, 2023; Finocchi *et al.*, 2020; Mokánszki *et al.*, 2020; Joshi *et al.*, 2023). Combining the results of these and other studies, Pantos et al., managed to suggest the main pathogenetic mechanisms underlying this relationship (Pantos *et al.*, 2021). Thus, according to the authors, decreased expression of microRNA-34/449 may lead to impaired ciliogenesis in the efferent ducts of the testis, which in turn leads to decreased reabsorption of seminal fluid, to aggregation and agglutination of spermatozoa, thereby leading to obstruction of efferent tubules and increased hydrostatic pressure in the testes, which ultimately leads to impaired semen quality ranging from oligospermia to azoospermia. Another postulated mechanism may be the significant involvement of the miR-34/449 microRNA family in the regulation of spermatogenesis.

When the expression of these microRNAs decreases, the cell cycle of spermatogenesis is disrupted, cell differentiation is impaired, sperm flagellum formation slows down or completely stops, and cell apoptosis increases. The results of our study demonstrate that miR-449c expression is significantly lower in the group of patients with established male factor infertility. At the same time, miR-34b, miR-449c, and miR-449c from the miRNA-34/449 family show a significant effect on the efficiency of conception and embryo development in ART programs.

Experimental data of many scientific works reveal similar patterns. Thus, Eikmans et al. noted a significant decrease in miRNA 34b expression in patients with oligozoospermia and asthenozoospermia ($p < 0.001$ and $p < 0.05$, respectively) (Eikmans *et al.*, 2020). Similar correlation has been reported by other studies in patients with oligoasthenozoospermia and with azoospermia (Khadhim *et al.*, 2023; Finocchi *et al.*, 2020; Mokánszki *et al.*, 2020). An interesting study by Momeni A et al. in which, in addition to the expression of miR-34 family, the degree of methylation of the promoter of the gene encoding the corresponding microRNA molecule was assessed. The results of the study demonstrate not only a significant decrease in expression in the group of infertile men ($P = 0.011$), but also a greater degree of promoter methylation (82.4% vs. 23.3%) (Momeni *et al.*, 2020). Similar conclusions regarding miR-449 were reached by Najafipour et al. (2021), thus, with significantly lower expression of miR-449-b ($p = 0.0001$), the frequency of promoter methylation was higher in the experimental group (60.8% vs. 23.3%) (Najafipour *et al.*, 2021). A recent study by Burgos CF et al. found that the effect of miR-34 expression on fertility may be due to the effect on the PI3K/AKT/mTOR signaling pathway (Burgos *et al.*, 2022). Another microRNA whose expression was analyzed in our work was miR-135a. The results of the study demonstrated not only a significant difference in the expression of the molecule in the experimental group, but also a significant effect on the embryological stage of ART programs. Among other scientific works evaluating the role of miR-135a we can highlight the study of Daneshmandpour Y et al. (2020), where in addition to the role of miRNA-34b, the association of miRNA-135a with oligozoospermia was noted, as well as the work of Li et al., 2021 in which the authors claim the association of miR-135a with asthenozoospermia (Daneshmandpour *et al.*, 2020; Li *et al.*, 2021). Al-Mawlah et al. also confirmed the presence of a significant difference in the expression of miRNA-135 in the ejaculate of infertile men (Al-Mawlah *et al.*, 2022). The above work also highlighted and putative mechanism of miR-135a's effect on male fertile function, particularly spermatogenesis. It is believed that miR-135a, along with other microRNAs, is involved in sperm maturation, development, motility, proliferation and survival by regulating

the expression of the Foxo1 gene, which is localized in spermatogonia stem cells. In turn, the O1 Foxo1 protein is a transcription factor that is upregulated in the stem cell nucleus.

The last miR-20a microRNA evaluated in our study was selected based on the encouraging data of Cito et al. who found a significant change in miR-20a-5p expression in blood from patients with nonobstructive azoospermia (Cito *et al.*, 2020). This microRNA molecule is believed to be bound to genes associated with cell apoptosis. Moreover, the authors noted the possibility of using the assessment of the expression of miR-20a-5p as a predictor of the effectiveness of testicular sperm extraction, which is of great clinical importance, given the invasiveness of the manipulation. Unfortunately, the results of our study do not allow us to confirm the conclusions obtained by Cito G. et al, as no significant correlation of expression changes in ejaculate of experimental and control groups was found.

Conclusion

Thus, idiopathic male infertility remains one of the major challenges facing modern medicine on the way to improving in vitro fertilization techniques, and understanding this problem is crucial to give couples in need a chance to have a child. The microRNA molecules selected for the study not only demonstrated their potential ability to be used as a diagnostic marker of male infertility, but also showed the ability to reflect the efficiency of fertilization and embryo formation processes. However, to fully exploit the potential of small nucleic acids, more research is needed to identify the types of microRNAs involved in these processes, their mechanism of action, and how modulation of their expression may alter the outcome for these patients.

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