SHORT COMMUNICATION

Could hsa-miR-29c and hsa-miR-30c be Used as Biomarkers for Day 3 Healthy Human Embryos in In-Vitro Fertilization?

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ABSTRACT

Embryo's quality is closely linked to miRNA regulation, which plays a critical role in gene expression and protein synthesis. In this prospective cohort observational study, extracellular miRNAs were tested using the miRCURY Locked Nucleic Acid (LNA) system, which used culture media from twenty-two singly transferred embryos. Study samples were collected on day three and embryos were transferred on day five. Based on earlier publications, hsa-miR-29c-3p and hsa-miR-30c-5p, among other miRNAs (not published), were chosen and assessed. The negative-implantation group had higher expression of hsa-miR-30c-5p regarded as statistically significant (P.value <0.001) than the other tested miRNAs. Contrary, there was no significant link between pregnancy outcome and expressed hsa-miR-29c-3p. Study findings, in comparison with findings from other reports, reveal the absence of hsa-miR-29c-3p and hsa-miR30c-5p from day 3 implanting embryos in contrast with day 5 where they are abundant, suggesting specificity to the embryo's development from cleavage to morula, blastocyst, and implantation stages. These findings could open the gate toward potential biomarkers for embryo development and implantation outcome.

Keywords: Human Embryo; IVF; MicroRNA; Non-Invasive Biomarker; Spent Culture Media.

1. INTRODUCTION

Identification of the most promising embryo for a successful IVF treatment is of greatest significance. Early reproduction-failure prevention comes from the successful alliance between a healthy embryo, a hospitable endometrium, and an effective embryo-endometrial conversation (Craciunas *et al*., 2019). Current identification methods for healthy embryos rely mainly on microscopic examination, which does not necessarily reflect the embryo's molecular makeup (Sanchez *et al*., 2017). Unfortunately, only 50% of top-quality singly transferred blastocyst implant successfully (Gardner and Balaban, 2016). With some aneuploid embryos showing good morphological scores under microscopic examination, a need for a non-invasive diagnostic substitute is impelling (Acuña-González *et al*., 2021). MicroRNAs were introduced recently as potential molecular biomarkers to shed some light on the genetical and physiological turnover of the embryos by non-invasive detectable means. These conserved epigenetic modulators bind to messenger RNA (mRNA) to influence gene expression and protein synthesis. They engage in nearly all biological processes and are detected in almost all body fluids including spent culture medium (SCM) collected from different cell lines (O'Brien *et al*., 2018). This study aims to attain if miRNAs, released by human embryos in spent culture medium, can be used as non-invasive biomarkers for achieving better embryo selection and improving pregnancy outcome rates.

2. MATERIALS AND METHODS

This pilot study is a prospective cohort observational study that includes embryo's spent culture media obtained from couples undergoing Intracytoplasmic Sperm Injection (ICSI) treatment at the fertility clinic unit of Ain Shams University Maternity Hospital in Egypt. After meeting the initial exclusion and inclusion criteria, twenty-two couples were asked to participate and written consents were obtained. All ICSI cycles were conducted according to the identical approved fertility clinic unit treatment protocol, i.e., the long mid-luteal gonadotropins releasing hormone (GnRH) agonist down-regulation protocol and standard control ovarian stimulation protocols. Embryos were cultured in single droplets until sample collection was conducted on the third day of the embryo's development. Elective single embryo transfer back to the mother was carried out on the fifth day when all embryos reached the blastocyst stage. In the SCM, miRNAs expression was evaluated between the positive and negative implantation groups. At least twenty microliters of the embryo's SCM were treated to isolate total RNA, including miRNAs, using the miRNeasy Micro Kit (Cat. No. 217084, Qiagen, USA). The miRCURY LNA RT kit (Cat. No. 339340, Qiagen, USA) was used to proceed to the reverse transcription step. The synthesized cDNA was then used as a template for real-time quantitative PCR using the miRCURY LNA SYBR Green PCR kit (Cat. No. 339345, Qiagen, USA) with primer sets (miRCURY LNA miRNA PCR Assay, Cat. No. 339306, Qiagen, USA) specific for the investigated miRNAs. PCR cycles were launched using a qPCR thermocycler (Agilent Aria Mx, software version 1.8, USA) and the results were statistically analyzed. Out of the twenty-two understudy cases, twelve resulted in negative pregnancies, while ten resulted in positive pregnancies. The expression levels show that hsa-miR-30c-5p was significantly higher in the negative pregnancy group ($p<0.001$). While hsa-miR-29c-3p was non-significant between both pregnancy groups (Table 1). Curiously, studying the correlations between expressed miRNAs with each other, the negative pregnancy group showed a significant positive correlation between hsa-miR-29c-3p and hsa-miR-30c-5p (Table 2).

3. RESULTS AND DISCUSSION

According to Abu-Halima *et al.* (2017), miR-29c was found to be the most significant differentially expressed miRNA and was correlated with a positive implantation outcome. The group also found hsa-miR-29c-3p and hsa-miR-30c-5p significant in day 5 blastocyst culture medium (Abu-Halima *et al.* (2017)). In a different approach, Viswanathan *et al.* (2009) found hsa-miR-29c, among other miRNAs, was proposed to be involved in trophectoderm specification by inducing embryonic stem cells to differentiate into trophectodermal cells Viswanathan *et al.* (2009). Battaglia *et al.,* (2019) pointed out that the miR-29 family are vital epigenetic regulators during human somatic cell reprogramming as they help to maintain the pluripotency of embryonic cells (Battaglia *et al.,* (2019)). Rosenbluth *et al.* (2014) found hsa-miR-30c-5p abundant in day 5 SCM samples (Rosenbluth *et al.* (2014)). Capalbo *et al.* (2016) conducted a comprehensive analysis of miRNA profiles and found hsa-miR-30c-5p presented significantly higher expression in SCM obtained from implanted blastocysts and were specific to the blastocyst stage. Interestingly, the group also found hsa-miR-30c-5p detection rate was 92.9% amongst samples that did not implant successfully, which is in line with this study's findings[10]. Results from these studies show the

significance of these miRNAs from day 5 sample collection day which is different from this study's day 3 collection day. In this study, the significant presence of hsa-miR-30c-5p and hsa-miR-29c-3p from day 3 cleavage embryos in the SCM is suggested to be associated with failed pregnancies as their early release is correlated with bad prognosis. These findings suggest a stage-specific role of these two miRNAs synchronizing the turnover of the embryo's development from the cleaved stage to the morula, blastocyst, and implanting stages of the embryos. Indeed, further investigations are needed to support this hypothesis.

Table 1. showing relations between expressed miRNAs and outcome of pregnancy

MicroRNA Name	Statistical Distribution	Negative Pregnancy	Positive Pregnancy	Test value	P-value
hsa-miR-29c-3p	**Mean± SD**	1.19± 0.67	0.74± 0.60	T= 1.66	0.113
	Median (IQR)	1.19 (0.84- 1.20)	0.60 (0.32- 0.84)		
	Range	0.40- 2.33	0.17- 1.93		
hsa-miR-30c-5p	**Mean± SD**	1.08± 0.24	0.46± 0.18	T= 6.91	<0.001
	Median (IQR)	1.08 (1.06- 1.08)	0.44 (0.28- 0.54)		
	Range	0.72- 1.45	0.27- 0.77		

$P \leq 0.05$ is considered statistically significant, $P \leq 0.01$ is considered highly statistically significant, SD: standard deviation, comparison between groups done by Independent-Samples Kruskal-Wallis Test, IQR: interquartile range.

Table 2. showing correlations between expressed hsa-miR-29c-3p and hsa-miR-30c-5p with each other among cases with negative and positive pregnancies

	hsa-miR-30c-5p			
hsa-miR-29c-3p	**Negative Pregnancy**		**Positive Pregnancy**	
	r	0.800	**r**	-0.714
	P-value	0.005	**P-value**	0.009

$P \leq 0.05$ is considered statistically significant, $P \leq 0.01$ is considered highly statistically significant, r=correlation coefficient of spearman correlation test.

6. REFERENCES

Abu-Halima, M., Häusler, S., Backes, C., Fehlmann, T., Staib, C., Nestel, S., Nazarenko, I., Meese, E. and Keller, A., 2017. Micro-ribonucleic acids and extracellular vesicles repertoire in the spent culture media is altered in women undergoing in vitro fertilization. *Scientific reports, 7*(1), pp.1-11.

Acuña-González, R.J., Olvera-Valencia, M., López-Canales, J.S., Lozano-Cuenca, J., Osorio-Caballero, M. and Flores-Herrera, H., 2021. MiR-191-5p is upregulated in culture media of implanted human embryo on day fifth of development. *Reproductive Biology and Endocrinology, 19*(1), pp.1-7.

Battaglia, R., Palini, S., Vento, M.E., La Ferlita, A., Lo Faro, M.J., Caroppo, E., Borzì, P., Falzone, L., Barbagallo, D., Ragusa, M. and Scalia, M., 2019. Identification of extracellular vesicles and characterization of miRNA

expression profiles in human blastocoel fluid. *Scientific reports, 9*(1), pp.1-11.

Capalbo, A., Ubaldi, F.M., Cimadomo, D., Noli, L., Khalaf, Y., Farcomeni, A., Ilic, D. and Rienzi, L., 2016. MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment. *Fertility and sterility, 105*(1), pp.225-235.

Craciunas, L., Gallos, I., Chu, J., Bourne, T., Quenby, S., Brosens, J.J. and Coomarasamy, A., 2019. Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Human reproduction update, 25*(2), pp.202-223.

Gardner, D.K. and Balaban, B., 2016. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important?. *MHR: Basic science of reproductive medicine, 22*(10), pp.704-718.

O'Brien, J., Hayder, H., Zayed, Y. and Peng, C., 2018. Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Frontiers in endocrinology, 9*, p.402.

Rosenbluth, E.M., Shelton, D.N., Wells, L.M., Sparks, A.E. and Van Voorhis, B.J., 2014. Human embryos secrete microRNAs into culture media—a potential biomarker for implantation. *Fertility and sterility, 101*(5), pp.1493-1500.

Sanchez, T., Seidler, E.A., Gardner, D.K., Needleman, D. and Sakkas, D., 2017. Will noninvasive methods surpass invasive for assessing gametes and embryos?. *Fertility and Sterility, 108*(5), pp.730-737.

Viswanathan, S.R., Mermel, C.H., Lu, J., Lu, C.W., Golub, T.R. and Daley, G.Q., 2009. microRNA expression during trophectoderm specification. *PloS one, 4*(7), p.e6143.