The Correlation of Chronological Age and Micro Ribonucleic Acid-135b Expression in Spent Culture Media of In vitro Fertilisation Patient

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Abstract

Background: The lack of accuracy in embryo viability assessment methods still remains a challenge to increase the in vitro fertilisation (IVF) success rate. The chronological age and micro ribonucleic acid (RNA)-135b influence the quality of the embryo since microRNA-135b expresses stably in the spent culture media. Therefore, microRNA-135b has the potential to become a non-invasive biomarker of IVF embryo quality. Aims: (1) The aim of this study is to determine the chronological age and microRNA-135b expression distribution of IVF patients. (2) to determine the correlation between chronological age and microRNA-135b expression in spent culture media of IVF patients.

Study Setting and Design: An observational study was conducted in Yasmin IVF clinic. Materials and Methods: The chronological age data were collected from the medical records and 31 spent culture media samples from 11 IVF patients were taken on day 5 of embryo culture. We also collected the basal media sample as the control group. The microRNA-135b expression was analysed using quantitative real-time polymerase chain reaction (qPCR) analysis.

Statistical Analysis Used: The data analysis was performed using IBM SPSS Statistics 25. Results: The chronological age and microRNA-135b expression were distributed abnormally. There was a significant positive correlation with moderate statistical power between chronological age and microRNA-135b expression in spent culture media. MicroRNA-135b expression increased 4.9-fold in spent culture media than basal media of IVF. Conclusions: The increase of chronological age is followed by the rise of microRNA-135b expression in spent culture media of IVF patients. The microRNA-135b is a potential biomarker to predict IVF embryo quality.

Keywords: Chronological age, in vitro fertilisation, micro ribonucleic acid -135b expression, spent culture media

INTRODUCTION

According to the Centers for Disease Control and Prevention and the European Society of Human Reproduction and Embryology, there are only a few top clinics that have more than 40% success rate of in vitro fertilisation (IVF).[1] Most of the clinics have a success rate between 10% and 40% and few others have success rate <10%.[1] IVF success rate is influenced by several factors such as oocyte quality, sperm cell quality, embryo quality, endometrial receptivity and embryo transfer quality.[2] This study focuses on embryo quality because it is one of the biggest challenges for increasing the IVF success rate. An important reason is the lack of accuracy in embryo viability assessment methods.[3] Embryo quality is influenced by chronological age and micro ribonucleic acid (RNA).[4,5] MicroRNA-135b is a
potential non-invasive embryo quality biomarker because it is expressed consistently in spent culture media.⁷

Chronological age and microRNA-135b, which have a positive correlation with aneuploid embryos in IVF form the basis of this study.⁷,⁸ This study aims to determine: (i) the distribution and (ii) correlation between chronological age and microRNA-135b expression in IVF patients. The hypothesis is the higher chronological age, the higher microRNA-135b will be expressed in IVF spent culture media.

**Materials and Methods**

This study has been approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (Nomor: KET-175/UN2.F1/ETIK/PPM.00.02/2020). This study was conducted in Yasmin IVF Clinic, Dr. Cipto Mangunkusumo General Hospital, and Human Reproduction, Infertility, and Family Planning Cluster, Indonesia Reproductive Medicine Research and Training Center, Jakarta, Indonesia Institute, from January 2020, to September 2020. This study was retrospective cross-sectional study. The patient who underwent at least one complete IVF cycle was included in this study. This study used primary data from spent culture media sample and secondary data from the patients’ medical records. The patients had given their informed consent so their anonymised data from their medical records and their discarded material from IVF lab can be used for research or educational purposes. This study committed to the Helsinki Declaration. The spent culture media samples were taken on the 5th day of embryo development in culture media. The amount of spent culture media that was taken is 20 µl, as well as 20 µl basal media sample was also taken. The sample size calculation was performed. The minimum sample size was 23 culture media.

The microRNA isolation and purification procedures were performed using mirVana™ PARIS™ RNA and Native Protein Purification Kit (AM1556, Thermo Fisher, US) using the manufacturer’s protocol. The microRNAs were eluted in a final volume of 20 µl to concentrate them in the smallest possible volume. Then, the cDNA was then generated using Taqman™ MicroRNA Reverse Transcription Kit (4366596, Thermo Fisher, US) according to the manufacturer’s instructions. MicroRNA-135b expression level was assessed by qPCR with the following primers UAAUGGCUUUUAUACACUGUGA. The primers were taken from https://mirbase.org website. MicroRNAs expression was evaluated using Taqman Fast Advanced Master Mix (4444557, Thermo Fisher, US) under the following thermal cycling conditions: 95°C for 20 s followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. U6 snRNA expression was used as internal control with the following sequence GGTGCTTCGCTTCGGCACACATATACTAAATTTGAGACGATAAGAGATTTGACATGGCCCTGTGCGCAAGGATGACGCAAAATTCGTAAGCGTGCTTCATATTTT. The microRNA-135b expression was valued by relative quantification of polymerase chain reaction (PCR) method using LIVAK method with 2−ΔΔCt formula.

**Statistical analysis**

The data analysis was performed using SPSS version 25 SPSS Inc, Chicago, Illinois, United States. First, the normality of data was analysed using the Shapiro–Wilk test. If the data distribution was normal with P > 0.05, the Pearson’s test for correlation was used. If the data distribution was not normal with P < 0.05, the Spearman test for correlation was used.

**Results**

**Subject characteristics**

There were 31 spent culture media samples from 11 IVF patients. The chronological age (P = 0.004) and microRNA-135b expression (P < 0.001) distribution were not normal (P < 0.05). The data distribution characteristic is presented in [Table 1] and the detail of chronological age data is presented in [Table 2].

MicroRNA-135b expression measured by real-time PCR machine. The maximum cycle value that was used in this study is 40 cycles. The amplification curve was presented in [Figure 1].

The relative quantification of PCR analysis showed an increase of 4.9-fold of microRNA-135b expression in spent culture media.

| Characteristic                  | Result (n=31) |
|---------------------------------|---------------|
| Chronological age (years)       | 34 (26-39)    |
| MicroRNA-135b expression (fold) | 0.384 (0.003-71.012) |

The values are median between the range of maximum and minimum.

| Table 1: Data distribution characteristic |
|------------------------------------------|
| Characteristic                  | Result (n=31) |
|---------------------------------|---------------|
| Chronological age (years)       | 34 (26-39)    |
| MicroRNA-135b expression (fold) | 0.384 (0.003-71.012) |

The values are median between the range of maximum and minimum.

| Table 2: Chronological age data       |
|---------------------------------------|
| Patient | Chronological age (years) |
|---------|--------------------------|
| 1       | 26                       |
| 2       | 31                       |
| 3       | 32                       |
| 4       | 32                       |
| 5       | 32                       |
| 6       | 34                       |
| 7       | 35                       |
| 8       | 35                       |
| 9       | 36                       |
| 10      | 37                       |
| 11      | 39                       |
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The comparison of the relative microRNA-135b expression in spent culture media and basal media is presented in [Figure 2].

**The correlation of chronological age and micro ribonucleic acid-135b expression**

The result of the Spearman test showed that there was a significant positive correlation with moderate statistical power between chronological age and microRNA-135b expression ($R = 0.463; P = 0.004$). The result of the Spearman test is presented in [Table 3].

**DISCUSSION**

**Subject characteristics**

This study showed that the embryo produces microRNA-135b in spent culture media. This result also supported by Rosenbluth et al. and Capalbo et al. who also found that microRNA is secreted by IVF embryo to culture media.$^{[9,10]}$

Fertility decline in women is seen in mid-thirties and beyond due to a decreasing number of oocytes.$^{[11]}$ This is also supported by Wiweko et al. that found a decrease in antral follicle numbers when women aged between 34 and 35 years old.$^{[4]}$ In this study, almost half of the study participants included women category that already experienced the decline of fertility. Therefore, the chronological age variable in this study is considered enough to represent either the good or poor women fertility category based on the chronological age.

The result of this study showed accordance with the hypothesis. This study showed that chronological age and microRNA-135b expression in spent culture media have a significant positive correlation with moderate statistical power. The result is supported by several studies about the relation of either IVF aneuploidy embryo or chronological age and microRNA-135b expression. Rosenbluth et al. found that microRNA in IVF spent culture media correlates with aneuploidy embryo incidence.$^{[9]}$ This is also supported by Febri et al. who found that the expression of microRNA-135b is higher in aneuploidy spent culture media than euploidy spent culture media in IVF.$^{[7]}$ On the other hand, Vagnini et al. found that aneuploidy embryo incidence has a positive correlation with IVF patients’ age.$^{[8]}$ It can be explained by the McCallie study which found that the increase of chronological age also increases the microRNA expression.$^{[12]}$ The increase of microRNA expression affects the target gene that plays a role in the oxidative stress defence mechanism.$^{[12]}$ The decrease of oxidative stress defence mechanism will cause the increase of oxidative stress that will disturb the growth and cell proliferation signal.$^{[12]}$ At the end, it will reduce the embryo quality.

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*[Figure 1: The amplification curve of micro ribonucleic acid-135b expression]*

*[Figure 2: The relative expression of micro ribonucleic acid-135b chart]*

**Table 3: The correlation between chronological age and microRNA-135b expression**

| MicroRNA-135b expression | Chronological age |
|--------------------------|------------------|
| Correlation coefficient  | 0.463            |
| Significant (one-tailed) | 0.004*           |
| $n$                      | 31               |

*Significant at $P<0.05$
CONCLUSIONS

The chronological age has a significant positive correlation with microRNA-135b expression in IVF spent culture media.

To our knowledge, this is the first study about the correlation between chronological age and microRNA-135b expression in IVF spent culture media. Therefore, we hope this study can support microRNA-135b expression development as a non-invasive biomarker for embryo quality. However, the limitation of this study is confounding variables which was not well controlled. The confounding variables were ovarian reserve and gonadotropin dose.

We recommend further research about other factors that influence the microRNA-135b expression in IVF spent culture media to support the use of microRNA-135b as an IVF embryo quality non-invasive biomarker. The IVF clinicians should also consider the chronological age in predicting the success rate.

Data availability
Data are not available for third-party use.

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Conflicts of interest
There are no conflicts of interest.

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