The levels of follicular fluid cell-free mitochondrial DNA could serve as a biomarker for pregnancy success in patients with small ovarian endometriosis cysts: A case-control study

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Abstract
Ovarian endometriosis cyst (OEC) is caused by the growth of ectopic endometrium into the ovarian cortex, leading to disrupted ovarian cortical structures and infertility. Large OECs are usually surgically removed, and assisted reproductive technology (ART) is required for future pregnancy. The oocyte reserve and development of patients with small non-surgical OECs are unknown. In this study, we compared mitochondrial abnormality, ATPase and IF1 mRNA expression levels, and OXPHOS complex proteins between OEC vs control mural granulosa cells (mGCs).

OEC mGCs show fewer mitochondria per cell, a higher proportion of aberrant morphology, lower ATPase mRNA levels, higher IF1 mRNA levels, and impaired expression of 3 of the 5 critical proteins involved in the OXPHOS complex, compared with control mGCs. Cell-free mitochondrial DNA (cfmtDNA) levels are higher in the follicular fluid of patients with OEC and were inversely associated with the expression of mtDNA in mGCs and cumulus granulosa cells (cGCs).

Taken together, this study indicates that small non-surgical OECs lead to poor quality of oocytes and subsequent embryos during ART compared with control, which was accompanied by mGC mitochondrial dysfunction. mGC and cGC mtDNA and FF cfmtDNA might serve as efficient biomarkers for the non-invasive prediction of pregnancy outcomes in patients with OEC undergoing ART.

Abbreviations: ART = assisted reproductive technology, cfDNA = circulating free DNA, cfmtDNA = cell-free mitochondrial DNA, cfmtDNA = cell-free nuclear DNA, cGCs = cumulus granulosa cells, Ems = endometriosis, FF = follicular fluid, GCs = granulosa cells, ICSI = intracytoplasmic sperm injection, IF1 = ATPase inhibitory factor 1, IVF = in vitro fertilization, mGCs = mural granulosa cells, mRNA = messenger RNA, mtDNA = mitochondrial DNA, OEC = ovarian endometriosis cyst, OXPHOS = oxidative phosphorylation, PBS = phosphate-buffered saline, PCOS = polycystic ovary syndrome, qRT-PCR = quantitative real-time polymerase chain reaction, ROS = reactive oxygen species, TEM = transmission electronic microscopy.

Keywords: biomarker, cell-free mitochondrial DNA, mitochondria, ovarian endometriosis cyst, pregnancy

1. Introduction
Endometriosis (Ems) is one of the most common diseases among women of childbearing age. About 10% to 25% of the patients with Ems also have infertility, requiring assisted reproductive technology (ART) for pregnancy.\textsuperscript{[1]} Most Ems lesions occur in the pelvic cavity, especially in the ovaries. The typical pathological manifestation includes the growth of the ectopic endometrium into the ovarian cortex, periodic bleeding, and formation of single or multiple ovarian endometriosis cysts (OECs). Patients with multiple large OECs or after surgery for EOC demonstrate significantly disrupted ovarian cortical structures, which seriously impact the ovarian reserve and oocyte development, leading to adverse pregnancy outcomes. On the other hand, how severely the ovarian reserve and oocyte development are affected in patients with small non-surgical OECs remains unknown.

The ovarian parenchyma is composed of oocytes and granulosa cells (GCs). The GCs include the mural GCs (mGCs) that form the follicle wall and the cumulus granulosa cell (cGCs) that surround the oocytes.\textsuperscript{[2]} Given that GCs are associated with oocyte development,\textsuperscript{[3]} mitochondrial protein turnover required in GC proliferation is critical in facilitating follicular development.\textsuperscript{[4]} The quality of GC mitochondria and oocytes decreases over age.\textsuperscript{[5]} The age-dependent instability of mitochondrial DNA
ATPase and IF1 and investigated the role of cfmtDNA in affected in terms of pregnancy outcomes during ART. We shed light on ART strategies for patients with small-size non-invasive tests. In the in vitro cultured oocyte-corona-cumulus complex, cfmtDNA is released into the culture media and high abundance and is considered a potential biomarker for survival of the oocytes and GCs. The composition of the FF has disrupted mtDNA results in mitochondrial dysfunction.

The follicular fluid (FF) provides a critical environment for the survival of the oocytes and GCs. The composition of the FF has an impact on the quality of the oocytes, fertilization, early embryonic development, and implantation. Higher oxidative stress in the FF leads to an excessive amount of reactive oxygen species (ROS) produced during ATP synthesis by the mitochondria, which may cause oxidative damage, mutations, and deletions to the mtDNA. Circulating free DNA (cfDNA) is a type of nucleic acid that exists outside of the cells and circulates in the fluids such as the bloodstream, FF, and embryo culture media. cfDNA includes circulating cell-free nuclear DNA (cfnDNA) and circulating cell-free mitochondrial DNA (cfmtDNA). The cfmtDNA has a relatively simple structure and high abundance and is considered a potential biomarker for non-invasive tests. In the in vitro cultured oocyte-corona-cumulus complex, cfmtDNA is released into the culture media by the GCs in response to mitochondrial dysfunction.

This study aims to examine how patients with small OECs are affected in terms of pregnancy outcomes during ART. We explored the mechanism of GC mitochondria in regulating ATPase and IF1 and investigated the role of cfmtDNA in reflecting the GC mitochondrial dysfunction and oocyte quality of patients with OEC. The results of this study could shed light on ART strategies for patients with small-size non-surgical OECs.

2. Methods

2.1. Study design and subjects

For the observation of the patient characteristics and mitochondrial function: the subjects (OEC, n=30; control, n=30) undergoing in vitro fertilization (IVF) were recruited at the Reproduction Center of the No. 4 Hospital of Shijiazhuang from April 2018 to December 2018. OEC was diagnosed as unilateral or bilateral OEC by multiple ultrasound examinations (OEC n ≤ 2 per side and diameter < 4 cm). The OEC group included 30 patients of 23 to 37 years of age (mean of 31.1 ± 3.9 years). Thirty patients with tubal factor infertility were included as controls; they were 24 to 35 years of age (mean of 29.3 ± 3.6 years).

For the investigation of the cfmtDNA and GC mtDNA: the subjects (OEC, n=30; control, n=30) were also recruited during the same period at the reproduction center of the No. 4 Hospital of Shijiazhuang, including 30 unilateral or bilateral OEC patients (OEC n ≤ 2 per side and OED diameter < 4 cm) of 24 to 36 years of age (mean of 30.6 ± 3.5 years) and 30 controls of 25 to 37 years of age (mean of 30.5 ± 3.3 years). The control subjects were patients undergoing ART using intracytoplasmic sperm injection (ICSI). Therefore, they had a normal ovarian function, but the couples had male factor infertility.

The inclusion criteria were:

1. before treatment, the basal hormone levels (on day 2–3 of the menstrual cycle) was within the normal range, 2. the subjects showed normal menstrual cycle without hyperandrogenemia or polycystic ovary syndrome (PCOS), 3. the subjects were physically healthy without physiological defects, congenital genital malformations, or other chronic and endocrine diseases, 4. no steroid medication was administrated within the last 3 months, and 5. the 2 mates had normal chromosomes.

The study was approved by the ethics committee of No. 4 Hospital of Shijiazhuang, Hebei, China.

2.2. Sample collection

2.2.1. Collection of FF supernatant and mGC samples. The FF was collected on the same day as the oocyte-corona-cumulus (OCC) complex. The samples significantly contaminated by blood were discarded. A two-step centrifugation protocol was applied. The FF samples were first centrifuged at 3000 rpm for 15 minutes, followed by another round of centrifugation at 16,000 rpm for 10 minutes. The supernatant was transferred into a 1.5-ml tube and stored at -80°C. The pellet was resuspended in 3 ml of phosphate-buffered saline (PBS) and slowly added to the upper layer of 3 ml of human lymphocyte separation medium (1:1). The mixture was centrifuged at 2500 rpm for 20 minutes. The GCs were seen in the white-membrane-like middle layer. The GCs were slowly aspirated by a pipette and transferred into another tube. If contaminated by membrane-like substances, the GC layer was isolated and mixed with 2 ml of PBS before being added slowly to the upper layer of another 2 ml of human lymphocyte separation medium (Shanghai Yanjin Biotechnology Co., Ltd., 1:1) and centrifuged at 2500 rpm for 10 minutes. The membrane-free GCs were isolated again using a pipette and washed twice with PBS at 6000 rpm for 5 minutes. To remove the red blood cells, the GCs were incubated with a red blood cell lysis buffer (Beijing Suo Laibao Technology Co., Ltd.) for 2 minutes at 4°C and then washed twice with PBS at 6000 rpm for 5 minutes.

Four samples with a relatively large cell number were selected from the control and OEC group, respectively. The majority of cells were fixed in 4% glutaraldehyde, and the remaining cells were stored at -80°C.

2.2.2. Collection of cGC samples. The OCC complexes were isolated from the FF when the OCC complexes were harvested for ICSI. The complexes were cultured in saturated humidity with 6% CO2 and 65% O2 at 37°C for 2 hour and then dissociated with 37°C preheated hyaluronic acid for 1 minute to remove most of the GCs. The remaining cGCs were resuspended several times in MOPS microdrops and centrifuged at 6000 rpm for 5 minutes. The pellet was washed once with PBS and saved for later use.

2.3. Experimental procedures

2.3.1. Transmission Electronic Microscopy (TEM). Freshly prepared mGCs were fixed in 4% glutaraldehyde for at least 2 hour and stored at 4°C for up to 1 week before TEM. Prior to TEM, the samples were washed 3 times with 1/15 M phosphate buffer for 10 minutes and fixed in 1% antimony tetroxide (Sigma-Aldrich, USA) for 2 hour. After another wash, gradient dehydration in acetone (Sigma-Aldrich, USA), embedding, and polymerization, the samples were sectioned into ultrathin slices of approximately 50 nm thickness using an ultra-thin slicer. The slices were stained in uranyl acetate (Sigma-Aldrich, USA) for...
30 minutes, followed by lead citrate (Sigma-Aldrich, USA) staining for 15 minutes. The mitochondrial structure was observed using an H-7500 TEM (HITACHI, Japan). The numbers of total and abnormal mitochondria, referring to mitochondria with fractured or fused cristae or swollen mitochondria with vacuoles, were counted. Four samples from both the OEC and control groups were examined. Five fields were taken in each sample at 8000 ×, and over 200 mitochondria were examined. The abnormality rates were analyzed.

2.3.2. Quantitative real-time PCR (qRT-PCR). mGCs were incubated in 1 ml of Trizol lysis buffer (Invitrogen, USA), and RNA was extracted according to the manufacturers instruction. The RNA concentration was determined by a UV spectrophotometer. The total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Thermo Fisher, USA) followed by PCR amplification. ATPase and IF1 mRNAs were examined by qRT-PCR, with a program of 95°C for 5 minutes, followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 second, 95°C for 15 second, 60°C for 1 minutes, and 95°C for 30 seconds, before the final extension at 72°C for 5 minutes. The samples were tested in triplicate for each experiment. The fold changes were calculated by the 2^ΔΔCT manner, and the results are presented as mean ± standard deviation. The primers were synthesized by Generay Co., Ltd. (Shanghai). Primer sequences:

- ATPase: F 5'-CTATTTCGCTGATACGTC-3' R 5'-GGAAAGCTATAGGGATCGC-3' 281bp
- FI: F 5'-GGAGGCATTGAGAAGAAG-3' R 5'-TAAGCTGCGCTGGTTC-3' 188bp
- β-actin: F 5'-CTGCGGTCTGGAAGGATTC-3' R 5'-CTGGGGTGTTGAAGGTTGC-3' 129bp

2.3.3. Western blot assay. mGCs were lysed in RIPA buffer supplemented with protease inhibitor. After incubation on ice for 1 hour, the lysate was centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatant was collected, and the protein concentration was determined by a BCA measurement kit. Equal amounts of proteins were loaded onto an SDS-PAGE gel and underwent electrophoresis to separate protein bands. The proteins were transferred onto a PVDF or natural cellulose membrane for immunoblotting, according to the manufacturers instruction. The blot was imaged and analyzed using an Odyssey far-red imaging system. The relative expression of the target protein was presented as the grayscale of the target protein band divided by the grayscale of the β-actin (loading control) band. The experiment was repeated twice. The primary antibody was mouse anti-total OXPHOS antibody (Abcam, 1:250). The secondary antibody was anti-mouse HRP (Shanghai Generay Biotech Co., Ltd., China).

2.3.4. Detection of cfMTDNA in FF. Protease K (100 μl), 1 ml of FF, and 800 μl of lysis buffer were added in a 15 ml tube. The mixture was vortexed at maximal speed and incubated at 55°C for 10 minutes. Then, 0.5 ml of isopropanol was added, followed by a 30-s vortex. Magnetic bead suspension (100 μl) was added, followed by vortexing at maximal speed. After 10 minutes of incubation, the 15-ml tube was placed on the magnetic isolator for 2 minutes. The supernatant was discarded by pipetting. The magnetic beads were washed and dried, and the purified cfMTDNA was eluted before PCR amplification. The primers were designed and synthesized by Generay Co., Ltd. (Shanghai) and diluted in ddH2O to a final concentration of 10 μM. The primer sequences were:

- β-actin:
  - Forward: 5’-CAGAGCAAGAGAGGATCC-3’
  - Reverse: 5’-CTGGGGTGTTGAAGGTTGC-3’
- ND1:
  - Forward: 5’-CCTAGGCCTTTACTCA4CTC-3’
  - Reverse: 5’-TGATGCTAGGCGTACTCAT-3’

2.3.5. Detection of mtDNA in the GCs. The cells were placed in 400 μl of ACL solution and vortexed for 1 minutes. Protease K (20 μl) was added into each tube before 55°C water bath incubation for 10 to 20 minutes. The DNA was extracted according to the kit manual, and the concentration was determined by spectrometry. OD_{A260nm}=1 suggested approximately 50 μg/ml of double-stranded DNA. A 0.7% agarose gel was run to determine the integrity and purity of the DNA samples, followed by PCR amplification. The primers were the same as the abovementioned for cfMTDNA detection.

2.4. Statistical analysis

The continuous data are presented as means ± standard deviations. The comparison between groups was performed using a t-test and SPSS 22.0. The categorical data are presented as a percentage and were tested by the Chi-Squared test. The correlation analysis was performed using the Spearman correlation coefficient (rs). At α=0.05, P values <.05 are considered statistically significant.

3. Results

3.1. OEC patients exhibit poorer embryo quality and pregnancy outcomes

The OEC and control groups show similar basic statuses such as age, years of infertility, BMI, and reasons for infertility (Table 1), but OEC patients show significantly reduced numbers of obtained oocytes and decreased induced MI (both number and %), which possibly led to fewer numbers of 2PN (Table 2). The absolute number of embryos undergoing cleavage is fewer in OEC, although the total percentage remains similar between OEC and controls. The OEC group shows significantly reduced numbers and the percentage of qualified embryos, resulting in a lower rate of implantation (Table 2).

3.2. Mitochondrial function is impaired in mGCs from OEC patients compared to controls

To evaluate whether the poor outcome of fertilization is due to differences in mitochondria between OEC and control mGCs, we prepared the oocytes for TEM imaging. The TEM results showed that the number of mitochondria in the mGCs of patients with OEC is significantly lower than that of the control patients (Fig. 1A). Mitochondria of different sizes and shapes and with extended, fractured, or fused cristae or swollen mitochondria with vacuoles, which were considered as abnormal, are significantly more often observed in OEC mGCs (21.1 ± 5.5%) compared with control (9.9 ± 2.2%) (Fig. 1B).

Next, we went to evaluate the levels of ATPase, as a direct marker of mitochondrial function, in the mGCs. The mitochondrial
Table 1
The characteristics of subjects receiving IVF in OEC and control groups.

|                   | OEC            | Control        | T value | P value |
|-------------------|----------------|----------------|---------|---------|
| n                 | 30             | 30             |         |         |
| Age (years)       | 31.1 ± 3.9     | 29.3 ± 3.6     | 1.845   | >.05    |
| Infertility (years)| 3.1 ± 2.1      | 3.1 ± 2.2      | −0.119  | >.05    |
| BMI (kg/m²)       | 22.5 ± 3.0     | 23.2 ± 3.0     | −0.831  | >.05    |
| Primary/secondary infertility | 15/15 | 15/15 | 0.000 | >.05 |
| AFC (n)           | 10.4 ± 4.4     | 11.6 ± 3.5     | −1.736  | >.05    |
| bFSH (mU/ml)      | 5.7 ± 1.6      | 5.1 ± 1.8      | 1.335   | >.05    |
| CA125 (IU/L)      | 34.4 ± 9.0     | 29.9 ± 8.6     | 1.997   | >.05    |
| AMH (ng/ml)       | 2.5 ± 0.9      | 2.6 ± 0.8      | −1.292  | >.05    |
| Gn usage (IU)     | 2642.5 ± 883.7 | 2495.5 ± 729.9 | 0.702  | >.05 |
| Gn duration (d)   | 12.1 ± 2.3     | 11.8 ± 2.4     | 0.703   | >.05 |
| Endometrium thickness (mm) | 12.0 ± 3.2 | 12.4 ± 2.3 | −0.661 | >.05 |
| E2 (pg/ml)        | 2951.6 ± 1428.4 | 3541.9 ± 1397.0 | −1.618 | >.05 |
| P (ng/ml)         | 1.2 ± 1.0      | 0.9 ± 0.7      | 1.165   | >.05    |
| LH (mU/ml)        | 1.1 ± 0.9      | 0.8 ± 0.5      | 1.067   | >.05    |

BMI = body mass index; AFC = antral follicular count; bFSH = basal follicle-stimulating hormone; CA125 = cancer antigen 125; AMH = anti-Müllerian hormone; Gn = gonadotropin; E2 = estradiol; P = progesterone; LH = luteinizing hormone.

ATPase levels are significantly lower in the OEC group than that of the control group (17.06 ± 4.68 vs 22.49 ± 4.90, P < .01) (Fig. 2A). In contrast, the IF1 levels are significantly higher in OEC vs control (1.47 ± 0.46 vs 0.77 ± 0.23, P < .01) (Fig. 2B).

To examine whether the lower levels of ATPase are due to the abnormal assembly of the mitochondrial OXPHOS complex, we performed a western blot assay in OEC and control mGCs. We found that, among 5 components, the protein levels of ATP5A1, MTCO1, and UQCR2 proteins are significantly lower in the OEC group than in the controls (P < .05) (Fig. 3A and B), while there are no significant differences in the levels of NDUFB8 and SDHB proteins (P > .05).

Taken together, these results indicate that in OEC patients, malfunction of the mGC mitochondria is present as increased aberrant mitochondria percentage, lower ATPase, higher IF1, and lower protein levels of critical components of the OXPHOS complex, compared with controls.

3.3. High levels of follicular fluid (FF) cfmtDNA are associated with OEC

Given that the cfmtDNA could serve as a non-invasive biomarker perpetuating mitochondrial dysfunction, we explored the cfmtDNA expression levels in the FF from ICSI-facilitated OEC patients and controls. The OEC patients are quite similar to controls in terms of general characteristics such as age and years of infertility (Table 3). When comparing oocyte and embryo quality, OEC displayed significantly fewer numbers/percentages of implantable or qualified embryos vs. control (Table 4).

The cfmtDNA levels in OEC FF are higher than that of the control group (0.53 ± 0.22 vs 0.42 ± 0.16) (Fig. 4A). We tested
mtDNA expression in mGCs and cGCs, and found that both mtDNA are lower in the OEC than in the control group (1.09 ± 0.30 vs 1.55 ± 0.34, mGC, Fig. 4B, and 1.95 ± 0.62 vs 2.47 ± 0.60, cGC, Fig. 4C) (P < .05 and P < .01).

Further analysis suggested that the cfmtDNA expression in the FF is negatively correlated with the mtDNA levels in the mGCs and cGCs (rs of −0.798 and −0.690, respectively, P < .01), as shown in Figure 4D-E, respectively. This suggests that the levels of FF cfmtDNA could faithfully reflect a decrease of mtDNA in both mGCs and cGCs. Combined with the mitochondrial function data, cfmtDNA might be used as a marker of poor mitochondrial function and implantation failure when considering ART in a clinical setting.

4. Discussion

Due to the limitations of the available studies, the infertility issues of patients with small non-surgical OEC were long-neglected. In this study, we show that small non-surgical OECs affect oocyte

| Table 3 |
| --- |
| The characteristics of subjects receiving IVF in ICSI and control groups. |
|  | OEC | Control | T value | P value |
| --- | --- | --- | --- | --- |
| n  | 30  | 30  |  |  |
| Age (years) | 30.6 ± 3.5 | 30.5 ± 3.3 | 0.283 | >.05 |
| Infertility (years) | 4.2 ± 3.0 | 4.1 ± 1.8 | 0.016 | >.05 |
| BMI (kg/m²) | 23.1 ± 3.0 | 22.9 ± 3.0 | 0.183 | >.05 |
| AFC (n) | 9.4 ± 3.7 | 10.2 ± 4.3 | −0.738 | >.05 |
| bFSH (mIU/mL) | 5.8 ± 1.5 | 5.6 ± 1.7 | 0.196 | >.05 |
| CA125 (IU/L) | 35.3 ± 10.9 | 30.2 ± 9.0 | 1.973 | >.05 |
| AMH (ng/ml) | 2.9 ± 1.0 | 2.5 ± 0.9 | −1.114 | >.05 |
| Gn usage (IU) | 2534.4 ± 607.6 | 2360.4 ± 620.8 | 1.091 | >.05 |
| Gn duration (d) | 11.7 ± 2.6 | 10.5 ± 2.3 | 1.773 | >.05 |
| Endometrium thickness (mm) | 12.2 ± 3.0 | 12.5 ± 2.8 | −0.116 | >.05 |
| E2 (pg/ml) | 3536.5 ± 1528.5 | 3489.6 ± 1469.3 | 0.751 | >.05 |
| P (ng/ml) | 1.1 ± 0.5 | 0.9 ± 0.4 | 0.217 | >.05 |
| LH (mIU/ml) | 1.2 ± 0.6 | 1.0 ± 0.5 | 0.263 | >.05 |

BMI = body mass index; AFC = antral follicular count; bFSH = basal follicle-stimulating hormone; CA125 = cancer antigen 125; AMH = anti-Müllerian hormone; Gn = gonadotropin; E2 = estradiol; P = progesterone; LH = luteinizing hormone.
and embryo quality, resulting in low efficiency of implantation during ART.

The developmental potential of the embryos depends on the capacities of the oocyte, but it is difficult to obtain in-depth information other than the morphology of the oocyte without disrupting its integrity, developmental capacity, and subsequent implantation. The GCs are important components of follicles and play critical roles in female reproduction. Reduced GC quality or enhanced GC apoptosis may affect the secretion of hormones and cytokines or even the oocyte development and the quality of the subsequent embryos.\(^{11}\) cGCs form a functionally connected syncytium with the oocyte and participate in the follicle development through complex mechanisms underlying intercellular gap junctions.\(^{12}\) Therefore, in this study, we mainly focused on investigating GCs role in regulating oocyte and subsequent embryo quality in patients with small non-surgical OEC.

The cGCs surrounding the oocyte is considered one of the best non-invasive way to study and evaluate the quality of the oocyte and the developmental potential of the embryo. The mtDNA copy number in the cGCs is a good predictor of the quality of the embryos, with positive and negative predictive values of 84.4% and 82.1%, respectively.\(^{13}\) Desquiret-Dumas et al\(^{14}\) used real-time fluorescent quantitative PCR to examine average mtDNA levels in the cGCs of 452 OCCs from 62 patients undergoing ICSI and found that the mtDNA levels in the cGCs are correlated with the quality of the embryos. High-quality embryos have significantly more mtDNA copies than the lower quality ones. Taugourdeau et al\(^{15}\) found that the cGCs from the successfully implanted embryos have a higher mtDNA expression, suggesting that a higher level of mtDNA indicates better implantation

| Table 4 | Comparison of oocyte and embryo development indicators in subjects receiving ICSI. |
|---------|--------------------------------------|
|         | OEC | Control | \( \chi^2 \) value | \( P \) value |
| n       | 30  | 30      | –                  | –          |
| Obtained oocytes (n) | \( 8.1 \pm 3.6 \) | \( 9.3 \pm 5.4 \) | –1.010     | >.05 |
| MII (n) | \( 7.3 \pm 3.2 \) | \( 8.5 \pm 4.9 \) | –0.416     | >.05 |
| MII (%) | 90.5 | 92.1    | 0.235              | >.05 |
| 2PN (n) | \( 6.3 \pm 2.7 \) | \( 7.5 \pm 4.5 \) | –1.293     | >.05 |
| 2PN (%) | 85.1 | 87.5    | 0.153              | >.05 |
| Cleavage (n) | \( 6.7 \pm 2.6 \) | \( 7.3 \pm 4.1 \) | –1.196     | >.05 |
| Cleavage (%) | 85.4 | 85.6    | 0.000              | >.05 |
| Implantable (n) | \( 3.9 \pm 1.7^* \) | \( 5.2 \pm 3.2 \) | –2.017     | <.05 |
| Implantable (%) | 61.2 | 70.4    | 3.500              | <.05 |
| Qualified (n) | \( 2.5 \pm 1.4^* \) | \( 3.6 \pm 2.5 \) | –2.201     | <.05 |
| Qualified (%) | 39.4 | 49.5    | 3.849              | <.05 |

**Figure 4.** The cfmtDNA of follicular fluid was negatively correlated with mtDNA expression of mGCs and cGCs.
potential for embryos of the same quality. Thus, mtDNA in the cGCs can be considered as a biomarker of embryo implantation capacity. In this case, we identified reduced mtDNA expression in both mGCs and cGCs from OEC patients against control, confirming that mtDNA expression levels are truly useful in predicting oocyte and embryo quality, as well as following implantation success.

The development of molecular biology in recent years has advanced the research of cfDNA and cfmtDNA in the body fluids. Scalici et al.\(^\text{[16]}\) found that the amount of cfDNA in the FF was associated with the embryonic debris derived from the oocyte. It was also positively correlated with the apoptosis of cGCs and negatively correlated with the capacity of the oocyte to reach the blastocyst stage.\(^\text{[17]}\) Given that mtDNA has the characteristics of short gene length, simple molecular structure, and numerous copy number,\(^\text{[18]}\) cfmtDNA shows the potential as a plasma biomarker of rich information and high sensitivity.\(^\text{[19,20]}\) In the in vitro cultured OCC complex, cfmtDNA is released into the culture media by the GCs in response to mitochondrial dysfunction. Kazuki et al.\(^\text{[21]}\) found that cfmtDNA can be detected in the FF, and mitochondrial malfunction of the OCCs increases the secretion of cfmtDNA from the GCs. In this study, we also observed elevated cfmtDNA expression in the FF from patients with OEC, which is in accordance with previous findings that the presence and enrichment of cfmtDNA in the FF are due to dysfunctional mitochondrial function in GCs.

Since OEC has a strong effect on patients quality of life and pregnancy, most patients with Ems choose to have the OECs removed by surgery once they are diagnosed. Therefore, it is critical for granulosa cell proliferation and differentiation in antral follicles. Biochim Biophys Acta Mol Basis Dis 2017;1863:1539–55.

In summary, our research shows for the first time how small non-surgical OECs affect oocyte quality and subsequent embryos during ART. The underlying mechanism would involve dysfunctional mitochondria in the GCs, exhibiting fewer numbers, aberrant morphology, and impaired function. These affected GCs would further secrete cfmtDNA into the FF, which could serve as a non-invasive biomarker to predict pregnancy outcome for OEC patients.

Author contributions

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