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Effects of Oocyte Cytoplasmic Central Granulation on Embryonic Development, Blastocyst Formation, and Pregnancy Outcome in Technology and Its Mechanism

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ABSTRACT

Abstract: To investigate whether oocyte centrally located cytoplasmic granulation (CLCG) affects embryonic development, blastocyst formation, and pregnancy outcomes in assisted reproductive technology, fifty patients with CLCG in all oocytes were selected as the CLCG group. Then, 150 patients with no abnormal oocyte morphology were randomly recruited as the control group. Both groups underwent laparoscopy, hysterosalpingography (HSG), vaginal ultrasound, and male semen examination. The down-regulation regimen was selected regarding the patient's ovarian reserve. The egg maturation rate, normal fertilization rate, cleavage rate, available embryo rate, and high-quality embryo rate in the CLCG group were greatly inferior to those in the control group, with remarkable differences ($P< 0.05$). CLCG grading of oocytes after 30 cycles in the CLCG group indicated that there were 36 cases in the mild group and 14 cases in the severe group. The egg maturation rate in the mild group was lower than that in the severe group, with a notable difference ($P< 0.05$). The fertilization rate, cleavage rate, available embryo rate, and high-quality embryo rate in the mild group were higher than those in the severe group, with a notable difference ($P< 0.05$). Compared with the control group, there was no considerable difference in the implantation rate, clinical pregnancy rate, and abortion rate between the CLCG group ($P> 0.05$). In summary, oocyte CLCG may affect fertilization, embryonic development, and blastocyst formation, but not pregnancy outcomes

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Introduction

Oocyte cytoplasmic granulation is a common oocyte morphology, and granulation is when the cytoplasm of the whole oocyte is thick, showing a granular state, and it can also show central cytoplasmic granulation (1). The oocyte centrally located cytoplasmic granulation (CLCG) is characterized by a darkened, rough central cytoplasm, and localized granules thickening, which is clearly demarcated from the surrounding normal cytoplasm (2-5). During the natural cycle, the maturation of the oocyte nucleus and cytoplasm is usually synchronized. When ovulation is promoted, the nucleus and cytoplasm may be in different maturation stages, which may be one of the mechanisms of cytoplasmic granulation. In addition, some studies suggested that the patient's age, genetic defects, nutritional conditions, environmental factors, and ovulation induction programs may be related to the formation of CLCG (6,7). The degree of granulation is mainly distinguished by the size of the granulated area and the depth of the damage. Virant-Klun et al. (2018) (8) classified the granulation area as greater than 50% of the cytoplasmic area, and the surface was crater-like, which was classified as severe central granulation. If the granulated area was less than 50%, and there was no clearly distinguishable boundary, it was classified as slightly granulated.

Currently, there is no consensus in the field of reproductive medicine regarding the relationship between CLCG and clinical outcomes of assisted reproductive therapy. Several studies indicated that patients with granulated oocytes have the same fertilization rate, high-quality embryo rate, blastocyst formation rate, implantation rate, clinical pregnancy rate, and survival rate compared with patients with normal oocyte cytoplasm (9-11). Therefore, it was believed that cytoplasmic granulation may be the normal morphology of oocytes and can't be used as a key indicator to assess the quality of oocytes. However, some other studies suggested that the fertilization rate, high-quality embryo rate, and

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blastocyst formation rate of cytoplasmic granulated oocytes were remarkably reduced, suggesting that CLCG may affect its fertilization and early embryo development (12-14). The differences in the results of these studies may be due to factors such as sample size, research methods, and the particularity and complexity of the patients themselves.

In this study, patients with no abnormal oocyte morphology were selected as the control group, and patients with CLCG in all oocytes were selected as the CLCG group. The differences in fertilization, embryonic development, and clinical outcomes between the two groups were compared, to further evaluate the effect of CLCG on oocyte fertilization, embryonic development, and pregnancy outcomes and improve the safety and efficacy of in vitro fertilization-embryo transplantation (IVF-ET) treatment.

Materials and methods

Research objects

A retrospective analysis was conducted on patients who underwent routine IVF-ET at Jingzhou Central Hospital Reproductive Center from April 2019 to December 2021. Fifty patients with CLCG of all oocytes were selected as the CLCG group. 150 patients with normal oocyte morphology were randomly selected as the control group. Both groups underwent laparoscopy, hysterosalpingography (HSG), vaginal B ultrasound, reproductive endocrine examination, and male semen examination. Confirmed causes of infertility included tubal pelvic factors, endometriosis, male oligospermia, and polycystic ovary syndrome (PCOS). Patients whose eggs were frozen due to egg donation or sperm collection failure were excluded.

Oocyte evaluation and embryo grading

Hyaluronidase degranulation was performed 1 h after egg retrieval (D0), and IVF-ET was performed 4-6 h later. The morphological characteristics of oocytes were recorded under an inverted microscope (Nikon, × 200 magnification) equipped with a Hoffman modulation phase contrast system and a thermostatic hot stage. Figure 1 showed human oocytes at different maturation stages. Germinal vesicles can be seen in the cytoplasm of immature oocytes (GV stage). The germinal vesicles in the cytoplasm of oocytes in the middle mature stage (MI stage) disappeared, but the first polar body was not seen in the periovular space. In mature oocytes (MII stage), germinal vesicles in the cytoplasm disappeared, and the first polar body was seen in the periovular space.

The morphology of the cleavage-stage embryos was assessed at 42-44 h after fertilization (D2) and 66-68 h after fertilization (D3), and the number of embryos, fragmentation ratio, and cell size was observed. Early embryos were classified into four grades regarding morphological features. Embryos of grades I and II were high-quality embryos, and embryos of grades I to III were available embryos and can be transferred or frozen. Figure 2 showed the grading of cleavage stage embryos. Grade I: the embryo developed at a normal rate, the blastomere was uniform in size, and the fragment content was within 5%. Grade II: the embryo developed at a normal rate, the blastomere size was slightly uneven, and the fragment content was 6%-20%. Grade III: the embryonic development speed was generally normal, the blastomere size was uneven and obvious, and the fragment content was 21%-50%. Grade IV: the embryo developed abnormally, the blastomere size was very uneven, and the fragment content was more than 50%.

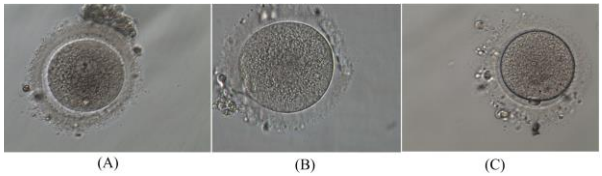

Figure 1. Human oocytes at different maturation stages. A: immature oocyte (GV stage); B: intermediate mature oocyte (MI stage); C: mature oocyte (MII stage).

Grading evaluation criteria for CLCG of oocytes

Oocyte CLCG was graded into mild and severe regarding the degree of central cytoplasmic darkening, roughness, and localized granule thickening (15). Mild meant that the CLCG area had a significant boundary with the surrounding normal cytoplasm, the area of the CLCG area was less than 1/2 of the cytoplasm area, the degree of local particle thickening was light, and the degree of blackening is light.

Severe referred to the obvious boundary between the CLCG area and the surrounding normal cytoplasm, the area of the CLCG area was larger than 1/2 of the cytoplasm area, the local granules were thicker, and the central cytoplasm was obviously darkened. Figure 3 showed the graded assessment of oocyte CLCG.

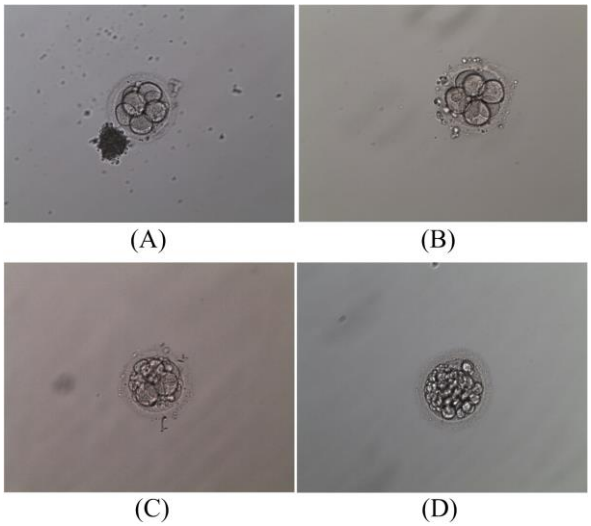

Figure 2. Grading of cleavage stage embryos. A (grade I): the embryo developed at a normal rate, and the fragment content was within 5%; B (grade II): the embryo developed at a normal rate, and the fragment content was 6%-20%; C (grade III): the embryonic development speed was generally normal, and the fragment content was 21%-50%; D (grade IV): the embryo developed abnormally, and the fragment content was more than 50%.

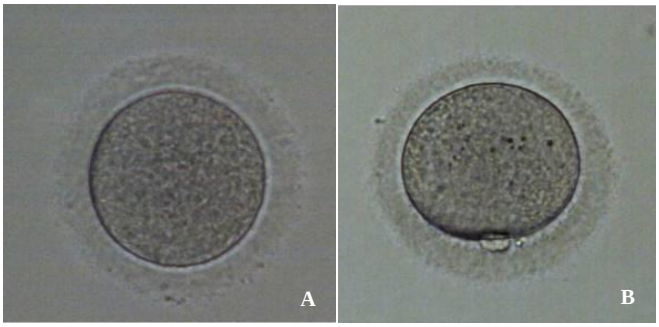

Figure 3. Grading assessment of oocyte CLCG. A: mild oocyte CLCG; B: severe oocyte CLCG**.**

Controlled superovulation program

The down-regulation program was selected regarding the patient's ovarian reserve, that was, the number of antral follicles on the third day of menstruation, basal endocrine, and age.

A short-acting gonadotropin-releasing hormone agonist (GnRH-a, Beijing Lebo Biotechnology Co., Ltd., China) was used in the mid-luteal phase of the cycle before the initiation of the long regimen. One week later, the transvaginal B-ultrasound was performed for reexamination. If there was a physiological cyst, puncture and drainage should be performed immediately under the guidance of vaginal B-ultrasound. Gonadotropin (Gn, Serono, Switzerland) was injected from the 3rd to 6th day of menstruation regarding the pituitary downregulation and ovarian reserve. Vaginal ultrasound was performed to detect the growth of follicles, and the Gn dose was adjusted at any time. When the diameter of more than one follicle was ≥18mm, 10000IU of HCG was injected, and the eggs were retrieved under the guidance of vaginal ultrasound after 36-38 hours.

Short regimen: GnRH-a was utilized on day 2 of menstruation, and Gn was injected on day 3.

Antagonist regimen: Gn was injected on the third day of menstruation, and when the largest follicle diameter reached 12-14 mm, a gonadotropin-releasing hormone antagonist (GnRH-ant, Ferring Pharmaceuticals Co., Ltd., China) was added.

Ultra-long regimen: long-acting GnRH-a was injected on the 2nd day of the menstrual cycle or mid-luteal phase, 28 days/1 times, with a total of 2 to 3 times; on the 29th day of the last injection, B-ultrasound was performed to monitor the situation to determine the Gn injection time.

Blastocyst scoring

The blastocysts were scored according to the Gardner scale (16), and the blastocysts were scored on a scale of 1-6 according to the expansion and hatching status of the blastocysts. The blastocysts of grades 3-6 were assessed according to the inner cell mass (ICM) and trophectoderm (TE) and were classified into grades A, B, and C. Blastocysts with grade B or better ICM and TE were selected for vitrification or transfer as available blastocysts.

Observation indicators

The blastocyst rate, implantation rate, clinical pregnancy rate, live birth rate, miscarriage rate, average number of eggs retrieved, egg maturation rate, normal fertilization rate, cleavage rate, available embryo rate, high-quality embryo rate, clinical pregnancy rate, implantation rate, and miscarriage rate were calculated in the following equations.

[1]

$$Available\ blastocyst\ rate = \frac{number\ of\ frozen\ blastocysts}{number\ of\ embryos\ continuously\ cultured}$$

[2]

$$Implantation\ rate = \frac{number\ of\ gestational\ sac}{number\ of\ embryos\ transferred}$$

[3]

$$Clinical\ pregnancy\ rate = \frac{number\ of\ clinical\ pregnancy\ cycles}{number\ of\ embryo\ transfer\ cycles}$$

[4]

$$Live\ birth\ rate = \frac{number\ of\ delivery\ cycles\ of\ live\ birth}{number\ of\ embryo\ transfer\ cycles}$$

[5]

$$Abortion\ rate = \frac{abortion\ cycles}{clinical\ pregnancy\ cycles}$$

[6]

$$Average\ number\ of\ eggs\ retrieved = \frac{total\ number\ of\ eggs\ retrieved}{number\ of\ egg\ retrieval\ cycles} \times 100\%$$

[7]

$$Maturity\ rate\ of\ eggs = \frac{number\ of\ MII\ eggs}{total\ number\ of\ eggs\ obtained} \times 100\%$$

[8]

$$Normal\ fertilization\ rate = \frac{2PN}{MII\ eggs} \times 100\%$$

[9]

$$Cleavage\ rate = \frac{number\ of\ cleavage\ embryos}{2PN\ number} \times 100\%$$

[10]

$$Available\ embryo\ rate = \frac{I + II + III\ embryos}{number\ of\ eggs\ obtained} \times 100\%$$

[11]

$$High-quality\ embryo\ rate = \frac{I + II\ embryos}{number\ of\ eggs\ obtained} \times 100\%$$

[12]

$$Clinical\ pregnancy\ rate = \frac{number\ of\ clinical\ pregnancies}{number\ of\ transplant\ cycles} \times 100\%$$

[13]

$$Implantation\ rate = \frac{number\ of\ intrauterine\ gestational\ sacs}{number\ of\ transplanted\ embryos} \times 100\%$$

[14]

$$Abortion\ rate = \frac{number\ of\ abortions}{number\ of\ clinical\ pregnancies} \times 100\%$$

Statistical methods

Excel was utilized to establish a database for all data in this study, and SPSS 19.0 version statistical software was employed for data analysis. Mean ± standard deviation ($\bar{x}\pm s$) was used for measurement data, χ^2 test was used for counting data, and percentage (%) was used for counting data. The difference was statistically significant at $P< 0.05$.

Results

Comparison of general conditions of the two groups of patients

In the CLCG group, there were 31 cases of primary infertility and 19 cases of secondary infertility, while there were 115 cases of primary infertility and 35 cases of secondary infertility in the control group. There were no considerable differences in the mean age, years of infertility, basal follicle-stimulating hormone (FSH), and endometrial thickness between the two groups ($P> 0.05$). Table 1 showed that the mean body mass index (BMI) and mean Gn usage days were statistically considerable ($P< 0.05$).

Table 1. Comparison of the basic conditions of the two groups of patients ($\bar{x}\pm s$)

	Case number (n)	Average age (years old)	Infertility years (years)	BMI (kg/m^2)	Gn intake duration (d)	Basal FSH (mIU/m)	Endometrial thickness (mm)	Number of follicles > 14mm
Control group	150	31.65±4.81	6.61±3.27	20.62±2.67	10.27±2.61	7.28±2.65	9.58±1.84	10.25±4.81
CLCGgroup	50	30.28±5.26	5.27±3.61	22.65±2.38*	11.45±2.81*	7.15±2.31	9.47±1.62	10.83±4.28
P		0.261	0.872	0.002	0.000	0.853	0.418	0.526

Note: * indicated that compared with the control group, the difference was statistically considerable, $P< 0.05$.

Comparison of fertilization, cleavage, and embryonic development between two groups

In the CLCG group, 356 eggs were retrieved, including 325 eggs in the meiotic metaphase (MII stage), and the egg maturation rate was 91.29%. In the control group, 648 eggs were retrieved, including 617 MII eggs, and the egg maturity rate was 95.21%. The egg maturity rate, normal fertilization rate, cleavage rate, available embryo rate, and high-quality embryo

rate in the CLCG group were greatly inferior to those in the control group ($P< 0.05$, Figure 4).

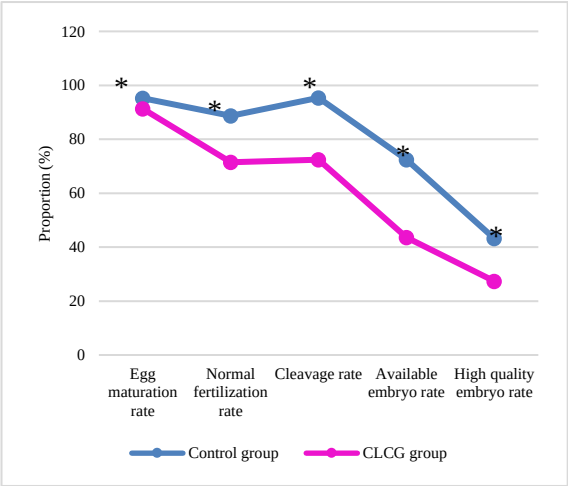

Figure 4. Comparison of fertilization, cleavage, and embryonic development between the two groups.
Note: * indicated that compared with the CLCG group, the difference was statistically considerable, $P< 0.05$.

The effect of oocyte CLCG degree on fertilization, cleavage, and embryonic development

CLCG grading of oocytes after 30 cycles in the CLCG group suggested that there were 36 cases in the mild group and 14 cases in the severe group. The egg maturation rate of the mild group was greatly inferior to that of the severe group, with a notable difference ($P< 0.05$). Figure 5 showed that the fertilization rate, cleavage rate, available embryo rate, and high-quality embryo rate in the mild group were remarkably higher than those in the severe group ($P< 0.05$).

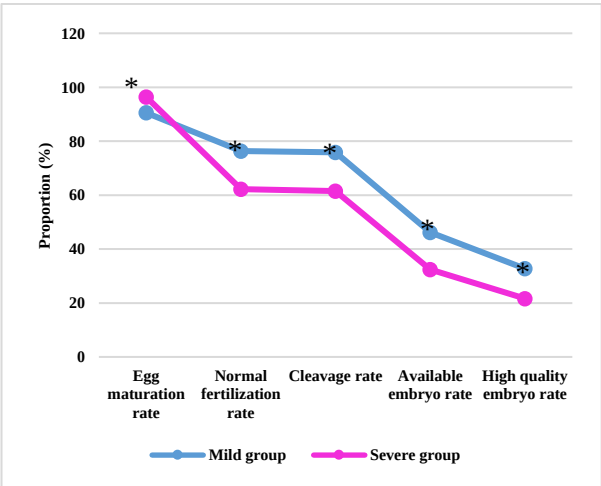

Figure 5. Comparison of fertilization, cleavage, and embryo development in mild and severe groups.
Note: * indicated that compared with the severe group, the difference was statistically considerable, $P< 0.05$.

Comparison of pregnancy outcomes between the two groups

The CLCG group was transplanted for 26 cycles (3 cycles without embryo transfer after thawing), and the control group was transplanted for 185 cycles (6 cycles without embryo transfer after thawing). The average number of embryos transferred between the CLCG group and the control group was 2.56±0.54 VS 2.15±0.52, and there was no statistical difference ($P> 0.05$). Compared with the control group, the implantation rate, clinical pregnancy rate, and abortion rate of the CLCG group had no statistical significance ($P> 0.05$, Figure 6).

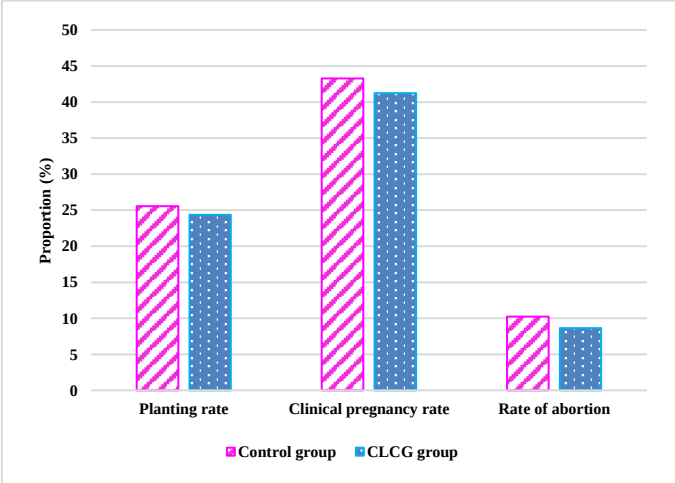

Figure 6. Comparison of pregnancy outcomes between the two groups

Analysis of clinical factors of oocyte CLCG

In the control group, there were 118 cases of the long regimen (accounting for 78.66% of the total cycle) and 32 cases of the short regimen (accounting for 21.33% of the total cycle). In the CLCG group, there were 38 cases of the long regimen (accounting for 76% of the total cycle) and 12 cases of the short regimen (accounting for 24% of the total cycle). The average Gn usage days and average Gn total in the CLCG group were remarkably higher than those in the control group, with substantial differences ($P< 0.05$).

According to the comparison of the controlled superovulation regimen, the average Gn usage days and the average Gn total use in the CLCG group were higher than those in the control group, with substantial differences ($P< 0.05$). The average Gn usage days of the short regimen in the CLCG group was higher than

that in the control group, with a notable difference (P< 0.05). Figure 7 showed that there was no statistical difference between the total amount of Gn used and the control group (P> 0.05).

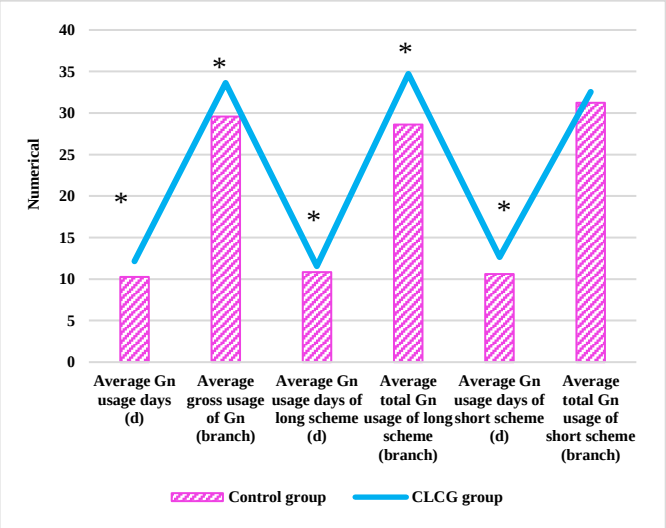

Figure 7. Comparison of Gn usage in two groups of patients with different regimens. Note: * indicated that compared with the control group, the difference was statistically considerable, P< 0.05.

Discussion

Oocyte CLCG is one of the common oocyte morphological abnormalities, and the cause and mechanism of its formation are still unclear (17-20). Studies revealed that in the natural cycle, the maturation of the oocyte nucleus and cytoplasm often occurs synchronously, but the development of the two may be asynchronous in the ovulation cycle (21-23). During nuclear maturation, the cytoplasm may be in different maturation stages, possibly resulting in abnormal cytoplasmic morphology (24,25). In this study, four special patients were found. In the first cycle, three patients had no oocyte CLCG, but in the second cycle, all oocytes showed CLCG. In the fourth patient, all oocytes showed CLCG in the first cycle, but no CLCG was found in the second cycle. Two patients were treated with a short regimen and an antagonist regimen, respectively, the third patient was treated with a long regimen twice, and the fourth patient was treated with a short regimen and an ultra-long regimen. The amount of Gn used in each cycle of these four patients was different, which indicated that the down-regulation regimen or other relevant links of assisted reproductive therapy were closely related to the formation of CLCG in oocytes.

At present, there is no unified conclusion on whether oocyte CLCG affects fertilization, early embryonic development, and pregnancy (26). Brouillet et al. (2020) (27) studied more than 500 oocyte CLCG and found that there was no considerable difference in fertilization rate, embryonic development potential, and successful pregnancy outcome between oocyte CLCG and normal cytoplasmic patients. Therefore, it was believed that oocyte CLCG was not an indicator of adverse outcomes in IVF, but a normal oocyte morphology. Wald et al. (2019) (28) reported that 1,299 oocytes with cytoplasmic CLCG were studied. It was found that compared with oocytes with normal cytoplasm, the fertilization rate, high-quality embryo rate, and available embryo rate of cytoplasmic CLCG were remarkably lower. Furthermore, the blastocyst formation rate of oocyte CLCG was remarkably reduced, which further indicated that oocyte CLCG might affect oocyte fertilization and embryonic development. In this study, the sample size was increased and 325 oocytes with cytoplasmic CLCG were studied in 150 patients with cytoplasmic CLCG. The results showed that compared with oocytes with normal cytoplasm, the fertilization rate and available embryo rate were remarkably lower in patients with cytoplasmic CLCG. The rate of available blastocysts in the CLCG group was greatly inferior to that in the normal cytoplasmic group, indicating that the morphology of oocyte CLCG may affect oocyte fertilization, embryonic development, and the formation of available blastocysts.

Studies reported that oocyte CLCG can reduce the embryo implantation rate and clinical pregnancy rate of patients. However, Nagyová et al. (2021) (29) reported that compared with patients with normal cytoplasm, the clinical pregnancy rate of oocyte CLCG patients was not remarkably different, but the miscarriage rate was remarkably higher. The results of this study showed that the embryo implantation rate, clinical pregnancy rate, and live birth rate of the CLCG group were lower than those of the normal cytoplasmic morphology group, but the difference was not statistically considerable (P> 0.05). In the later stage, the sample size of oocyte CLCG pregnant patients needs to be increased for further research.

Excessive use of Gn may interfere with oocyte developmental regulation and synchronization. If the

nuclear and cytoplasmic maturation is not synchronized, the maturation rate of the oocyte will be greatly reduced, resulting in an increased incidence of chromosomal abnormalities. It indicates that the basal FSH value, ovarian dysfunction, Gn usage days, and dosage of assisted reproductive therapy may be related to the formation of oocyte CLCG. There were no considerable differences in age, basal FSH value, and average infertility years between the two groups of patients selected in this study. It showed that the unreasonable down-regulation program may lead to the long use of Gn and the increase of the total dose, which may lead to the formation of oocyte CLCG.

Conclusion

In this research, 50 patients with CLCG of all oocytes were selected as the CLCG group, and patients with completely normal oocytes were selected as the control group. After elimination of the interference of other morphologically abnormal oocytes on the outcome, the fertilization, early embryonic development, blastocyst formation, and pregnancy outcome of oocyte CLCG were systematically analyzed. The results confirmed that oocyte CLCG may affect fertilization, embryonic development, and blastocyst formation, but not pregnancy outcomes. However, there are certain shortcomings in this study. Due to the limitation of study duration, the sample size is too small. Therefore, it is necessary to increase the sample size of oocyte CLCG in pregnant patients in the later stage to further verify the conclusions of this study. In conclusion, it is believed that this research can provide some ideas and experimental support for increasing the success rate of IVF-ET.

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