

The Activation of Residual Dormant Follicles by Human Chorionic Gonadotropin (HCG) *in Vivo*: a Novel Treatment for Premature Ovarian Insufficiency

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Abstract

Background

With the influence of factors such as ovarian surgery, high-dose radiotherapy and chemotherapy, environmental degradation, and bad living habits, the occurrence of premature ovarian insufficiency (POI) is getting younger and younger, and many young women's ovaries have entered the aging stage earlier. While many studies have investigated the patients with POI, which is still a challenge in reproductive medicine as the treatments available now are not ideal. POI patients have varying amounts of residual dormant follicles in the ovaries. Therefore, it is critical to further our understanding of primordial follicle activation in order to treat. This study aimed to investigate the activation of residual follicles in POI patients with injection of HCG, whether they could obtain embryos and become pregnant.

Methods

Four patients with POI were pretreated with dehydroepiandrosterone, Coenzyme Q10, estrogen and medroxyprogesterone. The prescribed amounts of estrogen and medroxyprogesterone were adjusted to maintain the level of FSH at ≤ 15 mIU/ml and the level of LH ≤ 10 mIU/ml. When the treatments failed to induce the appearance of follicles after 3 months, the patients received treatment with 10000 IU of HCG.

Results

The residual dormant follicles in POI patients can be activated using our approach to obtain embryos and conceive by injection of HCG.

Conclusions

POI patients may conceive their own genetic children by activating dormant follicles *in vivo*. These findings may represent a new simple and feasible solution for the treatment of patients with POI to conceive their own genetic children.

Introduction

With the influence of factors such as ovarian surgery, high-dose radiotherapy and chemotherapy, environmental degradation, and bad living habits, the occurrence of POI is getting younger and younger, and many young women's ovaries have entered the aging stage earlier. POI is a disease in which the ovarian follicles rapidly decay resulting in low numbers of residual follicles in women before the age of 40 years (1). Indeed, in these patients, the progressive reduction in ovarian reserve due to early loss of primordial follicles eventually leads to POI. POI is associated with hypoestrogenism and the loss of residual follicles, both of which lead to menstrual abnormalities, pregnancy failures, and decreased health-related quality of life. The current diagnostic criteria for POI from the European Society of Human Reproduction and Embryology (ESHRE) diagnostic include amenorrhoea or oligomenorrhoea for at least four months and increased follicle-stimulating hormone (FSH) levels > 25 IU/l measured on two occasions (with a four-week interval) (2).

POI patients have varying amounts of residual dormant follicles in the ovaries. When the number of residual follicles declines below a specific threshold (< 1000 follicles), they can no longer be activated and follicle development becomes arrested leading to anovulation and amenorrhoea (3). Currently, there are a lack of effective treatments for infertility that can increase the rate of conception in POI patients except for oocyte donation in which patients do not

conceive their own genetic children. Reproductive medicine continues to develop potential therapeutics to allow POI patients to conceive with their own oocytes using approaches to reactivate the ovaries by rescuing dormant follicles or generating functional oocytes *in vitro*.

The *in vitro* activation (IVA) of follicles followed by autologous transplantation has enabled POI patients to successfully give birth(4,5) but follicular growth was not observed until one year after transplantation of the ovaries. Recent studies have demonstrated ovarian rejuvenation following the injection of platelet-rich plasma (PRP) or stem cells into artificial gametes and ovaries, using ovarian transplantation, and also with mitochondrial replacement therapy (6). Whilst these infertility treatments may improve the probability of successful birth, they are expensive and the precise mechanisms underpinning their effects remain to be fully understood. Currently, no studies have reported on the activation of residual dormant follicles *in vivo*.

The injection of human chorionic gonadotropin (HCG) on trigger day induces a surge in luteinizing hormone (LH) to promote the maturation of oocytes and ovulation. The administration of HCG might trigger the resumption of meiosis and nuclear maturation of immature oocytes.(7). Introducing HCG into mammalian and human eggs *in vitro* has been shown to promote oocyte maturation and improve developmental potential (7-10). These studies have demonstrated the important role of HCG in the development and maturation of follicles. Recently, we developed a simple and feasible new infertility treatment that has enabled four POI patients to obtain viable embryos using HCG-activated residual dormant follicles *in vivo*. One patient subsequently gave birth to a healthy baby boy, and another patient became pregnant. In this review, we summarize the potential use of HCG as a new infertility treatment in POI patients.

Patients And Methods

Four POI patients all presented with amenorrhoea or oligomenorrhoea for at least four months and embarked on intermittent hormone therapy for several years, they were exploring the possibility of pursuing a pregnancy through IVF treatment. The follicles were monitored continuously for 1 year and no follicles were visualized before they were referred to our center. During the first appointment, the detailed reproductive examinations were performed, including the ultrasound reports(Fig 1), the hormone and anti-mullerian hormone(AMH) profiles assessment(Table 1-4). Karyotyping all showed 46 XX chromosomal in four patients. In our center, they were pretreated with dehydroepiandrosterone (DHEA), Coenzyme Q10, estrogen (Progynova, Bayer) and medroxyprogesterone (Duphaston, Abbott Biologicals B.V) for 3 months, and the ultrasound monitored follicles continuously every 1-2 weeks. The prescribed amounts of estrogen and medroxyprogesterone were adjusted to maintain the level of FSH at ≤ 15 mIU/ml and the level of LH ≤ 10 mIU/ml. When the treatments failed to induce the appearance of follicles for 30 days, the cycle was cancelled, the medication was stopped and the patients proceeded to the next cycle of medication after menstruation.

In this study where estrogen/medroxyprogesterone treatment lasted for 3 months and no follicles were observed in the bilateral ovaries, then the patient received HCG 10000 IU (Chorionic Gonadotrophin, Livzon) and continued to receive estrogen/progesterone to reduce the levels of endogenous gonadotropin. They also received weekly or bi-weekly ultrasound examinations. When the diameter of the dominant follicle was ≥ 18 mm and the Estradiol(E_2) level was ≥ 150 pg/ml, the egg retrieval was activated with 250IU of rhCG (Recombinant Human Choriogonadotropin alfa solution, Merck Serono S.p.A) and 0.1 ml of Tritorelin Acetate (Ipsen Pharma Biotech). The oocytes were trans-vaginally collected under ultrasound guidance at 36h after triggering and cultured *in vitro* for 3 days. All embryos were vitrified for cryopreservation.

Before preparing for frozen embryos transplantation (FET), all patients underwent hysteroscopy and ensured there were no adhesions or polyps in the uterine cavity. In the artificial cycle protocol, the endometrium was prepared with estradiol valerate (2 mg three times daily, Estrofem, Novo Nordisk, Turkey) beginning on day 3 of menses. If endometrial thickness was ≥ 7 mm and the serum E_2 level was >150 pg/mL, vaginal progesterone gel (90 mg/day, Progestan, Serono Rome, Italy) was started, the embryos were thawed after using vaginal progesterone gel for 3 days. Two patients transferred D3 embryos, and the other two patients embryos continued culture for 2 days to obtain blastocysts for transplantation. 90 mg of vaginal progesterone gel was used once a day for luteum supplementation after transplantation.

The first patient, aged 31, was diagnosed as being prematurely menopausal at the age of 26. Laboratory findings in Jun 2016 reported FSH levels of 50.23 mIU/ml, LH levels of 13.39 mIU/ml and E_2 levels of 40.09 pg/ml. In Aug 2016 laboratory findings reported FSH levels of 45.75 mIU/ml, LH levels of 12.87 mIU/ml and E_2 levels of 7.00 pg/ml. At 38 days after HCG injection, a 9.5 mm follicle was observed in the right ovary (Table 1). The patient did not receive an LH antagonist as the LH levels were low when the follicle from 13.5 mm to 18.0 mm. Exogenous gonadotropin was not given. One metaphase II oocyte was obtained under ultrasound guidance.

The second patient was 31 years old who presented with oligomenorrhoea since 23. At the age of 26, she presented with hot flushes, insomnia, dyspareunia, and emotional lability. Laboratory findings in April 2017 showed FSH levels of 85.13 mIU/ml, LH levels of 34.25 mIU/ml and E_2 levels of 18 pg/ml. In Aug 2017 laboratory findings reported FSH levels of 95.65 mIU/ml, LH levels of 29.75 mIU/ml and E_2 levels of 12 pg/ml. At 43 days after HCG injection, a 18.5 mm follicle presented in the left ovary (Table 2) and a metaphase II oocyte was obtained.

The third patient was 38 years old who presented with oligomenorrhoea since 30. The laboratory findings in Apr 2013 reported FSH levels of 32.5 mIU/ml, LH levels of 13.5 mIU/ml and E_2 levels of 17.0 pg/ml. In Aug 2013 laboratory findings reported FSH levels of 54.52 mIU/ml, LH levels of 23.52 mIU/ml and E_2 levels of 4.0 pg/ml. The patient presented with hot flashes and insomnia since 2018. At 29 days after the HCG injection, a 14.0 mm follicle was observed in the left ovary (Table 3). The patient received an LH antagonist (Cetrorelix Acetate, Merck Serono S. pA) for 4 days as the LH levels were high when the follicle reached a diameter of 14.0 mm. Exogenous gonadotropin was not given. One metaphase II oocyte was obtained under ultrasound guidance.

The fourth patient was a 34 year old who presented with oligomenorrhoea since the age of 29. The laboratory findings in Oct 2016 reported FSH levels of 66.13 mIU/ml, LH levels of 34.26 mIU/ml and E_2 levels of 19.61 pg/ml. In Feb 2017 laboratory findings reported FSH levels of 76.15 mIU/ml, LH levels of 23.7 mIU/ml and E_2 levels of 38.89 pg/ml. The patient presented with vaginal dryness, difficulties during intercourse, and developed symptoms of hot flashes, palpitation, insomnia and depression since 2019. At 29 days after the HCG injection, a 16.0 mm follicle was observed in the left ovary (Table 4). The patient received Cetrorelix Acetate for 4 days as the LH levels were high when the follicle reached a diameter of 16.0 mm. Exogenous gonadotropin was not given. One metaphase II oocyte was obtained under ultrasound guidance.

Results

For the first patient, an 8C1 embryo was preserved by vitrification (Fig 2). The patient continued estrogen/medroxyprogesterone treatment for 4 months after oocyte collection and B-ultrasound did not detect any antral follicles. She received a 3BB blastocyst transplantation. At 10 days after implantation, the level of β -HCG was 215.21 mIU/ml, and at 13 days after implantation, the level of β -HCG was 820 mIU/ml. At 24 days after implantation,

B-ultrasound showed a gemmule and the vitellus bag had a primitive heart tube pulse. At 71 days after implantation, early pregnancy screening reported that the fetal crown-rump length (CRL) was 6.49 cm and the Nuchal Translucency(NT) was 0.9 cm. Non-invasive Prenatal Testing(NIPT) showed that trisomy of chromosomes 21,18 and 13 were all low-risk (Fig 3).

An 8c1 embryo was vitrified for the second patient (Fig 2). The patient continued estrogen/medroxyprogesterone treatment after oocyte collection and no antral follicles were observed in the ovaries at 87 days after HCG injection. A second injection of HCG10000 IU was required. At 7 months after the injection there were no antral follicles in both ovaries. The couple transferred a 3BB blastocyst. At 10 days after the implantation, the level of β -HCG was 387.8 mIU/ml. After 3 days, the β -HCG level was 2077.00 mIU/ml. At 31 days after the implantation, B-ultrasound detected a gemmule and the vitellus bag had a primitive heart tube pulse. 77 days after implantation, early pregnancy screening showed that the fetal CRL was 7.2 cm and the NT was 0.12 cm. NIPT showed that trisomy of chromosomes 21,18 and 13 were all low-risk (Fig 4). On April 21st, 2021, the patient delivered a 3.65 kg healthy baby boy by cesarean section at term.

For the third patient, an 8C1 embryo was finally obtained and persevered by vitrification (Fig 2). Two months after oocyte collection, the 8C1 embryo was transferred. At 13 d after the implantation, the level of β -HCG was 161.5 mIU/ml, and at 16 d after implantation, the level of β -HCG was 459.84 mIU/ml. At 22 d after implantation, the level of β -HCG was 1362.49 mIU/ml. The patient experienced vaginal bleeding and eventually had a miscarriage. The B-ultrasound examination did not detect any antral follicle for 3 months after the miscarriage. Then she received a 10000 U HCG injection again, with re-injection after 62 days. A 15.5 mm follicle was observed in the left ovary, the level of E_2 was 113pg/ml, the level of LH was 14.47 mIU/ml, and the level of FSH was 16.99 mIU/ml. The patient did not receive Cetorelix Acetate for two days and the follicle ovulated.

For the fourth patient, a 7C1 embryo was finally obtained and preserved by vitrification(Fig 2). She continued to receive estrogen/medroxyprogesterone treatment after oocyte collection. A 5.5 mm antral follicle in the left ovary was observed at 79 d after HCG injection. At this time level of E_2 was 30 pg/ml, FSH was 7.39 mIU/ml, and LH was 6.27 mIU/ml. 5 days later, the diameter of the antral follicle did not increase and the E_2 level was 31pg/ml, FSH was 7.54 mIU/ml, and LH was 5.84 mIU/ml. The patient received injections of 225 IU rFSH (Recombinant Human Follitropin Alfa Solution, Merck Serono S. pA) for 8 days, however, the antral follicle disappeared. After 2 months, the patient transferred the 7C1 embryo, but was not pregnant.

Discussion

Different drug interventions have been recommended to improve ovarian function in POI patients. Androgen potentiates the expression of FSH receptor and supports preantral follicle development in mice(11). DHEA promotes follicular development and granulosa cell proliferation by increasing the androgen levels in the ovaries and it may have a role as a treatment for premature ovarian insufficiency(12). Coenzyme Q10 significantly increases the number of antral follicles in aged mice and effectively improves ovarian reserves and mitochondrial function (13). DHEA combined with Coenzyme Q10 can significantly increase the number of antral follicles and improve the ovarian response (14).

The occurrence (although rare) of a spontaneous pregnancy during cyclic estrogen and progestin therapy(15). It is generally accepted that FSH > 40 IU/L is associated with sterility and that induction of ovulation in POI patients is ineffective(16). It was hypothesized that the hypergonadotropic condition alone may reduce ovarian responsiveness. Estrogen-induced decrease in serum FSH improves the responsiveness of remnant ovarian follicles by the induction

of an upregulation of FSH receptors in granulosa cells(17). First in 1996, Taylor showed a spontaneous ovulation in 46% of POI patients treated with estrogens (18). The four patients in this study did not have any antral follicles with 1 year of continue estrogen and progesterone therapy and the serum levels of FSH remained at a high level at the first visit to our center. In POI patients, Tartagni et al. reported that ovulation is only possible when the level of FSH is \geq 15 IU/L(19). Sara et al. confirmed that ovulation induction was satisfactory only in women whose FSH levels at the beginning of COH were lower than 15 IU/L(17). Reducing the levels of FSH whilst reducing LH to within the normal range can increase the ovulation rate of POI patients (20). Vaishali et al. reported that the normal range of LH in POI patients is 3-14 IU/L (21). High progesterone levels can avoid the appearance of endogenous LH peak (19). Also, Yanping et al. reported that the use of medroxyprogesterone during ovulation induction does not increase the frequency of birth defects in newborns (22).

In this study, after estrogen and medroxyprogesterone treatment for 3 months, no follicles were observed in the bilateral ovaries. The patients did not want to wait for other treatments and so received HCG 10000 U. Follicles were detected in the four patients at 29, 38, 43, 62, 79 days after HCG treatment. Under normal conditions, it takes 150 days for human primordial follicles to develop into primary follicles, 120 days for primary follicles to develop into secondary follicles, and 85 days for secondary follicles to develop into mature follicular phase follicles (it takes 60 days to develop from secondary follicles into antral follicles 2 mm in diameter, and 25 days from antral follicles to develop into mature follicles) (23). We hypothesize that HCG may activate and accelerate the development of immature follicles from dormant follicles which would otherwise remain fixed at the stage of secondary follicle development.

The growth of antral follicles is mainly regulated by ovarian regulatory factors and hormones. Epidermal growth factor (EGF) is a key growth factor in the regulation of follicular development and maturation. Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are derived from oocytes which play important roles in the development of preantral follicles into antral follicles (24). GDF-9 is expressed in 96% of human secondary follicles and promotes the expansion of the mouse cumulus (25,26) and the growth of human and goat preantral follicles *in vitro* (27,28). BMP-15 stimulates the proliferation of granulosa cells (29), regulates the sensitivity of HGC to FSH and increases oocyte development (30,31).

The response of follicles to HCG stimulation is mediated by EGF-like growth factors produced by granulosa cells. HCG binds to the LH receptors in the parietal granulosa cells to rapidly induce the expression of EGF expression and activate EGF receptors in granulosa and cumulus cells through autocrine and paracrine pathways. These changes then further activate the key factors that promote cell proliferation and differentiation through the mitogen-activated protein kinase (MAPK)(32).

MAPK has been shown to stimulate granulosa cell proliferation and induce cumulus expansion and oocytes maturation (33). The effect of HCG on follicles also requires the involvement of oocyte-derived paracrine factors. The LH surge after HCG injection can increase the secretion of GDF-9 and BMP-15 to promote cumulus expansion and oocytes maturation (34). The surge in GDF-9 can also significantly enhance FSH-induced preantral follicles growth *in vitro* (27). Moreover, according to the two-cell and two-gonadotropin theory, HCG stimulates follicular membrane cells to produce sex hormones. Androgens diffuse to adjacent granulosa cells and are aromatized to estrogen. Aromatization is accelerated with the estrogen microenvironment which depends on FSH stimulation of the granulosa cells. Guelkli et al. found that 10000 IU of HCG increases the maturation rates of oocytes *in vitro* but higher doses of HCG did not improve the maturation rate of oocytes (35).

Conclusions

IVA follicles, ovarian injection and transplantation, and mitochondrial replacement therapy are expensive forms of treatment for POI patients and their clinical applications require further validation. Currently, there are no convenient, effective and economical treatments that can improve ART outcomes in POI patients. In this study, we report a breakthrough infertility treatment for POI patients that can activate dormant follicles *in vivo*, and maybe allow them to conceive their own genetic children. However, the mechanism of HCG activation of residual dormant follicles remains unclear and requires mechanistic investigations.

Abbreviations

Human chorionic gonadotropin (HCG)

Premature ovarian insufficiency(POI)

European Society of Human Reproduction and Embryology (ESHRE)

Follicle-stimulating hormone (FSH)

In vitro activation (IVA)

Platelet-rich plasma (PRP)

Luteinizing hormone (LH)

Anti-mullerian hormone(AMH)

Dehydroepiandrosterone (DHEA)

Estradiol(E₂)

Frozen embryos transplantation (FET)

Crown-rump length (CRL)

Nuchal Translucency(NT)

Non-invasive Prenatal Testing(NIPT)

Epidermal growth factor (EGF)

Growth differentiation factor 9 (GDF-9)

Bone morphogenetic protein 15 (BMP-15)

Mitogen-activated protein kinase (MAPK)

Declarations

Ethical Approval and Consent to participate

This project was approved by the Medical Ethics Committees of The Fourth Affiliated Hospital of Zhejiang University School of Medicine. Written informed consent for publication of clinical details was obtained from individual patients.

Consent for publication

All data generated or analysed during this study are included in this published article.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional figures and tables.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xiao Chen, Keda Yu, Hong Liu and Chen Chen wrote the main manuscript text; Yuanyuan Yu, Yingying Hu and Youjiang Li prepared figures 1-4 and Tables 1-4; Jian Xu edited the article. All authors read and approved the final manuscript.

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Tables

Table 1. Hormone levels in the first patient who received HCG therapy.

Time	Dose of HCG(IU/day)	Size of follicle(mm)	AMH (ng/ml)	Hormone levels			
				FSH (mIU/ml)	LH (mIU/ml)	Oestradiol (pg/ml)	Progesterone (ng/ml)
2018.12.12			0.02	64.83	22.33	8.00	0.31
2019.03.30	10000			14.13	8.01	30.00	0.30
2019.04.10				44.53	12.60	6.00	0.01
2019.04.16				51.02	14.80	11.00	0.00
2019.04.25				21.97	12.14	49.00	0.00
2019.05.06		9.50		12.98	6.91	107.40	0.10
2019.05.09		13.50		10.11	6.19	128.64	0.10
2019.05.12		15.50		9.43	6.60	174.58	0.20
2019.05.15		18.00		6.32	5.79	219.00	0.30

Table 2. Hormone levels in the second patient who received HCG therapy.

Time	Dose of HCG(IU/day)	Size of follicle(mm)	AMH (ng/ml)	Hormone levels			
				FSH (mIU/ml)	LH (mIU/ml)	Oestradiol (pg/ml)	Progesterone (ng/ml)
2019.05.11			0.01	32.20	21.91	50.00	0.02
2019.08.29	10000			12.69	6.89	97.00	0.02
2019.09.12				10.79	5.60	92.00	0.01
2019.09.26				9.84	5.51	95.00	0.01
2019.10.10		18.50		4.36	4.44	261.00	0.36

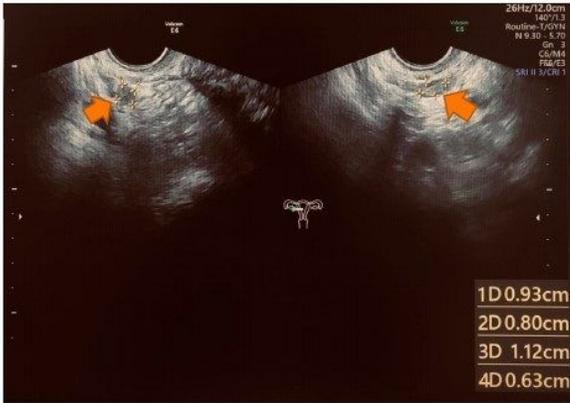
Table 3. Hormone levels in the third patient who received HCG therapy.

Time	Dose of HCG (IU/day)	Dose of Cetorelix Acetate (mg/day)	Size of follicle (mm)	AMH (ng/ml)	Hormone levels			
					FSH (mIU/ml)	LH (mIU/ml)	Oestradiol (pg/ml)	Progesterone (ng/ml)
2019.06.17				0.04	34.17	19.54	11.20	0.14
2019.09.30	10000				12.52	9.02	22.00	0.08
2019.10.14					21.91	11.84	19.00	0.10
2019.10.21					16.98	11.40	35.00	0.10
2019.10.28		0.25	14.00		9.65	12.26	105.00	0.08
2019.10.29		0.25						
2019.10.30		0.25	16.00		8.92	9.66	158.00	0.10
2019.10.31		0.25						
2019.11.01			18.00		/	7.20	206.00	0.10

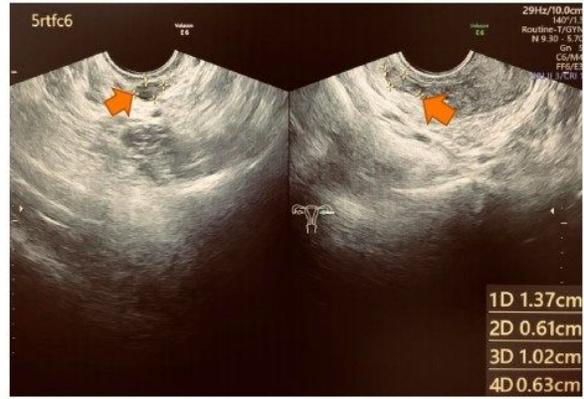
Table 4. Hormone levels in the fourth patient who received HCG therapy.

Time	Dose of HCG (IU/day)	Dose of Cetorelix Acetate (mg/day)	Size of follicle (mm)	AMH (ng/ml)	Hormone levels			
					FSH (mIU/ml)	LH (mIU/ml)	Oestradiol (pg/ml)	Progesterone (ng/ml)
2020.04.11				0.02	50.60	22.72	35.42	0.17
2020.07.30	10000				14.47	8.71	56.00	0.06
2020.08.12					46.31	18.40	19.00	0.09
2020.08.19					35.97	17.72	35.00	0.09
2020.08.27		0.25	16.00		18.89	15.02	105.00	0.06
2020.08.28		0.25	16.50		/	11.46	127.00	0.06
2020.08.29		0.25	17.00		/	7.68	145.00	0.06
2020.08.30		0.25						
2020.08.31			18.00		/	6.03	198.00	0.09

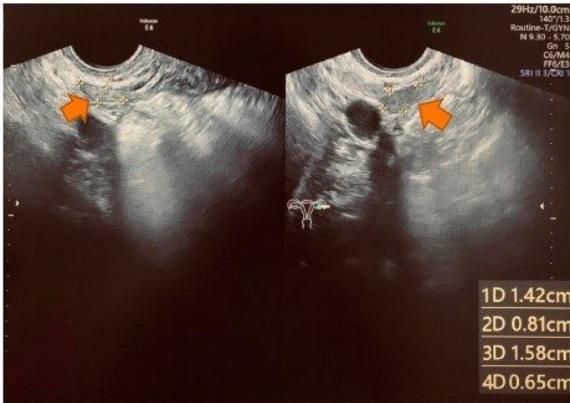
Figures



Patient 1



Patient 3



Patient 2



Patient 4

Figure 1

B-ultrasound examination of the four patients(The orange arrows point to the ovaries).

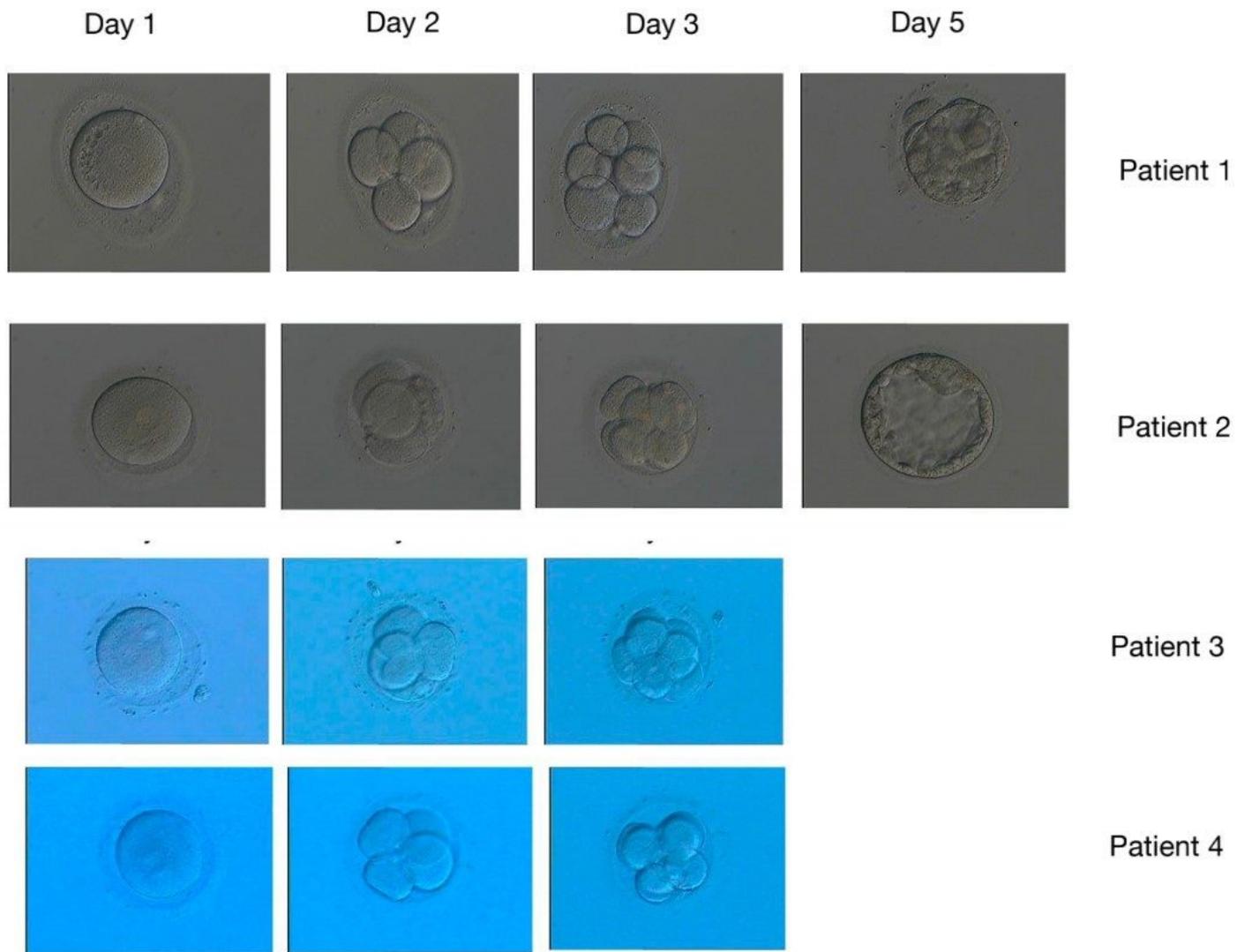


Figure 2

Imaging of the embryos and blastocysts in the four patients.



Screening Project	Detection Value	Normal Value	Result
Trisomy of chromosome 21	0.61	-0.3 – 3.0	Low-risk
Trisomy of chromosome 18	1.11	-0.3 – 3.0	Low-risk
Trisomy of chromosome 13	-1.16	-0.3 – 3.0	Low-risk

Figure 3

Imaging of the early pregnancy screening (NT and NIPT results) for the first patient.



Screening Project	Detection Value	Normal Value	Result
Trisomy of chromosome 21	0.09	-0.3 – 3.0	Low-risk
Trisomy of chromosome 18	0.53	-0.3 – 3.0	Low-risk
Trisomy of chromosome 13	-0.66	-0.3 – 3.0	Low-risk

Figure 4

Imaging of early pregnancy screening (NT and NIPT results) for the second patient.