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A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Title

A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Abstract

Introduction:

The success rate of in vitro fertilisation (IVF) treatment for couples with infertility remains low due to lack of a reliable tool in selecting euploid embryos for transfer. This study aims to compare the efficacy in embryo selection based on morphology alone compared to non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) and morphology in infertile women undergoing in vitro fertilisation (IVF).

Methods and analysis

This is a randomised double blind controlled trial conducted in two tertiary assisted reproduction centres. A total of 500 infertile women will be recruited and undergo IVF as indicated. They will be randomly assigned on day 6 after oocyte retrieval into two groups: the intervention group using morphology and niPGT-A and the control group based on morphology alone. In the control group, blastocysts with the best quality morphology will be replaced first. In the intervention group, blastocysts with the best morphology and euploid result of spent culture medium will be replaced first. The primary outcome is a live birth per the first embryo transfer. The statistical analysis will be performed with the intention to treat and per protocol.

Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the two participating units. All participants will provide written informed consent before joining the study. The results of the study will be submitted to scientific conferences and peer-reviewed journals.

Registration details

The study was registered under Clinical Trials Registry (trial number NCT04474522).

Strengths and limitations of this study

- This is a randomised double blind controlled clinical trial.
- There are no other similar studies registered.
- Our study is not designed to detect small differences in the live birth rates.

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Introduction

One in seven couples experience difficulty in conceiving. Many of them will require in vitro fertilisation (IVF) treatment (1). IVF involves hormone injections to stimulate a woman's ovaries to produce a number of oocytes which are collected by a minor operation and then mixed with sperm to form embryos in the laboratory. Usually, one or two embryos are transferred to the uterus 2-3 days (cleavage stage embryo transfer) or 5 days (blastocyst transfer) after oocyte retrieval. Despite advances in ovarian stimulation, culture medium and laboratory conditions, the pregnancy and birth rates remain 35% and 25% per transfer in Europe in 2014 (2). The corresponding rates in the United States in 2016 were 45% and 36% per transfer respectively (https://www.cdc.gov/art/reports/2016/national-summary.html).

The success of IVF depends on selection of the most competent embryos for transfer, which is still based on morphological criteria by examining the appearance of the embryos under a microscope. But it is well known that many women fail to achieve a pregnancy even after transfer of what are perceived to be good quality embryos. Therefore, some clinics replace multiple embryos in order to maximise pregnancy rates, a strategy which is associated with a high risk of multiple pregnancy.

Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. It is a major reason for failure of pregnancy, miscarriage and congenital anomalies following both natural conception and IVF pregnancies and increases exponentially with maternal age (3-5).

Our inability to assess embryo quality and select those with the highest potential for implantation on the basis of morphology has led to the use of preimplantation genetic testing for aneuploidy (PGT-A). PGT-A involves biopsy of a few cells from an embryo and assessment of the chromosome copy numbers. While PGT-A cannot create a healthy embryo or improve the quality of an embryo, it provides a method of selecting embryos with a normal number of chromosomes for transfer. This in turn has the potential to increase the chance of having a healthy live birth and reduce the risk of miscarriage or an abnormal fetus caused by an abnormal number of chromosomes.

Fluorescent in-situ hybridization (FISH) was first used in PGT-A studies but can only screen at most 9-12 chromosomes in multiple rounds of FISH with decreasing accuracy. A systematic review (6) of nine randomised controlled trials failed to demonstrate the benefit of the use of PGT-A with FISH.

Aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal i.e. comprehensive chromosome screening. Several small randomized controlled trials showed significantly higher pregnancy or live birth rate and lower miscarriage rate following the use of using chromosomal microarray analysis (7-9). The emergence of more advanced genome sequencing such as next generation sequencing (NGS) provides a reliable, high throughput

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approach for PGT-A (10). The turnaround time of PGT-A with NGS is about a week, thus it is not possible to transfer blastocyst in the stimulated cycle. All blastocysts will be frozen post biopsy and the blastocysts with normal genetic makeup will be thawed and replaced in a subsequent menstrual cycle. Cryopreservation of blastocysts and replacing the frozen blastocysts after thawing in subsequent cycles become a common practice with vitrification as the cryopreservation method (11).

A systematic review (12) of the clinical utility of PGT-A with comprehensive chromosome screening found that three small randomised controlled trials demonstrated benefit in young and good prognosis patients in terms of clinical pregnancy rates and the use of single embryo transfer. However, a recent large randomised controlled trial (13) of 661 women comparing PGT-A using NGS vs morphology showed PGT-A did not improve overall pregnancy outcomes in all women aged 25–40 years with at least two blastocysts that could be biopsied. It is possible that there is a detrimental effect of the biopsy of blastocysts on the embryo viability that nullifies the benefit of PGT-A.

The traditional PGT involves biopsy of a few cells from trophectoderm of a blastocyst, which requires skilful laboratory staff and additional instrumentation such as laser equipment. The trophectoderm biopsy is an invasive procedure and may lead to reduction in implantation potential, although the implantation potential is less affected when compared with blastomere biopsy from cleavage stage embryos (14). A non-invasive approach to PGT-A is definitely needed.

The demonstration of release of cell-free DNA from human embryos into the surrounding environment (15) opens up the possibility of non-invasive PGT for aneuploidy (niPGT-A). Collection of spent culture medium (SCM) requires no specialized training and imposes negligible risk to the embryo. SCM may be more representative of the whole blastocyst as embryonic DNA is released from both trophectoderm and inner cell mass while the invasive trophectoderm biopsy obtains embryonic DNA from trophectoderm only. Multiple recent studies (16-23) have demonstrated the ability to detect, extract, and amplify cell-free DNA from SCM at the cleavage and blastocyst stages. It was shown that 24–48 hr of contact with the embryo was sufficient to collect cell-free DNA from SCM (21). The origin of cell-free DNA can be embryonic or parental. It is proposed that the cell-free DNA is derived from cells discarded by the embryos as a corrective mechanism for aneuploidies (16). However, the amount of cell-free DNA was not significantly greater in SCM from aneuploid versus euploid embryos, ruling out this possibility (20). Maternal and paternal contamination in SCM can be minimized by performing thorough oocyte striping and intracytoplasmic sperm injection.

In a recent review (24), the amplification success rate using sequential culture media for SCM ranged between 90% and 100% while the concordance rate in general ploidy i.e. euploid vs aneuploidy between SCM and blastocyst biopsy can be as high as 100% with an average of 75%. The difference in the general ploidy between SCM and trophectoderm biopsy can be due to mosaicism, which can be revealed in

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trophectoderm biopsy but not in SCM due to the nature of the DNA source and relatively low embryonic DNA fraction.

All relevant studies on niPGT-A focus the amplification success and concordance rates between SCM and trophectoderm biopsy. A large clinical randomised trial is urgently needed to confirm its efficacy in embryo selection during IVF in terms of live birth and miscarriage rates.

Objective and hypothesis

This randomised double blind controlled trial aims to compare the efficacy in embryo selection based on morphology alone compared to niPGT-A and morphology in infertile women undergoing IVF.

Hypothesis to be tested include:

1. The embryo selection based on niPGT-A and morphology results in a higher live birth rate in IVF as compared with that based on morphology alone.
2. The embryo selection based on niPGT-A and morphology results in a lower miscarriage rate in IVF as compared with that based on morphology alone.

Methods and analysis

Trial design

This is a randomised double blind controlled trial. Eligible women seeking fertility treatment in the Assisted Reproduction Units in Department of Obstetrics & Gynaecology in Kwong Wah Hospital and Queen Mary Hospital will be recruited for the study and informed written consent will be obtained after counseling.

Ethics approval was sought from the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number: KC/KE-20-0098/FR-2). The study was registered in Clinical Trials Registry (Identifier NCT04474522). Written informed consent will be sought before joining the study.

Selection and Withdrawal of subjects:

The population for trial will be infertile women undergoing IVF.

Inclusion criteria:

Women admitted to the study will fulfil all of the following criteria:

- Age less than 43 years at the time of ovarian stimulation and
- Having at least two blastocysts suitable for freezing on day 6 after oocyte retrieval

Exclusion criteria:

Women should not be recruited in any of the following conditions:

- Having less than two blastocysts suitable for freezing on day 6 after oocyte

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retrieval;
- Undergoing PGT for monogenic diseases or structural rearrangement of chromosomes;
- Using donor oocytes;
- Having hydrosalpinx shown on pelvic scanning and not surgically treated

Withdrawal criteria
Participation in the study is totally voluntary. Recruited women can withdraw from the study at any time without giving any reasons and subsequently they will receive standard medical care.

Treatment of subjects:
Methods
Eligible women will be recruited for the study and informed written consent will be obtained after counseling on the day of oocyte retrieval.
IVF protocol
Infertile women will undergo IVF as clinically indicated. They will receive ovarian stimulation as in standard operation procedure. Ovarian stimulation with gonadotropin injections (150-300 IU daily depending on the antral follicle count) will be given. Medroxyprogesterone acetate 10mg daily will be started on the day 2 of ovarian stimulation or GnRH antagonist (Cetrorelix) 0.25mg daily will be started on the day 6 of ovarian stimulation to prevent premature ovulation. Ultrasound monitoring will be performed to monitor the growth of follicles. When three follicles reach >17 mm in diameter, human chorionic gonadotrophin (Ovidrel 0.25mg) or GnRH agonist (Decapeptyl 0.3mg) will be administered. Oocyte retrieval will be scheduled 36 hours after the trigger under transvaginal ultrasound guidance.

Oocytes will be fertilized and normal fertilization will be assessed and confirmed by the presence of two pronuclei. Embryos will be grown individually to the blastocyst stage, usually day 5 or 6 after oocyte retrieval, in a monophasic medium. On Day 3, embryos will be rinsed briefly in fresh culture medium. The culture medium will be replenished and culture will be continued at 37°C and 6% CO_2 in reduced oxygen tension (5%). No fresh transfer of blastocysts will be performed in the stimulated cycle.

Grading of blastocyst by morphology
Blastocysts are graded according to Gardner's classification (25). Each blastocyst will be cryopreserved on day 6 by vitrification individually and its SCM (~8 μl) will be frozen at -80^{0}C separately and individually. The embryologist will grade the morphology of blastocysts according to Gardner's criteria .

Then, on day 6 after oocyte retrieval, women will then be randomly assigned by a PGT laboratory staff into one of the following two groups according to a computer-generated randomization list with a 1:1 ratio and a block size of 10. The

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randomization list will be prepared by a research nurse, who is not involved in the clinical care of these women.

1. the intervention group using morphology and niPGT-A and
2. the control group based on morphology alone.

The women, clinicians and embryologists in the IVF laboratory will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory will be aware of the group assignment.

niPGT-A of spent culture medium (SCM)

In the intervention group, comprehensive chromosome screening using NGS will be performed according to the recommendations of the company in all SCM samples. In the control group, the measurement will be done retrospectively after a live birth or when all blastocysts are replaced without a live birth.

A commercially available NI-PGT kit (PG-Seq Rapid Non-Invasive PGT kit, PerkinElmer) will be used to analyse the SCM samples. The protocol has been previously optimized with non-invasive samples from 15 laboratories around the world. The kit follows a single tube workflow, two-steps PCR to whole genome amplification of the DNA in SCM and then attaches indexes and sequence-specific adapters to template DNA, resulting in sequencing ready samples.

After purification, equal molar concentration of indexed DNA from each sample will be pooled (96 samples) and then sequenced on a MiSeq system (Illumina) at 1x75 bp read length. On-board secondary analysis will be performed automatically by the MiSeq Reporter (Illumina) followed by the PG-Find Software (v 1.0, PerkinElmer). Reads aligning to anomalous, unstructured and highly repetitive sequences will be filtered from the analysis. A target bin size of 1,000 kb will be used, giving a minimum resolution of 10 Mb. All genomic positions will be referred to the human genome build NCBI 37.

According to the default setting of the PG-Find software, classification of aneuploidy is determined by CNV (copy number variation) value. CNV value >2.7 is considered as gain while CNV value <1.3 is considered as loss. Sample will be concluded as non-euploid when one or more of the chromosomes show gain/loss.

The niPGT-A result of the SCM sample can be euploid, non-euploid and non-informative. It will be used only to prioritize the sequence of embryo transfer. Blastocysts with non-euploid result in the niPGT-A report will not be discarded and will be transferred with lower priority.

Blinding

The embryologist will grade the morphology of blastocysts according to Gardner's criteria stated above and the grading of blastocysts will be entered into an online database, which will be managed by an IT technician. The laboratory staff in the PGT laboratory will enter the PGT result into an online database when the niPGT-

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A results are available. The IT technician will merge the data online to compile the sequence of embryo transfer according to a pre-determined algorithm which depends on the day of blastocyst development (day 5 better than day 6), blastocyst morphology and niPGT-A result of the intervention group. The IT technician will issue the sequence of embryo transfer which does not contain information on the grading of the blastocyst and the NIPGT result to the embryologists in the IVF laboratory. Therefore, the subjects recruited, the clinicians and the embryologists will be blinded to the group allocation.

A pilot study

A pilot study was conducted on 82 SCM from February to September 2020. Media cultured in parallel but without contact with embryos were collected as controls (n=8). Amplification was successful in 80 SCM (97.6%, 80/82) and 72 SCM resulted in conclusive result (90.0%, 72/80). All controls showed no amplification.

In this cohort, 40 SCM with conclusive results were collected from PGT cycles in which trophectoderm biopsies were also performed. 85.0% (34/40) of samples showed concordance results between trophectoderm biopsy and SCM.

Frozen embryo transfer (FET)

Blastocysts will be replaced in the natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. The embryologists in the IVF laboratory will thaw and transfer the blastocyst according to the sequence of embryo transfer generated and issued by the IT technician. In the control group, blastocysts which develop on day 5 after the oocyte retrieval and have the best grading will be replaced first. In the intervention group, blastocysts which develop on day 5 and have the best grading and euploid result will be replaced first. If no blastocysts have euploid result, those with non-informative followed by non-euploid results will be replaced.

Pregnancy

A urine pregnancy test will be performed 14 days after the transfer. If the pregnancy test is positive, transvaginal ultrasound will be performed two weeks later to locate the pregnancy and confirm foetal viability and the number of fetuses. Subsequent management will be the same as other women with early pregnancy. They will be referred for antenatal care when the ongoing pregnancy is 8-10 weeks.

Follow-up

Written consent regarding retrieval of pregnancy and delivery data will be sought from the recruited women at the time of study. The women will be contacted after delivery by phone to retrieve the information of the pregnancy outcomes. The outcome of the pregnancy (delivery, miscarriage), number of babies born, birth weights and obstetrics complications will be recorded.

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Women in both groups will continue to have blastocyst transfer until all the cryopreserved blastocysts are used up or they become pregnant within 6 months after randomisation. Cumulative live birth rate will be calculated (the number of live birth per couple within the study period). The pregnancy complication and congenital abnormalities of the pregnancies in the two groups will be traced through hospital records or patient contact by mail or phone of mail and compared.

Assessment of outcomes:
The primary outcome is live birth beyond 22 weeks of gestation per the first FET.

Secondary outcomes include

- Cumulative live birth rate: the number of pregnancies leading to live birth within 6 months of randomization.
- Time to pregnancy
- Positive urine pregnancy test
- Clinical pregnancy defined as presence of intrauterine gestational sac on scanning at gestational week 6.
- Ongoing pregnancy as presence of a fetal pole with pulsation at 8-10 weeks of gestation
- Miscarriage defined as a clinically recognized pregnancy loss before the 22 weeks of pregnancy and whose denominator is the clinical pregnancy.
- Multiple pregnancy: presence of more than one intrauterine sac at 6 weeks of gestation
- Ectopic pregnancy
- Pregnancy outcomes including preterm delivery, pre-eclampsia, gestational diabetes, congenital anomaly, perinatal mortality and birthweight of newborn.

Consistency of case management:
The same standardised study protocol will be adopted in the two study centres. The clinicians who manage the women in KWH had all been trained at QMH, and are now adopting the same clinical management protocols in their respective unit. Regular and frequent communication among the two participating centers will also ensure dissemination of updated information on recruitment and safety issues.

Patient and Public involvement
Patients or the public were not involved in the design, conduct, reporting or dissemination plans of this study.

Statistics
Sample size calculation
From the data in QMH and KWH, the live birth rate following transfer of one blastocyst based on morphology was about 35% in 2018 and 2019. We anticipate following blastocyst morphology and NIPGT-A, the live birth rate will increase from 35% to 50% i.e. 15% increase. The 50% live birth rate is based on the live birth rate observed following conventional PGT-A in the centre. The calculated sample size is 224 women in each group to give a power of 0.9 and type I error of 0.05. Assuming a 10% drop-out rate, the total sample size to be 500, 250 subjects in each group.

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A pre-specified subgroup analysis will be performed: women aged <35 years vs >=35 years.

Data analysis

Demographic features of women in the two groups will be compared. Comparison of quantitative variables will be performed using Student's t, while categorical variables will be compared using a Chi-square analysis. All statistical analyses of the data will be performed with the intention to treat and per protocol using the SPSS program V.26.0 (SPSS Inc, Chicago, Illinois, USA), and a p value <0.05 will be considered statistically significant.

The sensitivity, specificity, likelihood ratios and odd ratio of the niPGT-A result will be calculated.

Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number: KC/KE-20-0098/FR-2). All participants will provide written informed consent before randomization. The research findings from this study will be submitted to scientific conferences and peer-reviewed journals for publication so as to disseminate the results to other researchers and clinicians working in the field.

Discussion

The success rate of IVF has long been limited due to the inability to assess the implantation potential of each embryo accurately. Assessment by morphology using Gardner's classification could not reflect chromosomal abnormalities in embryos, which is the reason for failure of implantation in most of the cases. NGS offers several advantages over chromosomal microarray analysis by (i) reduced DNA sequencing cost by high throughput sequencing technologies and a high number of samples can be simultaneously sequenced in a single testing; (ii) enhanced detection of partial or segmental aneuploidies as a result of the increase in chromosomal analysis resolution to a few mega bases; (iii) increased dynamic range enabling enhanced detection of mosaicism in multicellular samples and (iv) automation of the sequencing library preparation and automation of the PGT-A diagnostic procedure. However, the beneficial effect of PGT-A has been nullified by the detrimental effect of embryo biopsy. If niPGT-A can be demonstrated to be a better blastocyst evaluation tool than the traditional morphology assessment during IVF treatment, it can potentially shorten the time-to-pregnancy in women with infertility.

Currently, all relevant studies on niPGT-A focused on the amplification success and concordance rates between SCM and trophectoderm biopsy but not on assessing its ability as a screening tool for blastocyst transfer prioritization. This study could

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provide valuable information on the potential novel use of niPGT-A as an adjunct for morphological assessment of blastocysts.

Trial status

The first subject was recruited on 1st July 2021 and 400 women were recruited up to the writing of this protocol paper.

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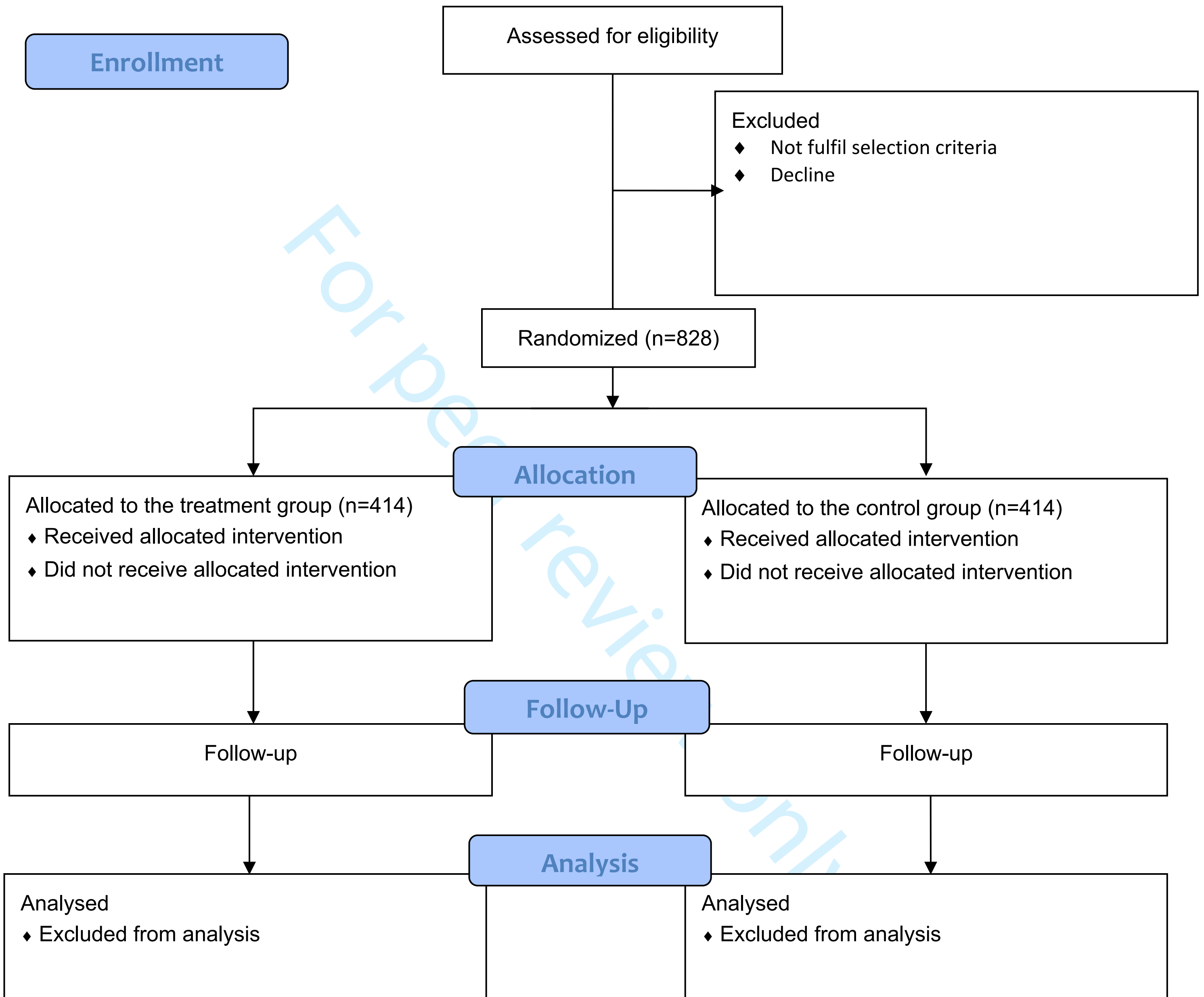
CONSORT 2010 Flow Diagram



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A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Title

A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Abstract

Introduction:

The success rate of in vitro fertilisation (IVF) treatment for couples with infertility remains low due to lack of a reliable tool in selecting euploid embryos for transfer. This study aims to compare the efficacy in embryo selection based on morphology alone compared to non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) and morphology in infertile women undergoing in vitro fertilisation (IVF).

Methods and analysis

This is a randomised double blind controlled trial conducted in two tertiary assisted reproduction centres. A total of 500 infertile women will be recruited and undergo IVF as indicated. They will be randomly assigned on day 6 after oocyte retrieval into two groups: the intervention group using morphology and niPGT-A and the control group based on morphology alone. In the control group, blastocysts with the best quality morphology will be replaced first. In the intervention group, blastocysts with the best morphology and euploid result of spent culture medium will be replaced first. The primary outcome is a live birth per the first embryo transfer. The statistical analysis will be performed with the intention to treat and per protocol.

Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the two participating units. All participants will provide written informed consent before joining the study. The results of the study will be submitted to scientific conferences and peer-reviewed journals.

Registration details

The study was registered under ClinicalTrials.gov (trial number NCT04474522).

Strengths and limitations of this study

- This is a randomised double blind controlled clinical trial.
- The recruitment takes place in a large representative stakeholder group from the field of reproductive medicine in Hong Kong.
- The intervention group of this study includes both morphology and niPGT-A, thereby investigating the sole effect of addition of niPGT-A on embryo prioritization alongside with morphology assessment
- One limitation is that our study is not designed to detect small differences in the live birth rates.

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Introduction

One in seven couples experience difficulty in conceiving. Many of them will require in vitro fertilisation (IVF) treatment (1). IVF involves hormone injections to stimulate a woman's ovaries to produce a number of oocytes which are collected by a minor operation and then mixed with sperm to form embryos in the laboratory. Usually, one or two embryos are transferred to the uterus 2-3 days (cleavage stage embryo transfer) or 5 days (blastocyst transfer) after oocyte retrieval. Despite advances in ovarian stimulation, culture medium and laboratory conditions, the pregnancy and birth rates remain 35% and 25% per transfer in Europe in 2014 (2). The corresponding rates in the United States in 2016 were 45% and 36% per transfer respectively (https://www.cdc.gov/art/reports/2016/national-summary.html).

The success of IVF depends on selection of the most competent embryos for transfer, which is still based on morphological criteria by examining the appearance of the embryos under a microscope. But it is well known that many women fail to achieve a pregnancy even after transfer of what are perceived to be good quality embryos. Therefore, some clinics replace multiple embryos in order to maximise pregnancy rates, a strategy which is associated with a high risk of multiple pregnancy.

Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. It is a major reason for failure of pregnancy, miscarriage and congenital anomalies following both natural conception and IVF pregnancies and increases exponentially with maternal age (3-5).

Our inability to assess embryo quality and select those with the highest potential for implantation on the basis of morphology has led to the use of preimplantation genetic testing for aneuploidy (PGT-A). PGT-A involves biopsy of a few cells from an embryo and assessment of the chromosome copy numbers. While PGT-A cannot create a healthy embryo or improve the quality of an embryo, it provides a method of selecting embryos with a normal number of chromosomes for transfer. This in turn has the potential to increase the chance of having a healthy live birth and reduce the risk of miscarriage or an abnormal fetus caused by an abnormal number of chromosomes.

Fluorescent in-situ hybridization (FISH) was first used in PGT-A studies but can only screen at most 9-12 chromosomes in multiple rounds of FISH with decreasing accuracy. A systematic review of nine randomised controlled trials failed to demonstrate the benefit of the use of PGT-A with FISH(6).

Aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal i.e. comprehensive chromosome screening. Several small randomized controlled trials showed significantly higher pregnancy or live birth rate and lower miscarriage rate following the use of using chromosomal microarray analysis (7-9). The emergence of more advanced genome sequencing such as next generation sequencing (NGS) provides a reliable, high throughput

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approach for PGT-A (10). The turnaround time of PGT-A with NGS is about a week, thus it is not possible to transfer blastocyst in the stimulated cycle. All blastocysts are frozen post biopsy and the blastocysts with normal genetic makeup are thawed and replaced in a subsequent menstrual cycle. Cryopreservation of blastocysts and replacing the frozen blastocysts after thawing in subsequent cycles become a common practice with vitrification as the cryopreservation method (11).

A systematic review of the clinical utility of PGT-A with comprehensive chromosome screening found that three small randomised controlled trials demonstrated benefit in young and good prognosis patients in terms of clinical pregnancy rates and the use of single embryo transfer (12). However, a recent large randomised controlled trial of 661 women comparing PGT-A using NGS vs morphology showed PGT-A did not improve overall pregnancy outcomes in all women aged 25–40 years with at least two blastocysts that could be biopsied (13). It is possible that there is a detrimental effect of the biopsy of blastocysts on the embryo viability that nullifies the benefit of PGT-A.

The traditional PGT involves biopsy of a few cells from trophectoderm of a blastocyst, which requires skilful laboratory staff and additional instrumentation such as laser equipment. The trophectoderm biopsy is an invasive procedure and may lead to reduction in implantation potential, although the implantation potential is less affected when compared with blastomere biopsy from cleavage stage embryos (14). A non-invasive approach to PGT-A is definitely needed.

The demonstration of release of cell-free DNA from human embryos into the surrounding environment opens up the possibility of non-invasive PGT for aneuploidy (niPGT-A)(15). Collection of spent culture medium (SCM) requires no specialized training and imposes negligible risk to the embryo. SCM may be more representative of the whole blastocyst as embryonic DNA is released from both trophectoderm and inner cell mass while the invasive trophectoderm biopsy obtains embryonic DNA from trophectoderm only. Multiple recent studies have demonstrated the ability to detect, extract, and amplify cell-free DNA from SCM at the cleavage and blastocyst stages(16-23). It was shown that 24–48 hr of contact with the embryo was sufficient to collect cell-free DNA from SCM (21). The origin of cell-free DNA can be embryonic or parental. It is proposed that the cell-free DNA is derived from cells discarded by the embryos as a corrective mechanism for aneuploidies (16,24). However, the amount of cell-free DNA was not significantly greater in SCM from aneuploid versus euploid embryos, ruling out this possibility (20). Maternal and paternal contamination in SCM can be minimized by performing thorough oocyte striping and intracytoplasmic sperm injection(25).

In a recent review, the amplification success rate using sequential culture media for SCM ranged between 90% and 100% while the concordance rate in general ploidy i.e. euploid vs aneuploidy between SCM and blastocyst biopsy can be as high as 100% with an average of 75% (26). The difference in the general ploidy between SCM and trophectoderm biopsy can be due to mosaicism, which can be revealed in

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trophectoderm biopsy but not in SCM due to the nature of the DNA source and relatively low embryonic DNA fraction.

All relevant studies on niPGT-A focus the amplification success and concordance rates between SCM and trophectoderm biopsy. A large clinical randomised trial is urgently needed to confirm its efficacy in embryo selection during IVF in terms of live birth and miscarriage rates.

Objective and hypothesis

This randomised double blind controlled trial aims to compare the efficacy in embryo selection based on morphology alone compared to niPGT-A and morphology in infertile women undergoing IVF.

Hypothesis to be tested include:

1. The embryo selection based on niPGT-A and morphology results in a higher live birth rate in IVF as compared with that based on morphology alone.
2. The embryo selection based on niPGT-A and morphology results in a lower miscarriage rate in IVF as compared with that based on morphology alone.

Methods and analysis

Trial design

This is a randomised double blind controlled trial. Eligible women seeking fertility treatment in the Assisted Reproduction Units in Department of Obstetrics & Gynaecology in Kwong Wah Hospital and Queen Mary Hospital will be recruited for the study and informed written consent will be obtained after counseling.

Ethics approval was sought from the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number: KC/KE-20-0098/FR-2). The study was registered in Clinical Trials Registry (Identifier NCT04474522). Written informed consent will be sought before joining the study.

Selection and Withdrawal of subjects:

The population for trial will be infertile women undergoing IVF.

Inclusion criteria:

Women admitted to the study will fulfil all of the following criteria:

- Age less than 43 years at the time of ovarian stimulation and
- Having at least two blastocysts suitable for freezing on day 6 after oocyte retrieval

Exclusion criteria:

Women should not be recruited in any of the following conditions:

- Having less than two blastocysts suitable for freezing on day 6 after oocyte

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retrieval;
- Undergoing PGT for monogenic diseases or structural rearrangement of chromosomes;
- Using donor oocytes;
- Having hydrosalpinx shown on pelvic scanning and not surgically treated

Withdrawal criteria

Participation in the study is totally voluntary. Recruited women can withdraw from the study at any time without giving any reasons and subsequently they will receive standard medical care.

Treatment of subjects:

Methods

Eligible women will be recruited for the study and informed written consent will be obtained after counseling on the day of oocyte retrieval by research nurse or clinicians.

IVF protocol

Infertile women will undergo IVF as clinically indicated. They will receive ovarian stimulation as in standard operation procedure. Ovarian stimulation with gonadotropin injections (150-300 IU daily depending on the antral follicle count) will be given. Medroxyprogesterone acetate 10mg daily will be started on the day 2 of ovarian stimulation or GnRH antagonist (Cetrorelix) 0.25mg daily will be started on the day 6 of ovarian stimulation to prevent premature ovulation. Ultrasound monitoring will be performed to monitor the growth of follicles. When three follicles reach >17 mm in diameter, human chorionic gonadotrophin (Ovidrel 0.25mg) or GnRH agonist (Decapeptyl 0.3mg) will be administered. Oocyte retrieval will be scheduled 36 hours after the trigger under transvaginal ultrasound guidance.

Oocytes will be fertilized conventionally or by intracytoplasmic sperm injection (ICSI) depending on the semen parameters in accordance with the standard operating procedures and normal fertilization will be assessed and confirmed by the presence of two pronuclei. Embryos will be grown individually to the blastocyst stage, up to day 6 after oocyte retrieval, in a monophasic medium. On Day 3, embryos will be rinsed briefly in fresh culture medium. The culture medium will be replenished and culture will be continued at $37^{o}C$ and 6% CO_2 in reduced oxygen tension (5%). No fresh transfer of blastocysts will be performed in the stimulated cycle.

Grading of blastocyst by morphology

Blastocysts are graded according to Gardner's classification (27). Each blastocyst will be cryopreserved on day 6 by vitrification individually and its SCM (~8 μl) will be frozen at $-80^{0}C$ separately and individually. The embryologist will grade the morphology of blastocysts according to Gardner's criteria .

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Then, on day 6 after oocyte retrieval, women will then be randomly assigned by a PGT laboratory staff into one of the following two groups according to a computer-generated randomization list with a 1:1 ratio and a block size of 10. The randomization list will be prepared by a research nurse, who is not involved in the clinical care of these women.

1. the intervention group using morphology and niPGT-A and
2. the control group based on morphology alone.

The women, clinicians and embryologists in the IVF laboratory will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory will be aware of the group assignment.

niPGT-A of spent culture medium (SCM)

In the intervention group, comprehensive chromosome screening using NGS will be performed according to the recommendations of the company in all SCM samples. In the control group, the measurement will be done retrospectively after a live birth or when all blastocysts are replaced without a live birth.

A commercially available NI-PGT kit (PG-Seq Rapid Non-Invasive PGT kit, PerkinElmer) will be used to analyse the SCM samples. The protocol has been previously optimized with non-invasive samples from 15 laboratories around the world. The kit follows a single tube workflow, two-steps PCR to whole genome amplification of the DNA in SCM and then attaches indexes and sequence-specific adapters to template DNA, resulting in sequencing ready samples.

After purification, equal molar concentration of indexed DNA from each sample will be pooled (96 samples) and then sequenced on a MiSeq system (Illumina) at 1x75 bp read length. On-board secondary analysis will be performed automatically by the MiSeq Reporter (Illumina) followed by the PG-Find Software (v 1.0, PerkinElmer). Reads aligning to anomalous, unstructured and highly repetitive sequences will be filtered from the analysis. A target bin size of 1,000 kb will be used, giving a minimum resolution of 10 Mb. All genomic positions will be referred to the human genome build NCBI 37.

According to the default setting of the PG-Find software, classification of aneuploidy is determined by CNV (copy number variation) value. CNV value >2.7 is considered as gain while CNV value <1.3 is considered as loss. Sample will be concluded as non-euploid when one or more of the chromosomes show gain/loss.

The niPGT-A result of the SCM sample can be euploid, non-euploid and non-informative. It will be used only to prioritize the sequence of embryo transfer.

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Blastocysts with non-euploid result in the niPGT-A report will not be discarded and will be transferred with lower priority.

Blinding

The embryologist will grade the morphology of blastocysts according to Gardner's criteria stated above and the grading of blastocysts will be entered into an online database, which will be managed by an IT technician. The laboratory staff in the PGT laboratory will enter the PGT result into an online database when the niPGT-A results are available. The IT technician will merge the data online to compile the sequence of embryo transfer according to a pre-determined algorithm which depends on the day of blastocyst development (day 5 better than day 6), blastocyst morphology and niPGT-A result of the intervention group. The IT technician will issue the sequence of embryo transfer which does not contain information on the grading of the blastocyst and the NIPGT result to the embryologists in the IVF laboratory. Therefore, the subjects recruited, the clinicians and the embryologists will be blinded to the group allocation.

A pilot study

A pilot study was conducted on 82 SCM from February to September 2020. Media cultured in parallel but without contact with embryos were collected as controls (n=8). Amplification was successful in 80 SCM (97.6%, 80/82) and 72 SCM resulted in conclusive result (90.0%, 72/80). All controls showed no amplification.

In this cohort, 40 SCM with conclusive results were collected from PGT cycles in which trophectoderm biopsies were also performed. 85.0% (34/40) of samples showed concordance results between trophectoderm biopsy and SCM.

Frozen embryo transfer (FET)

Blastocysts will be replaced in the natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. The embryologists in the IVF laboratory will thaw and transfer the blastocyst according to the sequence of embryo transfer generated and issued by the IT technician. In the control group, blastocysts which develop on day 5 after the oocyte retrieval and have the best grading will be replaced first. In the intervention group, blastocysts which develop on day 5 and have the best grading and euploid result will be replaced first. If no blastocysts have euploid result, those with non-informative followed by non-euploid results will be replaced.

Pregnancy

A urine pregnancy test will be performed 14 days after the transfer. If the pregnancy test is positive, transvaginal ultrasound will be performed two weeks later to locate the pregnancy and confirm foetal viability and the number of fetuses. Subsequent management will be the same as other women with

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early pregnancy. They will be referred for antenatal care when the ongoing pregnancy is 8-10 weeks.

Follow-up

Written consent regarding retrieval of pregnancy and delivery data will be sought from the recruited women at the time of study. The women will be contacted after delivery by phone to retrieve the information of the pregnancy outcomes. The outcome of the pregnancy (delivery, miscarriage), number of babies born, birth weights and obstetrics complications will be recorded.

Women in both groups will continue to have blastocyst transfer until all the cryopreserved blastocysts are used up or they become pregnant within 6 months after randomisation. Cumulative live birth rate will be calculated (the number of live birth per couple within the study period). The pregnancy complication and congenital abnormalities of the pregnancies in the two groups will be traced through hospital records or patient contact by mail or phone of mail and compared.

Assessment of outcomes:

The primary outcome is live birth beyond 22 weeks of gestation per the first FET.

Secondary outcomes include

- Cumulative live birth rate: the number of pregnancies leading to live birth within 6 months of randomization.
- Time to pregnancy
- Positive urine pregnancy test
- Clinical pregnancy defined as presence of intrauterine gestational sac on scanning at gestational week 6.
- Ongoing pregnancy as presence of a fetal pole with pulsation at 8-10 weeks of gestation
- Miscarriage defined as a clinically recognized pregnancy loss before the 22 weeks of pregnancy and whose denominator is the clinical pregnancy.
- Multiple pregnancy: presence of more than one intrauterine sac at 6 weeks of gestation
- Ectopic pregnancy
- Pregnancy outcomes including preterm delivery, pre-eclampsia, gestational diabetes, congenital anomaly, perinatal mortality and birthweight of newborn.

Consistency of case management:

The same standardised study protocol will be adopted in the two study centres. The clinicians who manage the women in KWH had all been trained at QMH, and are now adopting the same clinical management protocols in their respective unit. Regular and frequent communication among the two participating centers will also ensure dissemination of updated information on recruitment and safety issues.

Patient and Public involvement

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Patients or the public were not involved in the design, conduct, reporting or dissemination plans of this study.

Statistics

Sample size calculation

From the data in QMH and KWH, the live birth rate following transfer of one blastocyst based on morphology was about 35% in 2018 and 2019. We anticipate following blastocyst morphology and NIPGT-A, the live birth rate will increase from 35% to 50% i.e. 15% increase. The 50% live birth rate is based on the live birth rate observed following conventional PGT-A in the centre. The calculated sample size is 224 women in each group to give a power of 0.9 and type I error of 0.05. Assuming a 10% drop-out rate, the total sample size to be 500, 250 subjects in each group.

A pre-specified subgroup analysis will be performed: women aged <35 years vs >=35 years.

Data analysis

Demographic features of women in the two groups will be compared. Comparison of quantitative variables will be performed using Student's t or Mann-whitney U-test where appropriate, while categorical variables will be compared using a Chi-square analysis, multivariable logistic regression or the one-way ANOVA test if more than two categories will be compared. All statistical analyses of the data will be performed with the intention to treat and per protocol using the SPSS program V.26.0 (SPSS Inc, Chicago, Illinois, USA), and a p value <0.05 will be considered statistically significant.

The sensitivity, specificity, likelihood ratios and odd ratio of the niPGT-A result will be calculated.

Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number: KC/KE-20-0098/FR-2). All participants will provide written informed consent before randomization. The research findings from this study will be submitted to scientific conferences and peer-reviewed journals for publication so as to disseminate the results to other researchers and clinicians working in the field.

Discussion

The success rate of IVF has long been limited due to the inability to assess the implantation potential of each embryo accurately. Assessment by morphology using Gardner's classification could not reflect chromosomal abnormalities in embryos, which is the reason for failure of implantation in most of the cases. NGS offers several advantages over chromosomal microarray analysis by (i) reduced DNA sequencing cost by high throughput sequencing technologies and a high number of

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samples can be simultaneously sequenced in a single testing; (ii) enhanced detection of partial or segmental aneuploidies as a result of the increase in chromosomal analysis resolution to a few mega bases; (iii) increased dynamic range enabling enhanced detection of mosaicism in multicellular samples and (iv) automation of the sequencing library preparation and automation of the PGT-A diagnostic procedure. However, the beneficial effect of PGT-A has been nullified by the detrimental effect of embryo biopsy. If niPGT-A can be demonstrated to be a better blastocyst evaluation tool than the traditional morphology assessment during IVF treatment, it can potentially shorten the time-to-pregnancy in women with infertility.

Currently, all relevant studies on niPGT-A focused on the amplification success and concordance rates between SCM and trophectoderm biopsy but not on assessing its ability as a screening tool for blastocyst transfer prioritization. This study could provide valuable information on the potential novel use of niPGT-A as an adjunct for morphological assessment of blastocysts.

Trial status

The first subject was recruited on 1st July 2021 and 400 women were recruited up to the writing of this protocol paper.

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Competing interest: The authors report no conflicts of interests.

Contributorship Statement

All authors have substantial contribution to the protocol paper:

Cheng Hiu Yee Heidi: Drafting the manuscript and revising it critically for important intellectual content, responsible for seeking ethics approval and registration on ClinicalTrials.gov

Chow, Judy F.C: substantial contribution to designing the work flow and logistics of niPGT-A, responsible for generating niPGT-A report

Lai, Shui Fan: responsible for the recruitment of subjects and seeking ethics approval at the Kwong Wah Hospital

Lam, Kevin KW: responsible for collaborating with the PGT laboratory regarding logistics of collecting SCM and transferral. Responsible for IVF laboratory.

Ng, Ernest HY: Substantial contributions to the conception and design of the whole study, and final approval of the version to be published

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466 Yeung, William Shu Biu: Responsible for the design and worked on logistics of
467 the study, and final approval of the version to be published
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471 **Word Count:** 3387

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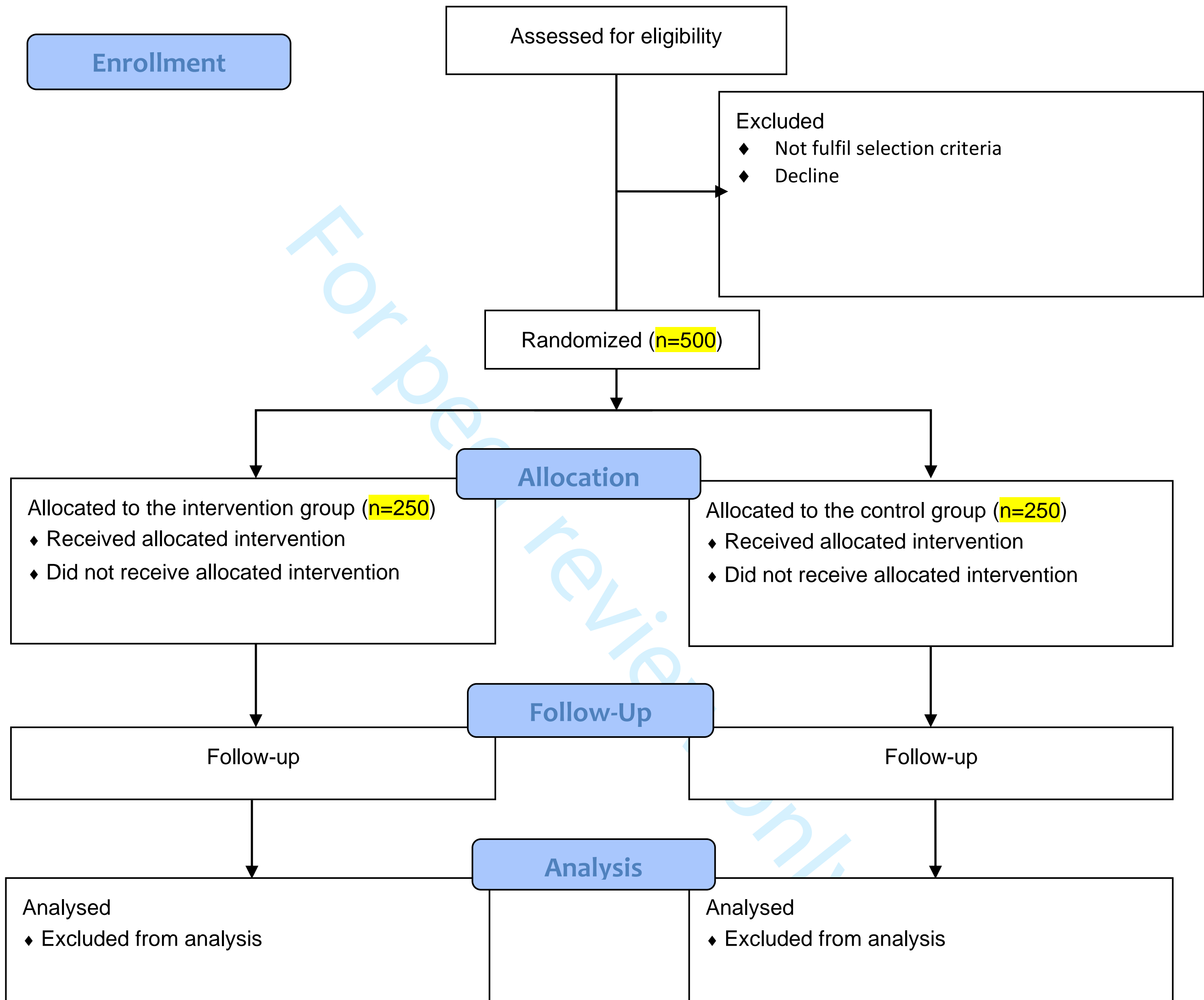
CONSORT 2010 Flow Diagram



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DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
THE UNIVERSITY OF HONG KONG

PATIENT INFORMATION AND CONSENT

PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization
Version 1.0: Dated 8 March 2020

You are cordially invited to participate in the above named research study. You need to decide whether you want to participate or not. Please take your time to make up your mind. Carefully read the following and feel free to ask the study doctor any question which you may have.

Why is this study being done?
Couples with difficulty conceiving will require test-tube baby or in vitro fertilization (IVF). Despite advances in technology, the pregnancy rate of IVF remained around 35% per transfer. Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. Chromosome aneuploidy is the major reason for failure of pregnancy and miscarriage.

The traditional preimplantation genetic testing for aneupoloidy (PGT-A) involves taking a few cells from a blastocyst, which requires skillful laboratory staff and laser equipment. Taking cells from a blastocyst is an invasive procedure and may lead to reduction in implantation potential. The demonstration of release of DNA from human embryos into the surrounding environment opens up the possibility of non-invasive PGT for aneuploidy (NIPGT-A). Collection of spent culture medium requires no specialized training and imposes negligible risk to the embryo. Spent culture medium may be more representative of the whole blastocyst.

The results of NIPGT-A will be able to prioritize the sequence of embryo transfer. There is still no good evidence to show the efficacy of NIPGT-A in IVF. The aim of this study is to compare with efficacy of embryo selection for replacement based on conventional method through embryo morphology versus morphology with additional input from result of NIPGT-A.

Who should be in this study?
You will be recruited if

1. You are aged less than 43 years at the time of ovarian stimulation, and
2. At least two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval

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You will not be included in this study if

1. Less than two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval;
2. Women undergoing PGT for monogenic diseases or structural rearrangement of chromosomes
3. Use of donor oocytes
4. Hydrosalpinx shown on pelvic scanning and not surgically treated

What will I be asked to do?

Recruited patients will undergo IVF as clinically indicated. Ovarian stimulation, ultrasound monitoring, and oocyte retrieval will follow the standard operating procedure of the unit.

Each blastocyst will be frozen individually and its spent culture medium (~8 μl) will be frozen at -80^{0}C separately and individually. The embryologist will prepare a sequence of blastocyst transfer based on the best morphology by Gardner's criteria.

On the day of blastocyst freezing, recruited women will then be randomly assigned into two groups using a randomization program.

1. the intervention group using morphology and NIPGT-A and
2. the control group based on morphology alone.

The women and doctors will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory and the embryologists in the IVF laboratory will be aware of the group assignment.

In the intervention group, comprehensive chromosome screening using NGS will be performed. In the control group, the measurement will be done in the spent culture medium of the blastocyst that is replaced in the first transfer. The NIPGT-A report is used only to prioritize the sequence of embryo transfer. Blastocysts with non-euploid result in the NIPGT-A report will not be discarded.

Blastocysts can be replaced in the subsequent natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. In the control group, blastocysts with the best quality morphology will be replaced first and the sequence of blastocyst transfer is decided prior to randomization. In the intervention group, blastocysts with the best morphology and euploid result will be replaced first as the sequence of blastocyst transfer will be modified after the NIPGT-A reports are available.

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How long will I be in the study?

1 IVF cycle

How many other people will be participating in the study?

We plan to recruit 500 women in this study.

Will I be paid?

No payment will be made to you for this study.

What adverse (bad) effects can happen to me by participating in the study?

There should be no major safety concern as all the embryos will be kept after prioritization regardless of the group recruited.

What benefit can I expect?

You may have a higher pregnancy rate per transfer, lower miscarriage rate and short time to pregnancy if you are randomized to the intervention group with NIPGT-A.

Can I refuse to be in the study?

Your participation in this study is voluntary. You can choose not to take part in the study, or you can quit at any time. You will not lose any benefit to which you are otherwise entitled. If you quit the study, you can receive the standard treatment as other patients in our Department.

Confidentiality and privacy

The investigators have always maintained a strict privacy policy. We never sell, trade or otherwise share your details with any sources. All correspondence to the department is held confidentially; furthermore, at no time will your personal and/or identifying information be shared outside of our organization, for any reason.

Subjects have the rights of access to personal data and known study results, if and when needed. Under the laws of the Hong Kong Special Administrative Region and, in particular, the Personal Data (Privacy) Ordinance, Cap 486, you enjoy or may enjoy rights for the protection of the confidentiality of your personal data, such as those regarding the collection, custody, retention, management, control, use (including analysis or comparison), transfer in or out of Hong Kong, non-disclosure, erasure and/or in any way dealing with or disposing of any of your personal data in or for this study. For any query, you should consult the Privacy Commissioner for Privacy Data or his office (2827 2827) as to the proper monitoring or

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supervision of your personal data protection so that your full awareness and understanding of the significance of compliance with the law governing privacy data is assured.

For questions about the study or reporting of adverse events, please call Dr Heidi Cheng at telephone no. 22553657. The phone number of the Institutional Review Board of The University of Hong Kong / Hospital Authority Hong Kong Wester cluster is 2255 4086.

If you consent to take part in the research, any of your medical records may be inspected by the research team for purposes of analysing the results. They may also be looked at by people from regulatory authorities to check that the study is being carried out correctly. Your name, however, will not be disclosed outside the hospital.

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DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
THE UNIVERSITY OF HONG KONG

PATIENT CONSENT FORM

PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization

Name of Principal Investigator: **Dr. Heidi Cheng**

1. I confirm that I have read and understood the patient information sheet for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals who are relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree that the spent culture medium may be saved for future research.

5. I agree to take part in the above study.

I understand that I will be given a signed copy of this Patient Information and Consent Form.

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____________________	____________________	__________
Subject's signature	Subject's name	Date
Investigator's signature	Investigator's name	Date
Witness' signature	Witness' name	Date

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description
Administrative information		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym P.1/line 2-3
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry P.2/47-48
	2b	All items from the World Health Organization Trial Registration Data Set P2/47-48
Protocol version	3	Date and version identifier Version 2.0. Date 15 April 2021
Funding	4	Sources and types of financial, material, and other support P.11/line 9-10
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors P.1/line 5-19
	5b	Name and contact information for the trial sponsor Not applicable
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities P11/443-444
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) not applicable
Introduction		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention P3/61 – P5/154
	6b	Explanation for choice of comparators P5/156-159
Objectives	7	Specific objectives or hypotheses P5/162-170

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Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) P5/162

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained P5/174-177
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) P.5/189 – P.6/202
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered P6/211-P9/349
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) P6/205-207
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) Not applicable
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial Not applicable
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended P.9/352-369
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) P6/211-P9/349
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations P10/383-393
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size P6

Methods: Assignment of interventions (for controlled trials)

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Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions P.7/243-247
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned P7/243-249
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions P7/243-249
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how P.7/251-253
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial P.6/204-207
Methods: Data collection, management, and analysis		
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol P.9/337-349
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols P9/337-349
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol Data will be stored and kept for 10 years, only accessible by investigators as written in the application during ethics approval
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol P.10/396-406
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses) P.10/396-406

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	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) P.10/396-406
Methods: Monitoring		
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed This is not a sponsored trial
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial No interim analyses will be carried out.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct P9/344-349
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor Not applicable
Ethics and dissemination		
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval P.10/408-415
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) not applicable
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) P6/211-213
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable Not applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial supplemental material
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site P11/447

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Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators supplemental material
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation Not applicable
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions supplemental material
	31b	Authorship eligibility guidelines and any intended use of professional writers no intended use of professional writers
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code no plans for granting public access
Appendices		
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates supplemental material
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable not applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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BMJ Open

A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Title

A randomised double blind controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilisation: a protocol paper

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Abstract

Introduction:

The success rate of in vitro fertilisation (IVF) treatment for couples with infertility remains low due to lack of a reliable tool in selecting euploid embryos for transfer. This study aims to compare the efficacy in embryo selection based on morphology alone compared to non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) and morphology in infertile women undergoing in vitro fertilisation (IVF).

Methods and analysis

This is a randomised double blind controlled trial conducted in two tertiary assisted reproduction centres. A total of 500 infertile women will be recruited and undergo IVF as indicated. They will be randomly assigned on day 6 after oocyte retrieval into two groups: the intervention group using morphology and niPGT-A and the control group based on morphology alone. In the control group, blastocysts with the best quality morphology will be replaced first. In the intervention group, blastocysts with the best morphology and euploid result of spent culture medium will be replaced first. The primary outcome is a live birth per the first embryo transfer. The statistical analysis will be performed with the intention to treat and per protocol.

Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the two participating units. All participants will provide written informed consent before joining the study. The results of the study will be submitted to scientific conferences and peer-reviewed journals.

Registration details

The study was registered under ClinicalTrials.gov (trial number NCT04474522).

Strengths and limitations of this study

- This is a randomised double blind controlled clinical trial.
- The recruitment takes place in a large representative stakeholder group from the field of reproductive medicine in Hong Kong.
- The intervention group of this study includes both morphology and niPGT-A, thereby investigating the sole effect of addition of niPGT-A on embryo prioritization alongside with morphology assessment
- One limitation is that our study is not designed to detect small differences in the live birth rates.

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Introduction

One in seven couples experience difficulty in conceiving. Many of them will require in vitro fertilisation (IVF) treatment (1). IVF involves hormone injections to stimulate a woman's ovaries to produce a number of oocytes which are collected by a minor operation and then mixed with sperm to form embryos in the laboratory. Usually, one or two embryos are transferred to the uterus 2-3 days (cleavage stage embryo transfer) or 5 days (blastocyst transfer) after oocyte retrieval. Despite advances in ovarian stimulation, culture medium and laboratory conditions, the pregnancy and birth rates remain 35% and 25% per transfer in Europe in 2014 (2). The corresponding rates in the United States in 2016 were 45% and 36% per transfer respectively (https://www.cdc.gov/art/reports/2016/national-summary.html).

The success of IVF depends on selection of the most competent embryos for transfer, which is still based on morphological criteria by examining the appearance of the embryos under a microscope. But it is well known that many women fail to achieve a pregnancy even after transfer of what are perceived to be good quality embryos. Therefore, some clinics replace multiple embryos in order to maximise pregnancy rates, a strategy which is associated with a high risk of multiple pregnancy.

Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. It is a major reason for failure of pregnancy, miscarriage and congenital anomalies following both natural conception and IVF pregnancies and increases exponentially with maternal age (3-5).

Our inability to assess embryo quality and select those with the highest potential for implantation on the basis of morphology has led to the use of preimplantation genetic testing for aneuploidy (PGT-A). PGT-A involves biopsy of a few cells from an embryo and assessment of the chromosome copy numbers. While PGT-A cannot create a healthy embryo or improve the quality of an embryo, it provides a method of selecting embryos with a normal number of chromosomes for transfer. This in turn has the potential to increase the chance of having a healthy live birth and reduce the risk of miscarriage or an abnormal fetus caused by an abnormal number of chromosomes.

Fluorescent in-situ hybridization (FISH) was first used in PGT-A studies but can only screen at most 9-12 chromosomes in multiple rounds of FISH with decreasing accuracy. A systematic review of nine randomised controlled trials failed to demonstrate the benefit of the use of PGT-A with FISH(6).

Aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal i.e. comprehensive chromosome screening. Several small randomized controlled trials showed significantly higher pregnancy or live birth rate and lower miscarriage rate following the use of using chromosomal microarray analysis (7-9). The emergence of more advanced genome sequencing such as next generation sequencing (NGS) provides a reliable, high throughput

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approach for PGT-A (10). The turnaround time of PGT-A with NGS is about a week, thus it is not possible to transfer blastocyst in the stimulated cycle. All blastocysts are frozen post biopsy and the blastocysts with normal genetic makeup are thawed and replaced in a subsequent menstrual cycle. Cryopreservation of blastocysts and replacing the frozen blastocysts after thawing in subsequent cycles become a common practice with vitrification as the cryopreservation method (11).

A systematic review of the clinical utility of PGT-A with comprehensive chromosome screening found that three small randomised controlled trials demonstrated benefit in young and good prognosis patients in terms of clinical pregnancy rates and the use of single embryo transfer (12). However, a recent large randomised controlled trial of 661 women comparing PGT-A using NGS vs morphology showed PGT-A did not improve overall pregnancy outcomes in all women aged 25–40 years with at least two blastocysts that could be biopsied (13). It is possible that there is a detrimental effect of the biopsy of blastocysts on the embryo viability that nullifies the benefit of PGT-A.

The traditional PGT involves biopsy of a few cells from trophectoderm of a blastocyst, which requires skilful laboratory staff and additional instrumentation such as laser equipment. The trophectoderm biopsy is an invasive procedure and may lead to reduction in implantation potential, although the implantation potential is less affected when compared with blastomere biopsy from cleavage stage embryos (14). A non-invasive approach to PGT-A is definitely needed.

The demonstration of release of cell-free DNA from human embryos into the surrounding environment opens up the possibility of non-invasive PGT for aneuploidy (niPGT-A)(15). Collection of spent culture medium (SCM) requires no specialized training and imposes negligible risk to the embryo. SCM may be more representative of the whole blastocyst as embryonic DNA is released from both trophectoderm and inner cell mass while the invasive trophectoderm biopsy obtains embryonic DNA from trophectoderm only. Multiple recent studies have demonstrated the ability to detect, extract, and amplify cell-free DNA from SCM at the cleavage and blastocyst stages(16-23). It was shown that 24–48 hr of contact with the embryo was sufficient to collect cell-free DNA from SCM (21). The origin of cell-free DNA can be embryonic or parental. It is proposed that the cell-free DNA is derived from cells discarded by the embryos as a corrective mechanism for aneuploidies (16,24). However, the amount of cell-free DNA was not significantly greater in SCM from aneuploid versus euploid embryos, ruling out this possibility (20). Maternal and paternal contamination in SCM can be minimized by performing thorough oocyte striping and intracytoplasmic sperm injection(25).

In a recent review, the amplification success rate using sequential culture media for SCM ranged between 90% and 100% while the concordance rate in general ploidy i.e. euploid vs aneuploidy between SCM and blastocyst biopsy can be as high as 100% with an average of 75% (26). The difference in the general ploidy between SCM and trophectoderm biopsy can be due to mosaicism, which can be revealed in

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trophectoderm biopsy but not in SCM due to the nature of the DNA source and relatively low embryonic DNA fraction.

All relevant studies on niPGT-A focus the amplification success and concordance rates between SCM and trophectoderm biopsy. A large clinical randomised trial is urgently needed to confirm its efficacy in embryo selection during IVF in terms of live birth and miscarriage rates.

Objective and hypothesis

This randomised double blind controlled trial aims to compare the efficacy in embryo selection based on morphology alone compared to niPGT-A and morphology in infertile women undergoing IVF.

Hypothesis to be tested include:

1. The embryo selection based on niPGT-A and morphology results in a higher live birth rate in IVF as compared with that based on morphology alone.
2. The embryo selection based on niPGT-A and morphology results in a lower miscarriage rate in IVF as compared with that based on morphology alone.

Methods and analysis

Trial design

This is a randomised double blind controlled trial. Eligible women seeking fertility treatment in the Assisted Reproduction Units in Department of Obstetrics & Gynaecology in Kwong Wah Hospital and Queen Mary Hospital will be recruited for the study and informed written consent will be obtained after counseling.

Ethics approval was sought from the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number: KC/KE-20-0098/FR-2). The study was registered in Clinical Trials Registry (Identifier NCT04474522). Written informed consent will be sought before joining the study.

Selection and Withdrawal of subjects:

The population for trial will be infertile women undergoing IVF.

Inclusion criteria:

Women admitted to the study will fulfil all of the following criteria:

- Age less than 43 years at the time of ovarian stimulation and
- Having at least two blastocysts suitable for freezing on day 6 after oocyte retrieval

Exclusion criteria:

Women should not be recruited in any of the following conditions:

- Having less than two blastocysts suitable for freezing on day 6 after oocyte

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retrieval;
- Undergoing PGT for monogenic diseases or structural rearrangement of chromosomes;
- Using donor oocytes;
- Having hydrosalpinx shown on pelvic scanning and not surgically treated

Withdrawal criteria

Participation in the study is totally voluntary. Recruited women can withdraw from the study at any time without giving any reasons and subsequently they will receive standard medical care.

Treatment of subjects:

Methods

Eligible women will be recruited for the study and informed written consent (See online supplemental material) will be obtained after counseling on the day of oocyte retrieval by research nurse or clinicians.

IVF protocol

Infertile women will undergo IVF as clinically indicated. They will receive ovarian stimulation as in standard operation procedure. Ovarian stimulation with gonadotropin injections (150-300 IU daily depending on the antral follicle count) will be given. Medroxyprogesterone acetate 10mg daily will be started on the day 2 of ovarian stimulation or GnRH antagonist (Cetrorelix) 0.25mg daily will be started on the day 6 of ovarian stimulation to prevent premature ovulation. Ultrasound monitoring will be performed to monitor the growth of follicles. When three follicles reach >17 mm in diameter, human chorionic gonadotrophin (Ovidrel 0.25mg) or GnRH agonist (Decapeptyl 0.3mg) will be administered. Oocyte retrieval will be scheduled 36 hours after the trigger under transvaginal ultrasound guidance.

Oocytes will be fertilized conventionally or by intracytoplasmic sperm injection (ICSI) depending on the semen parameters in accordance with the standard operating procedures and normal fertilization will be assessed and confirmed by the presence of two pronuclei. Embryos will be grown individually to the blastocyst stage, up to day 6 after oocyte retrieval, in a monophasic medium. On Day 3, embryos will be rinsed briefly in fresh culture medium. The culture medium will be replenished and culture will be continued at 37°C and 6% CO_2 in reduced oxygen tension (5%). No fresh transfer of blastocysts will be performed in the stimulated cycle.

Grading of blastocyst by morphology

Blastocysts are graded according to Gardner's classification (27). Each blastocyst will be cryopreserved on day 6 by vitrification individually and its SCM (~8 μl) will be frozen at -80^{0}C separately and individually. The embryologist will grade the morphology of blastocysts according to Gardner's criteria .

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Then, on day 6 after oocyte retrieval, women will then be randomly assigned by a PGT laboratory staff into one of the following two groups according to a computer-generated randomization list with a 1:1 ratio and a block size of 10. The randomization list will be prepared by a research nurse, who is not involved in the clinical care of these women.

1. the intervention group using morphology and niPGT-A and
2. the control group based on morphology alone.

The women, clinicians and embryologists in the IVF laboratory will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory will be aware of the group assignment.

niPGT-A of spent culture medium (SCM)

In the intervention group, comprehensive chromosome screening using NGS will be performed according to the recommendations of the company in all SCM samples. In the control group, the measurement will be done retrospectively after a live birth or when all blastocysts are replaced without a live birth.

A commercially available NI-PGT kit (PG-Seq Rapid Non-Invasive PGT kit, PerkinElmer) will be used to analyse the SCM samples. The protocol has been previously optimized with non-invasive samples from 15 laboratories around the world. The kit follows a single tube workflow, two-steps PCR to whole genome amplification of the DNA in SCM and then attaches indexes and sequence-specific adapters to template DNA, resulting in sequencing ready samples.

After purification, equal molar concentration of indexed DNA from each sample will be pooled (96 samples) and then sequenced on a MiSeq system (Illumina) at 1x75 bp read length. On-board secondary analysis will be performed automatically by the MiSeq Reporter (Illumina) followed by the PG-Find Software (v 1.0, PerkinElmer). Reads aligning to anomalous, unstructured and highly repetitive sequences will be filtered from the analysis. A target bin size of 1,000 kb will be used, giving a minimum resolution of 10 Mb. All genomic positions will be referred to the human genome build NCBI 37.

According to the default setting of the PG-Find software, classification of aneuploidy is determined by CNV (copy number variation) value. CNV value >2.7 is considered as gain while CNV value <1.3 is considered as loss. Sample will be concluded as non-euploid when one or more of the chromosomes show gain/loss.

The niPGT-A result of the SCM sample can be euploid, non-euploid and non-informative. It will be used only to prioritize the sequence of embryo transfer.

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Blastocysts with non-euploid result in the niPGT-A report will not be discarded and will be transferred with lower priority.

Blinding

The embryologist will grade the morphology of blastocysts according to Gardner's criteria stated above and the grading of blastocysts will be entered into an online database, which will be managed by an IT technician. The laboratory staff in the PGT laboratory will enter the PGT result into an online database when the niPGT-A results are available. The IT technician will merge the data online to compile the sequence of embryo transfer according to a pre-determined algorithm which depends on the day of blastocyst development (day 5 better than day 6), blastocyst morphology and niPGT-A result of the intervention group. The IT technician will issue the sequence of embryo transfer which does not contain information on the grading of the blastocyst and the NIPGT result to the embryologists in the IVF laboratory. Therefore, the subjects recruited, the clinicians and the embryologists will be blinded to the group allocation.

A pilot study

A pilot study was conducted on 82 SCM from February to September 2020. Media cultured in parallel but without contact with embryos were collected as controls (n=8). Amplification was successful in 80 SCM (97.6%, 80/82) and 72 SCM resulted in conclusive result (90.0%, 72/80). All controls showed no amplification.

In this cohort, 40 SCM with conclusive results were collected from PGT cycles in which trophectoderm biopsies were also performed. 85.0% (34/40) of samples showed concordance results between trophectoderm biopsy and SCM.

Frozen embryo transfer (FET)

Blastocysts will be replaced in the natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. The embryologists in the IVF laboratory will thaw and transfer the blastocyst according to the sequence of embryo transfer generated and issued by the IT technician. In the control group, blastocysts which develop on day 5 after the oocyte retrieval and have the best grading will be replaced first. In the intervention group, blastocysts which develop on day 5 and have the best grading and euploid result will be replaced first. If no blastocysts have euploid result, those with non-informative followed by non-euploid results will be replaced.

Pregnancy

A urine pregnancy test will be performed 14 days after the transfer. If the pregnancy test is positive, transvaginal ultrasound will be performed two weeks later to locate the pregnancy and confirm foetal viability and the number of fetuses. Subsequent management will be the same as other women with

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early pregnancy. They will be referred for antenatal care when the ongoing pregnancy is 8-10 weeks.

Follow-up

Written consent regarding retrieval of pregnancy and delivery data will be sought from the recruited women at the time of study. The women will be contacted after delivery by phone to retrieve the information of the pregnancy outcomes. The outcome of the pregnancy (delivery, miscarriage), number of babies born, birth weights and obstetrics complications will be recorded.

Women in both groups will continue to have blastocyst transfer until all the cryopreserved blastocysts are used up or they become pregnant within 6 months after randomisation. Cumulative live birth rate will be calculated (the number of live birth per couple within the study period). The pregnancy complication and congenital abnormalities of the pregnancies in the two groups will be traced through hospital records or patient contact by mail or phone of mail and compared.

Assessment of outcomes:

The primary outcome is live birth beyond 22 weeks of gestation per the first FET.

Secondary outcomes include

- Cumulative live birth rate: the number of pregnancies leading to live birth within 6 months of randomization.
- Time to pregnancy
- Positive urine pregnancy test
- Clinical pregnancy defined as presence of intrauterine gestational sac on scanning at gestational week 6.
- Ongoing pregnancy as presence of a fetal pole with pulsation at 8-10 weeks of gestation
- Miscarriage defined as a clinically recognized pregnancy loss before the 22 weeks of pregnancy and whose denominator is the clinical pregnancy.
- Multiple pregnancy: presence of more than one intrauterine sac at 6 weeks of gestation
- Ectopic pregnancy
- Pregnancy outcomes including preterm delivery, pre-eclampsia, gestational diabetes, congenital anomaly, perinatal mortality, Apgar score and birthweight of newborn.

Consistency of case management:

The same standardised study protocol will be adopted in the two study centres. The clinicians who manage the women in KWH had all been trained at QMH, and are now adopting the same clinical management protocols in their respective unit. Regular and frequent communication among the two participating centers will also ensure dissemination of updated information on recruitment and safety issues.

Patient and Public involvement

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Patients or the public were not involved in the design, conduct, reporting or dissemination plans of this study.

Statistics

Sample size calculation

From the data in QMH and KWH, the live birth rate following transfer of one blastocyst based on morphology was about 35% in 2018 and 2019. We anticipate following blastocyst morphology and NIPGT-A, the live birth rate will increase from 35% to 50% i.e. 15% increase. The 50% live birth rate is based on the live birth rate observed following conventional PGT-A in the centre. The calculated sample size is 224 women in each group to give a power of 0.9 and type I error of 0.05. Assuming a 10% drop-out rate, the total sample size to be 500, 250 subjects in each group (Figure 1 – Consort 2010 flow diagram).

A pre-specified subgroup analysis will be performed: women aged <35 years vs >=35 years.

Data analysis

Demographic features of women in the two groups will be compared. Comparison of quantitative variables will be performed using Student's t or Mann-whitney U-test where appropriate, while categorical variables will be compared using a Chi-square analysis, multivariable logistic regression or the one-way ANOVA test if more than two categories will be compared. All statistical analyses of the data will be performed with the intention to treat and per protocol using the SPSS program V.26.0 (SPSS Inc, Chicago, Illinois, USA), and a p value <0.05 will be considered statistically significant.

The sensitivity, specificity, likelihood ratios and odd ratio of the niPGT-A result will be calculated.

Ethics and dissemination

Ethics approval was sought from the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (IRB number: UW 20-248) and Research Ethics Committee, Kowloon Central/Kowloon East (IRB number: KC/KE-20-0098/FR-2). All participants will provide written informed consent before randomization. The research findings from this study will be submitted to scientific conferences and peer-reviewed journals for publication so as to disseminate the results to other researchers and clinicians working in the field.

Discussion

The success rate of IVF has long been limited due to the inability to assess the implantation potential of each embryo accurately. Assessment by morphology using Gardner's classification could not reflect chromosomal abnormalities in embryos, which is the reason for failure of implantation in most of the cases. NGS offers several advantages over chromosomal microarray analysis by (i) reduced DNA

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sequencing cost by high throughput sequencing technologies and a high number of samples can be simultaneously sequenced in a single testing; (ii) enhanced detection of partial or segmental aneuploidies as a result of the increase in chromosomal analysis resolution to a few mega bases; (iii) increased dynamic range enabling enhanced detection of mosaicism in multicellular samples and (iv) automation of the sequencing library preparation and automation of the PGT-A diagnostic procedure. However, the beneficial effect of PGT-A has been nullified by the detrimental effect of embryo biopsy. If niPGT-A can be demonstrated to be a better blastocyst evaluation tool than the traditional morphology assessment during IVF treatment, it can potentially shorten the time-to-pregnancy in women with infertility.

Currently, all relevant studies on niPGT-A focused on the amplification success and concordance rates between SCM and trophectoderm biopsy but not on assessing its ability as a screening tool for blastocyst transfer prioritization. This study could provide valuable information on the potential novel use of niPGT-A as an adjunct for morphological assessment of blastocysts.

Trial status

The first subject was recruited on 1st July 2021 and 400 women were recruited up to the writing of this protocol paper.

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Competing interest: The authors report no conflicts of interests.

Contributorship Statement

All authors have substantial contribution to the protocol paper:

Cheng Hiu Yee Heidi: Drafting the manuscript and revising it critically for important intellectual content, responsible for seeking ethics approval and registration on ClinicalTrials.gov

Chow, Judy F.C: substantial contribution to designing the work flow and logistics of niPGT-A, responsible for generating niPGT-A report

Lai, Shui Fan: responsible for the recruitment of subjects and seeking ethics approval at the Kwong Wah Hospital

Lam, Kevin KW: responsible for collaborating with the PGT laboratory regarding logistics of collecting SCM and transferral. Responsible for IVF laboratory.

Ng, Ernest HY: Substantial contributions to the conception and design of the whole study, and final approval of the version to be published

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Yeung, William Shu Biu: Responsible for the design and worked on logistics of the study, and final approval of the version to be published

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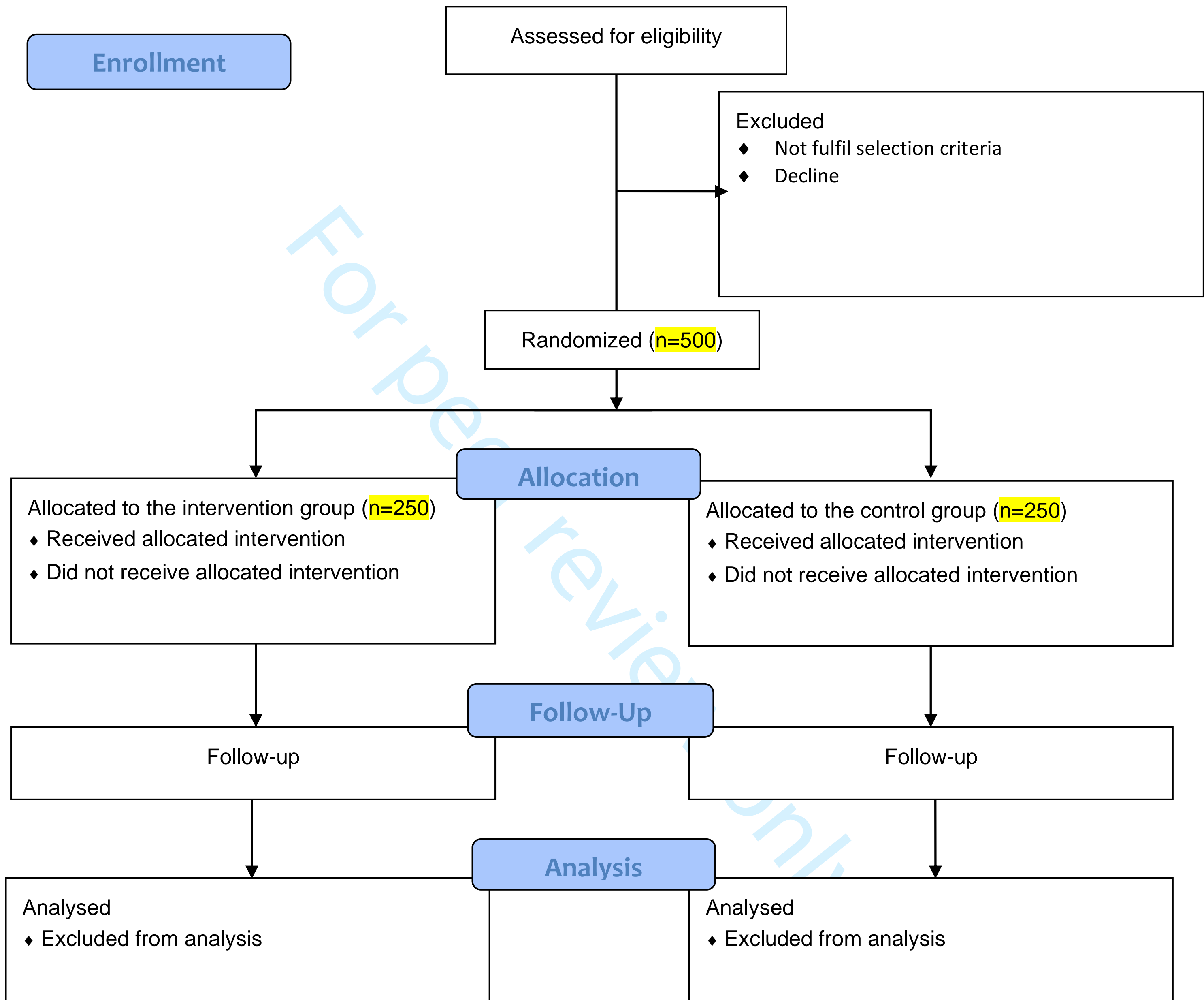
CONSORT 2010 Flow Diagram



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DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
THE UNIVERSITY OF HONG KONG

PATIENT INFORMATION AND CONSENT

PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization
Version 1.0: Dated 8 March 2020

You are cordially invited to participate in the above named research study. You need to decide whether you want to participate or not. Please take your time to make up your mind. Carefully read the following and feel free to ask the study doctor any question which you may have.

Why is this study being done?
Couples with difficulty conceiving will require test-tube baby or in vitro fertilization (IVF). Despite advances in technology, the pregnancy rate of IVF remained around 35% per transfer. Chromosome aneuploidy is an error in cell division that results in the "daughter" cells having the wrong number of chromosomes. In some cases there is a missing chromosome, while in others an extra one. Chromosome aneuploidy is the major reason for failure of pregnancy and miscarriage.

The traditional preimplantation genetic testing for aneupoloidy (PGT-A) involves taking a few cells from a blastocyst, which requires skillful laboratory staff and laser equipment. Taking cells from a blastocyst is an invasive procedure and may lead to reduction in implantation potential. The demonstration of release of DNA from human embryos into the surrounding environment opens up the possibility of non-invasive PGT for aneuploidy (NIPGT-A). Collection of spent culture medium requires no specialized training and imposes negligible risk to the embryo. Spent culture medium may be more representative of the whole blastocyst.

The results of NIPGT-A will be able to prioritize the sequence of embryo transfer. There is still no good evidence to show the efficacy of NIPGT-A in IVF. The aim of this study is to compare with efficacy of embryo selection for replacement based on conventional method through embryo morphology versus morphology with additional input from result of NIPGT-A.

Who should be in this study?
You will be recruited if
1. You are aged less than 43 years at the time of ovarian stimulation, and
2. At least two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval

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You will not be included in this study if

1. Less than two blastocysts suitable for freezing on day 5 or 6 after oocyte retrieval;
2. Women undergoing PGT for monogenic diseases or structural rearrangement of chromosomes
3. Use of donor oocytes
4. Hydrosalpinx shown on pelvic scanning and not surgically treated

What will I be asked to do?

Recruited patients will undergo IVF as clinically indicated. Ovarian stimulation, ultrasound monitoring, and oocyte retrieval will follow the standard operating procedure of the unit.

Each blastocyst will be frozen individually and its spent culture medium (~8 μl) will be frozen at -80^{0}C separately and individually. The embryologist will prepare a sequence of blastocyst transfer based on the best morphology by Gardner's criteria.

On the day of blastocyst freezing, recruited women will then be randomly assigned into two groups using a randomization program.

1. the intervention group using morphology and NIPGT-A and
2. the control group based on morphology alone.

The women and doctors will be blinded to the treatment groups they are assigned. Only the laboratory staff in the PGT laboratory and the embryologists in the IVF laboratory will be aware of the group assignment.

In the intervention group, comprehensive chromosome screening using NGS will be performed. In the control group, the measurement will be done in the spent culture medium of the blastocyst that is replaced in the first transfer. The NIPGT-A report is used only to prioritize the sequence of embryo transfer. Blastocysts with non-euploid result in the NIPGT-A report will not be discarded.

Blastocysts can be replaced in the subsequent natural or hormonal replacement cycles, depending whether the women have regular menstrual cycles or not. Only one blastocyst will be transferred each time. In the control group, blastocysts with the best quality morphology will be replaced first and the sequence of blastocyst transfer is decided prior to randomization. In the intervention group, blastocysts with the best morphology and euploid result will be replaced first as the sequence of blastocyst transfer will be modified after the NIPGT-A reports are available.

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How long will I be in the study?

1 IVF cycle

How many other people will be participating in the study?

We plan to recruit 500 women in this study.

Will I be paid?

No payment will be made to you for this study.

What adverse (bad) effects can happen to me by participating in the study?

There should be no major safety concern as all the embryos will be kept after prioritization regardless of the group recruited.

What benefit can I expect?

You may have a higher pregnancy rate per transfer, lower miscarriage rate and short time to pregnancy if you are randomized to the intervention group with NIPGT-A.

Can I refuse to be in the study?

Your participation in this study is voluntary. You can choose not to take part in the study, or you can quit at any time. You will not lose any benefit to which you are otherwise entitled. If you quit the study, you can receive the standard treatment as other patients in our Department.

Confidentiality and privacy

The investigators have always maintained a strict privacy policy. We never sell, trade or otherwise share your details with any sources. All correspondence to the department is held confidentially; furthermore, at no time will your personal and/or identifying information be shared outside of our organization, for any reason.

Subjects have the rights of access to personal data and known study results, if and when needed. Under the laws of the Hong Kong Special Administrative Region and, in particular, the Personal Data (Privacy) Ordinance, Cap 486, you enjoy or may enjoy rights for the protection of the confidentiality of your personal data, such as those regarding the collection, custody, retention, management, control, use (including analysis or comparison), transfer in or out of Hong Kong, non-disclosure, erasure and/or in any way dealing with or disposing of any of your personal data in or for this study. For any query, you should consult the Privacy Commissioner for Privacy Data or his office (2827 2827) as to the proper monitoring or

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supervision of your personal data protection so that your full awareness and understanding of the significance of compliance with the law governing privacy data is assured.

For questions about the study or reporting of adverse events, please call Dr Heidi Cheng at telephone no. 22553657. The phone number of the Institutional Review Board of The University of Hong Kong / Hospital Authority Hong Kong Wester cluster is 2255 4086.

If you consent to take part in the research, any of your medical records may be inspected by the research team for purposes of analysing the results. They may also be looked at by people from regulatory authorities to check that the study is being carried out correctly. Your name, however, will not be disclosed outside the hospital.

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DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
THE UNIVERSITY OF HONG KONG

PATIENT CONSENT FORM

PROTOCOL TITLE: A double blind randomized controlled trial of non-invasive preimplantation genetic testing for aneuploidy in in vitro fertilization

Name of Principal Investigator: **Dr. Heidi Cheng**

1. I confirm that I have read and understood the patient information sheet for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals who are relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree that the spent culture medium may be saved for future research.

5. I agree to take part in the above study.

I understand that I will be given a signed copy of this Patient Information and Consent Form.

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______________________	______________________	__________
Subject's signature	Subject's name	Date
______________________	______________________	__________
Investigator's signature	Investigator's name	Date
______________________	______________________	__________
Witness' signature	Witness' name	Date

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description
Administrative information		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym P.1/line 2-3
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry P.2/47-48
	2b	All items from the World Health Organization Trial Registration Data Set P2/47-48
Protocol version	3	Date and version identifier Version 2.0. Date 15 April 2021
Funding	4	Sources and types of financial, material, and other support P.11/line 9-10
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors P.1/line 5-19
	5b	Name and contact information for the trial sponsor Not applicable
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities P11/443-444
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) not applicable
Introduction		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention P3/61 – P5/154
	6b	Explanation for choice of comparators P5/156-159
Objectives	7	Specific objectives or hypotheses P5/162-170

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Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) P5/162

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained P5/174-177
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) P.5/189 – P.6/202
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered P6/211-P9/349
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) P6/205-207
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) Not applicable
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial Not applicable
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended P.9/352-369
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) P6/211-P9/349
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations P10/383-393
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size P6

Methods: Assignment of interventions (for controlled trials)

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Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions P.7/243-247
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned P7/243-249
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions P7/243-249
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how P.7/251-253
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial P.6/204-207
Methods: Data collection, management, and analysis		
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol P.9/337-349
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols P9/337-349
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol Data will be stored and kept for 10 years, only accessible by investigators as written in the application during ethics approval
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol P.10/396-406
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses) P.10/396-406

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	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) P.10/396-406
Methods: Monitoring		
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed This is not a sponsored trial
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial No interim analyses will be carried out.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct P9/344-349
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor Not applicable
Ethics and dissemination		
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval P.10/408-415
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) not applicable
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) P6/211-213
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable Not applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial supplemental material
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site P11/447

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Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators supplemental material
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation Not applicable
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions supplemental material
	31b	Authorship eligibility guidelines and any intended use of professional writers no intended use of professional writers
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code no plans for granting public access
Appendices		
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates supplemental material
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable not applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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