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Triple-arm Trial of pH (Tri-pH) Effect on Term Live birth After ICSI

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pH-Study Protocol

Title: Triple-arm Trial of pH (Tri-pH) Effect on Term Live birth After ICSI

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Abstract

Introduction

The pH of culture media for human in vitro fertilization (IVF) is a potential stressor that can affect pre- and post-implantation embryonic growth. There has been no clear evidence about the level that can support in vitro human embryo development optimally. Most manufactures of culture media have specified a range of 7.2 to 7.4, and routine practice is to use a level of 7.25 to 7.3 pH. However, these recommendations resulted from designers' wishes or experiments on mice models. There has been no randomised trial to search for the effect of pH level on live birth rate after IVF. The aim of this trial is to examine if there is an effect on live birth rate using three different levels of pH.

Methods and analysis

This multicentre randomised trial will involve centres specialised in IVF in Egypt. Eligible women amenable for intracytoplasmic sperm injection (ICSI) will be randomized to undergo in vitro culture in either 7.2, 7.3 or 7.4 pH level. The study is designed to detect 10% difference in live birth rate with 93% per cent power at 1% significance level.

Ethics of conduct

Ethics review boards of the participating centres approved the study and eligible women will sign written informed consent before enrolment. The study has established an independent data monitoring and safety committee from international experts in the field and in trial design.

Trial registration number NCT02896777.

Keywords

Embryo culture, pH level, culture media, blastocyst formation

Background

Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six cycles of *in vitro* fertilisation (IVF),[1] which is relatively suboptimal. In addition, IVF results in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared with the *in vivo* conception.[2] These outcomes can rely on factors relating to patients, stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200 variables have been identified as being potentially relevant to the cycle outcome.[3] One element that may influence embryo development *in vitro* is the pH level of a culture medium, which thus far has been determined by manufacturers of culture media without recourse to a well-powered randomized clinical trial (RCT).[4] The pH levels are potential stressors that vary between media brands and from batch-to-batch depending on the bicarbonate level in culture media and on the CO_2 level of incubators.[5] This would suggest that pH level can vary between incubators within the same laboratory if it is not well adjusted. Recommendations for measuring pH for embryo culture are variable between daily to monthly measurement.[4] Oocytes and embryos have intercellular (pHi), which is modulated by the extracellular pH (pHe).[6] The *in vitro* conditions including concentrations of bicarbonates, proteins, amino acids in culture media and the CO_2 of incubators affect the pHe, which is a potential stressor.[7] The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell division and differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal regulation, and microtubule dynamics.[7 8] Drifts in pHe translate into changes in pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt to restore pHi to a safe level.[8] The pHi can compensate through an active exchange among Na^+, $HCO3^-/Cl^-$ and Na^+/H^+ to maintain it between 7 to 7.3.[5 8] Denuded oocyte for ICSI through fertilization thereafter and vitrified-warmed embryos lack robust compensatory

mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios can significantly perturbate embryo development.[9-11]

That being said, an optimum level of pH for human embryo culture in vitro is still unknown.[4 9 12-15] Most recommendations rely on mice models or manufacturers of culture media. Theoretically, a wide range of pHe levels (7.0–7.5) are believed to support human embryo development *in vitro*. This multicentre, randomized, clinical trial aims to compare the influence of three commonly used levels of pH on term live birth rate after ICSI, in order to investigate the potential for optimisation.

Methods and Design

This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for human embryo culture in vitro on term livebirth after ICSI. This partially blind design represents that clinicians, participants and outcome assessor, not including the embryologists, will be unaware of the study arms. This multicentre trial will involve private IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre and Amshaj IVF Centre and others) with the study protocol in their hand before enrolment of participants. This trial obtained the approval from Ethics Review Board of Upper Egypt IVF Network relating to the participating sites (Approval No. 009/2016). An independent safety and monitoring committee formed of five experts in reproductive endocrinology, reproductive biology, embryo culture, biostatistics and trial methodology will oversee this trial. All participants will receive independent counselling from research instructors who are not involved in patient care or laboratory work. Participants who will accept to participate will sign a written informed consent before enrolment. Conducting this study will be in accordance with the Declaration of Helsinki.[16] The trial reporting will be according to the CONSORT statement,[17] unless other guideline will have higher ranking at that time. No plan

exists to amend this protocol and any amendments will be responsibility for the safety committee and will undergo detailed reporting on the trial registry and in the final manuscript.

Oocytes and embryos in the three arms will undergo continuous culture from day 0 through day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after ICSI in pHe of 7.2±0.02. Arm II The “Arm II” is to culture oocytes and resulting embryos after ICSI in pHe of 7.3±0.02. “Arm III” is to culture oocytes and resulting embryos after ICSI in pHe of 7.4±0.02. This trial will include intracytoplasmic sperm injection (ICSI) cycles.

Randomization and Masking

Using an online tool, participants will be randomised to the experimental arms with a 1:1:1 allocation ratio. The allocation sequence of participants will be generated using a permuted block randomization of 3, 6 and 9 block sizes with unique identifiers in random order, stratified by trial site. Randomization of participants and its storage in sequentially numbered, opaque, sealed envelopes will occur by a secretary with no involvement in patient care and will be provided to trial sites before enrolment of first participant. Eligible participants will be allocated to the relevant arms on the day of maturation trigger and allocation result will be communicated to the laboratory team. Participants, clinicians and outcome assessors for the clinical outcomes will be unaware of the allocation, while embryologists who will assess embryo development will be aware of the allocation.

Participants

The inclusion criteria include:

1) Women age of ≥ 18 to ≤ 40;

2) BMI of ≤ 31;

3) Anticipated normal responder by 10 basal ultrasound examination or AMH measurement);

4) Women who have ≥ 1 year of primary or secondary infertility;

5) Fresh ejaculate sperm of any count provided they have ≥ 1% normal forms and a motile fraction;

6) Women undergoing their first ICSI cycle or following a previous successful attempt;

7) Women with > 7 mm endometrial thickness at day of trigger;

and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g. submucosal myomas, polyps or septa).

Women will be excluded if they have:

1) Unilateral oophorectomy;

2) Abnormal karyotyping for them or their male partners;

3) History of repeated abortions or implantation failure;

4) Uncontrolled diabetes;

5) Liver or renal disease;

6) History of severe ovarian hyperstimulation;

7) History of malignancy or borderline pathology;

8) Endometriosis;

9) Plan for PGD-A;

10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;

and 11) A plan for a "freeze-all".

Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support

Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg, Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will continue to the day of maturation trigger. For Antagonist group, women will start the antagonist on day 6 of treatment cycle. All women will receive follicular stimulating

hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG; Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger shot (Choriomon, IBSA) or 250 µg rhCG (Ovitrelle, Merck Serono) for final oocyte maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily, starting on day 1 after retrieval ("day 1") to 12 weeks of gestation, unless negative pregnancy.

Sperm Preparation, Oocyte Denudation and ICSI

Semen samples will be processed through density gradient,[18] using Puresperm (Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in Global HEPES medium under inverted microscope as previously described.[19]

Incubator Management and pH Adjustment

Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to account for incubator as variable. If another brand of incubators will be used, we will ensure they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be used at some centres; however, we will adjust the analysis by trial site to account for

differences between centres. Incubators will undergo stringent control of temperature (36.9±0.1˚C). The temperature will be validated daily using a certified thermometer. Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2 and a proper CO_2 concentration to achieve the required pH. All the three measurements (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with installation of inline filters (Green, Lifeglobal, CooperSurgical).[20]

A minimum of 3 incubators of a single brand within each participating facility with different levels of pH representing the study arms is obligatory: Incubator A of 7.2±0.02 pH, Incubator B of 7.3±0.02 pH, and Incubator C of 7.4±0.02. The three incubators will undergo a strict adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of pH will occur after an overnight incubation of 1mL culture media in a central well dish covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for measuring pH as per the manufacturer protocol. Briefly, after switching on the device, calibration of the device automatically occurs. Next, we adjust the temperature to 37˚C, and select the sample as arterial. Next, we insert the card, which undergoes automatic calibration. Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the first droplet and smoothly inject the sample until the beep. We can see the results of pH, partial CO_2 and O_2 pressures thereafter. Each laboratory will report the results to also compare the resulting partial pressures of CO_2 and O_2 with the incubator display. pH will also be measured every new batch of a culture medium. The measurement of pH and CO_2 across

the centres will be performed using a one-brand equipment that will undergo periodic calibration together. To account for errors in measurement, one well-trained personnel will be assigned to measure the pH and double check the CO_2 level across the centres.

Culture Protocol and Embryo Scoring

Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil, Nidacon). If a decision to change culture media at any time point of the study conduct is made, this will be performed at the same time across the study sites. Dishes will undergo overnight incubation in the relevant incubator adjusted to the relevant pH as per the randomization. After ICSI, the injected oocytes will undergo washing in culture medium followed by incubation from day 0 through day 5 or 6 in the relevant arm of pH, except for the portion of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5/6, with removal of the unfertilized, abnormally fertilized or degenerated oocytes at fertilization check. Two embryologists will perform the fertilization check and embryo grading on day 1, 2 and 3 of culture as per the Istanbul Consensus.[21] All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable for transfer or vitrification on day 5 provided they are graded 311 as per the Istanbul Consensus.[21] Embryos utilized for transfer or cryopreservation will be pictured and recorded in the patient file. All the recorded pictures from all centres will undergo blind grading by two independent experienced embryologists.

Embryo Transfer

Women will undergo fresh embryo transfer by replacing one to two embryos on day 5 with those who replaced embryos on day 3 will be reported as per each centre protocol, except for

women with reduced uterine cavity or previous preterm birth, they will replace only one embryo. One participating centre will transfer majority of its cases on day 3. This issue will be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre standardized protocol. The rest of the utilizable embryos will undergo vitrification for transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted from fresh and vitrified-warmed transfer within one year of randomization. Women will test for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will confirm pregnancy at ≥ week 7 of gestation by detection of intrauterine sac with a heartbeat on ultrasound.

Outcome Measures

Each outcome will be calculated including all randomised participants in the arms to which they were allocated, with the exception of implantation rate, which will be interpreted cautiously due to concerns over its validity as a measure of treatment effect, and perinatal outcomes, which by definition are only available in the subset of participants achieving live birth. This study will adopt the COMMIT definitions of outcomes,[22] where appropriate.

Primary outcome

Live birth (delivery of one or more viable infants > 20th weeks of gestation).

Secondary outcomes

1) Biochemical pregnancy (positive βhCG ≥ 10 IU/L at 14 days after egg retrieval).

2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th weeks of gestation).

3) Ongoing pregnancy (continued viable pregnancy > 20th weeks of gestation).

4) Miscarriage (loss of a clinical pregnancy ≤ 20th weeks of gestation).

5) Term live-birth (i.e. delivery of one or more viable infants ≥37 weeks of gestation).

6) Preterm Birth (delivery of one or more viable infants < 37th weeks of gestation).

7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).

8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)

9) Congenital malformation (delivery of congenitally malformed babies).

10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).

11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-warmed within one year of randomization).

12) Fertilization (presence of 2 pronuclei 17±1 hr after ICSI).

13) Embryo cleavage (cleaved embryos per fertilized oocyte).

14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than 10% fragmentation by volume).

15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).

16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume; ≥ 311 grade per fertilized oocyte).

17) Cryopreservation (cryopreserved embryos per fertilized oocyte).

18) Live-birth-implantation rate (number of viable neonates per number of embryos transferred).

19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized oocyte).

20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst cryopreserved of 311 grade per fertilized oocyte).

Statistical Analysis

Sample size estimation

The primary analysis will test whether there is an increase or decrease in live birth rate as pH changes, using logistic regression. Supposing an increase from 25% to 35% live birth rate across the tested pH range, a sample size of 680 per group would give 93% per cent power to reject the null at a 1% significance level. If, on the other hand, live birth decreased (e.g. from 25% to 15%), then 680 participants per arm would grant more than 99% power at a 5% significance level and 98% power at a 1% level. This is before adjustment for prognostic covariates. This sample size is robust to 5% dropout, yielding > 90% for all scenarios discussed above. Therefore, we anticipate recruiting 2100 participants assigned to the groups at 1:1:1 ratio.

Analytical methods

The study conduct will be according to the intention-to-treat approach, where each participant randomised will be included in the analysis, regardless of protocol deviation. The primary analysis of term live-birth will be conducted using logistic regression, with term live birth event regressed on log(pH), adjusted for study site and participant age, which will be standardised before being entered as a covariate. A secondary analysis of live birth will compare the outcome between each pH group, by replacing log(pH) in the regression model with a dummy variable for treatment group. For secondary outcomes, binary variables will be analysed in an analogous fashion to the primary analysis. Count variables will be analysed using Poisson regression, with zero-inflated models wherever the outcome is undefined for some participants. Again, these will be adjusted for site and age. A 1% significance level will be employed. Due to the short treatment duration, it is anticipated that loss to follow up will be minimal, but if any loss does occur then these participants will be analysed as having negative status for the primary outcome.

Discussion

Given the lack of evidence for a superior pH level for human embryo culture and whether the pH level could make a difference in live birth after IVF, this trial is performed. This trial is expected to fill the gap in this area leaving the wishes of manufactures of culture media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1% significance level) to minimize the risk for uninformative results.

Strengths and limitations of this study

The study is randomised controlled which reduces the possibility of bias. The study has an independent data monitoring committee with access to the data with no involvement in the study conduct. A possible limitation is that the study will be conducted on ICSI cycles as ICSI is the preferred insemination method in the participating centres. The embryologists will be aware of the culture arms during the study conduct.

Funding and conflict of interest

The study receives no fund and the authors have no conflict of interest to declare.

References

1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi: 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update* 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First: 2012/07/26]
4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First: 2012/02/09]
5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation embryo culture media: effects of culture and batches. *Reprod Biomed Online* 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First: 2018/09/20]
6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]

7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi: 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development* 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First: 1998/06/10]
10. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi: 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009;18(6):799-810. [published Online First: 2009/06/06]
12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online First: 2010/11/12]
13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155 [published Online First: 1987/06/01]
14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First: 1999/01/14]
15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J [published Online First: 1998/07/21]
16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ* 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi: 10.1136/bmj.c332 [published Online First: 2010/03/25]
18. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization 2010.
19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-6736(92)92425-f [published Online First: 1992/07/04]
20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory environment and air quality: report of an expert meeting. *Reprod Biomed Online* 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First: 2018/04/17]
21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi: 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and implementing a core outcome set for infertility. *Hum Reprod Open* 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First: 2019/03/22]

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Reporting checklist for protocol of a clinical trial.

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	4
Protocol version	#3	Date and version identifier	4
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#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	13
Roles and responsibilities: committees	#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)	4
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	2 & 3
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	2 & 3
Objectives	#7	Specific objectives or hypotheses	3 & 4
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, non-inferiority, exploratory)	4
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	4

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)	5 & 6
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
Interventions: modifications	#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)	4 & 5
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	10 & 11
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)	8
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	11 & 12
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation: 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	5

		provided in a separate document that is unavailable to those who enrol participants or assign interventions	
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	5
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	5
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	5
Methods: Data collection, management, and analysis			
Data collection plan	#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	11
Data collection plan: retention	#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	11
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	11
Statistics: outcomes	#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	11 & 12

Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11 & 12
Statistics: analysis population and missing data	#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)	11 & 12
Methods: Monitoring			
Data monitoring: formal committee	#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	4
Data monitoring: interim analysis	#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	4, 11 & 12
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	4
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	4
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	4
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)	4
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	4

Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	4
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	4
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	4
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	4
Dissemination policy: trial results	#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	4
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	1
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	4
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	n/a

BMJ Open

Triple-arm Trial of pH (Tri-pH) Effect on Live birth After ICSI: Protocol of a randomised controlled trial

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pH-Study Protocol

Title: Triple-arm Trial of pH (Tri-pH) Effect on Live birth After ICSI: Protocol of A Randomised Controlled Trial

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Abstract

Introduction

The pH of culture media for human in vitro fertilization (IVF) is a potential stressor that can affect pre- and post-implantation embryonic growth. There has been no clear evidence about the level that can support in vitro human embryo development optimally. Most manufactures of culture media have specified a range of 7.2 to 7.4, and routine practice is to use a level of 7.25 to 7.3 pH. However, these recommendations resulted from designers' opinions or experiments on mice models. There has been no randomised trial to search for the effect of pH level on live birth rate after IVF. The aim of this trial is to examine if there is an effect on live birth rate using three different levels of pH.

Methods and analysis

This multicentre randomised trial will involve centres specialised in IVF in Egypt. Eligible women amenable for intracytoplasmic sperm injection (ICSI) will be randomized to undergo in vitro culture in either 7.2, 7.3 or 7.4 pH level. The study is designed to detect 10% difference in live birth rate with 93% per cent power at 1% significance level.

Ethics and dissemination

Ethics review boards of the participating centres approved the study and eligible women will sign written informed consent before enrolment. The study has established an independent data monitoring and safety committee from international experts in the field and in trial design.

Trial registration number NCT02896777.

Keywords

Embryo culture, pH level, culture media, blastocyst formation

Strengths and limitations of this study

- The study is randomised controlled which reduces the possibility of bias.
- The study has an independent data monitoring committee with access to the data with no involvement in the study conduct.
- A possible limitation is that the study will be conducted on ICSI cycles as ICSI is the preferred insemination method in the participating centres.
- The embryologists will be aware of the culture arms during the study conduct.

Background

Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six cycles of *in vitro* fertilisation (IVF),[1] which is relatively suboptimal. In addition, IVF results in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared with the *in vivo* conception.[2] These outcomes can rely on factors relating to patients, stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200 variables have been identified as being potentially relevant to the cycle outcome.[3] One element that may influence embryo development *in vitro* is the pH level of a culture medium, which thus far has been determined by manufacturers of culture media without recourse to a well-powered randomized clinical trial (RCT).[4] The pH levels are potential stressors that vary between media brands and from batch-to-batch depending on the bicarbonate level in culture media and on the CO_2 level of incubators.[5] This would suggest that pH level can vary between incubators within the same laboratory if it is not well adjusted. Recommendations for measuring pH for embryo culture are variable between daily to monthly measurement.[4] Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH (pHe).[6] The *in vitro* conditions including concentrations of bicarbonates, proteins, amino acids in culture media and the CO_2 of incubators affect the pHe, which is a potential stressor.[7]

The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell division and differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal regulation, and microtubule dynamics.[7 8] Drifts in pHe translate into changes in pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt to restore pHi to a safe level.[8] The pHi can compensate through an active exchange among Na^+, $HCO3^-/Cl^-$ and Na^+/H^+ to maintain it between 7 to 7.3.[5 8] Denuded oocyte for ICSI through fertilization thereafter and vitrified-warmed embryos lack robust compensatory mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios can significantly perturbate embryo development.[9-11]

That being said, an optimum level of pH for human embryo culture in vitro is still unknown.[4 9 12-15] Most recommendations rely on mice models or manufacturers of culture media. Theoretically, a wide range of pHe levels (7.0–7.5) are believed to support human embryo development *in vitro*. However, extreme pHe levels can adversely affect oocyte and embryo development.[4] An acidic range of pHe can negative affect the oocyte spindle leading to no fertilization, degeneration or blocking embryo development.[4] An alkaline shift in pHe has the similar adverse effects but lesser than the acidic shift.[4] Recently, there has been anecdotal evidence that culturing embryos at pHe away from 7.2 to 7.4 can lead to mitotic errors. Therefore, the optimal level of pHe for human embryo development is still to be determined. Given the anecdotal evidence that levels between 7.2 to 7.4 pHe can support embryo development with variable results, this multicentre, randomized, clinical trial aims to compare the influence of three commonly used levels of pH (7.2, 7.3 and 7.4) on live birth rate after ICSI, in order to investigate the potential for optimisation.

Methods and Design

This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for

human embryo culture in vitro on live birth after ICSI (Figure 1). This partially blind design represents that clinicians, participants and outcome assessor, not including the embryologists, will be unaware of the study arms. This multicentre trial will involve private IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre and Amshaj IVF Centre) with the study protocol in their hand before enrolment of participants. If other IVF facilities join this trial before recruitment, this will be reported in the study.

Ethics and dissemination

This trial obtained the approval from Ethics Review Board of Upper Egypt IVF Network relating to the participating sites (Approval No. 009/2016). An independent safety and monitoring committee formed of five experts in reproductive endocrinology, reproductive biology, embryo culture, biostatistics and trial methodology will oversee this trial. All participants will receive independent counselling from research instructors who are not involved in patient care or laboratory work. Participants who will accept to participate will sign a written informed consent before enrolment. Conducting this study will be in accordance with the Declaration of Helsinki.[16] The trial reporting will be according to the CONSORT statement,[17] unless other guideline will have higher ranking at that time. No plan exists to amend this protocol and any amendments will be responsibility for the safety committee and will undergo detailed reporting on the trial registry and in the final manuscript.

Intervention

Oocytes and embryos in the three arms will undergo continuous culture from day 0 through day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after ICSI in pHe of 7.2±0.02. Arm II The “Arm II” is to culture oocytes and resulting embryos after ICSI in pHe of 7.3±0.02. “Arm III” is to culture oocytes and resulting embryos after

ICSI in pHe of 7.4±0.02. This trial will include intracytoplasmic sperm injection (ICSI) cycles.

Patient and Public Involvement

No patient involved

Randomization and Masking

Using an online tool, participants will be randomised to the experimental arms with a 1:1:1 allocation ratio. The allocation sequence of participants will be generated using a permuted block randomization of 3, 6 and 9 block sizes with unique identifiers in random order, stratified by trial site. Randomization of participants and its storage in sequentially numbered, opaque, sealed envelopes will occur by a secretary with no involvement in patient care and will be provided to trial sites before enrolment of first participant. Eligible participants will be allocated to the relevant arms on the day of maturation trigger and allocation result will be communicated to the laboratory team. Participants, clinicians and outcome assessors for the clinical outcomes will be unaware of the allocation, while embryologists who will assess embryo development will be aware of the allocation.

Participants

The inclusion criteria include:

1) Women age of ≥ 18 to ≤ 40;

2) BMI of ≤ 31;

3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);

4) Women who have ≥ 1 year of primary or secondary infertility;

5) Fresh ejaculate sperm of any count provided they have ≥ 1% normal forms and a motile fraction;

6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous successful one;

7) Women with > 7 mm endometrial thickness at day of trigger;

and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g. submucosal myomas, polyps or septa).

Women will be excluded if they have:

1) Unilateral oophorectomy;

2) Abnormal karyotyping for them or their male partners;

3) History of repeated abortions or implantation failure;

4) Uncontrolled diabetes;

5) Liver or renal disease;

6) History of severe ovarian hyperstimulation;

7) History of malignancy or borderline pathology;

8) Endometriosis;

9) Plan for PGD-A;

10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;

and 11) Severe PCOS, hyper-responder, OHSS patients, and cycles with agonist trigger or any patient with a plan for a "freeze-all".

Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support

Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg, Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will continue to the day of maturation trigger. For Antagonist group, women will start the antagonist on day 6 of treatment cycle. All women will receive follicular stimulating hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG; Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles

measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger shot (Choriomon, IBSA) for final oocyte maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily, starting on day 1 after retrieval ("day 1") to 12 weeks of gestation, unless negative pregnancy.

Sperm Preparation, Oocyte Denudation and ICSI

Semen samples will be processed through density gradient,[18] using Puresperm (Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in Global HEPES medium under inverted microscope as previously described.[19]

Incubator Management and pH Adjustment

Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to account for incubator as variable. If another brand of incubators will be used, we will ensure they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be used at some centres; however, we will adjust the analysis by trial site to account for differences between centres. Incubators will undergo stringent control of temperature (36.9±0.1˚C). The temperature will be validated daily using a certified thermometer. Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2

and a proper CO_2 concentration to achieve the required pH. All the three measurements (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with installation of inline filters (Green, Lifeglobal, CooperSurgical).[20]

A minimum of 3 incubators of a single brand within each participating facility with different levels of pH representing the study arms is obligatory: Incubator A of 7.2±0.02 pH, Incubator B of 7.3±0.02 pH, and Incubator C of 7.4±0.02. The three incubators will undergo a strict adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of pH will occur after an overnight incubation of 1mL culture media in a central well dish covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for measuring pH as per the manufacturer protocol. Briefly, after switching on the device, calibration of the device automatically occurs. Next, we adjust the temperature to 37˚C, and select the sample as arterial. Next, we insert the card, which undergoes automatic calibration. Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the first droplet and smoothly inject the sample until the beep. We can see the results of pH, partial CO_2 and O_2 pressures thereafter. Each laboratory will report the results to also compare the resulting partial pressures of CO_2 and O_2 with the incubator display. pH will also be measured every new batch of a culture medium. The measurement of pH and CO_2 across the centres will be performed using a one-brand equipment that will undergo periodic calibration together. To account for errors in measurement, one well-trained personnel will be assigned to measure the pH and double check the CO_2 level across the centres.

Culture Protocol and Embryo Scoring

Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil, Nidacon). If a decision to change culture media at any time point of the study conduct is made, this will be performed at the same time across the study sites. Dishes will undergo overnight incubation in the relevant incubator adjusted to the relevant pH as per the randomization. After ICSI, the injected oocytes will undergo washing in culture medium followed by incubation from day 0 through day 5 or 6 in the relevant arm of pH, except for a small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5/6, with removal of the unfertilized, abnormally fertilized or degenerated oocytes at fertilization check. Two embryologists will perform the fertilization check and embryo grading on day 1, 2 and 3 of culture as per the Istanbul Consensus.[21] All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable for transfer or vitrification on day 5 provided they are graded 311 as per the Istanbul Consensus.[21] Embryos utilized for transfer or cryopreservation will be pictured and recorded in the patient file. All the recorded pictures from all centres will undergo blind grading by two independent experienced embryologists.

Embryo Transfer

Women will undergo fresh embryo transfer by replacing one to two embryos on day 5 with those who replaced embryos on day 3 will be reported as per each centre protocol, except for women with reduced uterine cavity or previous preterm birth, they will replace only one embryo. One participating centre will transfer majority of its cases on day 3. This issue will be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under

sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre standardized protocol. The rest of the utilizable embryos will undergo vitrification for transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted from fresh and vitrified-warmed transfer within one year of randomization. Women will test for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will confirm pregnancy at ≥ week 7 of gestation by detection of intrauterine sac with a heartbeat on ultrasound.

Outcome Measures

Each outcome will be calculated including all randomised participants in the arms to which they were allocated, with the exception of implantation rate, which will be interpreted cautiously due to concerns over its validity as a measure of treatment effect, and perinatal outcomes, which by definition are only available in the subset of participants achieving live birth. This study will adopt the COMMIT definitions of outcomes,[22] where appropriate.

Primary outcome

Live birth (delivery of one or more viable infants > 20th weeks of gestation).

Secondary outcomes

1) Biochemical pregnancy (positive βhCG ≥ 10 IU/L at 14 days after egg retrieval).

2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th weeks of gestation).

3) Ongoing pregnancy (continued viable pregnancy > 20th weeks of gestation).

4) Miscarriage (loss of a clinical pregnancy ≤ 20th weeks of gestation).

5) Term live-birth (i.e. delivery of one or more viable infants ≥37 weeks of gestation).

6) Preterm Birth (delivery of one or more viable infants < 37th weeks of gestation).

7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).

8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)

9) Congenital malformation (delivery of congenitally malformed babies).

10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).

11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-warmed within one year of randomization).

12) Fertilization (presence of 2 pronuclei 17±1 hr after ICSI).

13) Embryo cleavage (cleaved embryos per fertilized oocyte).

14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than 10% fragmentation by volume).

15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).

16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume; ≥ 311 grade per fertilized oocyte).

17) Cryopreservation (cryopreserved embryos per fertilized oocyte).

18) Live-birth-implantation rate (live birth per embryo transferred).

19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized oocyte).

20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst cryopreserved of 311 grade per fertilized oocyte).

Statistical Analysis

Sample size estimation

This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested is that adjusting the pH value to the edges of this range might result in improvements to the live birth rate, although we remain in equipoise as to whether higher or lower values will be optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and our goal is to investigate whether this is associated with varying pH.

The study has been powered for a global test of the effect of pH, calculated using plausible birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yields 98% power in this scenario, using a 5% significance level. This test makes no assumption about the ordering of the live birth rates in relation to the ordering of the pH values. The high power level has been adopted to allow for some leeway in relation to the minimum effect size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just seven percentage points) this sample size yields 85% power against a 5% significance level, and 65% at a 1% significance level. We have also been conservative in our inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to 680. In reality, we will conduct analysis on an intention to treat basis, including all randomised women. Women who do not complete treatment (for example, they do not undergo embryo transfer) will be counted as not having a live birth. The only exceptions to this will be participants who withdraw consent for their data to be used in the study. Our inflation for loss to follow up reflects this possibility.

We also note that adjustment for site and age in the analysis will increase power further.

Analytical methods

The study conduct will be according to the intention-to-treat approach, where each participant randomised will be included in the analysis, regardless of protocol deviation. The primary analysis of live birth will be conducted using logistic regression, with live birth event regressed on pH group, adjusted for study site and participant age, which will be standardised before being entered as a covariate. pH will be entered as a categorical covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate across the three groups to be performed. Secondary supportive analyses will be conducted to try to characterise the nature of any association. This will include a test of linear trend in live birth rates across pH

groups, which would imply an optimal pH level for the lowest or highest value, as well as pairwise comparisons between each group (again, these analyses will be adjusted for site and age). The pairwise comparisons will focus on size and precision of the odds ratios. Although it would be desirable to power the study for all pairwise comparisons as the primary outcome, this yields impracticable sample sizes (> 4000 participants) against realistic effects. The study has therefore been designed to represent the most informative test of the hypothesis that pH level affects live birth, that is practicable.

For secondary outcomes, binary variables will be analysed in an analogous fashion to the primary analysis. Count variables will be analysed using Poisson regression, with zero-inflated models wherever the outcome is structurally undefined for some participants. Again, these will be adjusted for site and age. In the analysis of number of usable embryos, implanted embryos arising from the day 3 transfer will be included as formed and good quality blastocysts, while those that do not implant in this portion will be considered blocked at day 3. The total of the number of embryos transferred and the formed blastocysts will be used to calculate number of utilizable embryos. A 1% significance level will be employed. Due to the short treatment duration, it is anticipated that loss to follow up will be minimal, but if any loss does occur then these participants will be analysed as having negative status for the primary outcome, unless consent to use data is withdrawn. The follow-up period is identified as one year from randomization of the last participant provided that all pregnant women have given birth.

Discussion

Given the lack of evidence for a superior pH level for human embryo culture and whether the pH level could make a difference in live birth after IVF, this trial is performed. This trial is expected to fill the gap in this area leaving the recommendations of manufactures of culture

media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1% significance level) to minimize the risk for uninformative results.

Funding and conflict of interest

The study receives no fund and the authors have no conflict of interest to declare.

Authors' contributions

Mohamed is the creator of the concept and design of the study, and is the principal investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across the sites and will make sure that data is periodically sent to for storage in independent database. Jack Wilkinson is the statistician of the study who revised the study design and calculated the sample size and power of the study and he will be responsible for the data analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a primary investigator at Banon IVF centre and a sub-investigator at Ibnsina Centre, and she participated in revising the trial protocol and will participate in trial reporting thereafter. Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the study that will make sure all male partners are in line with the inclusion criteria, and revised the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF Centre and participated revising the protocol and provided comments. Mohamed AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF Centre. Hazem Abdelghafar is a primary investigator at Ibnsina IVF Centre and participated in the trial design. All authors provided comments and agreed on the study design and protocol, and will participate in reporting this trial thereafter.

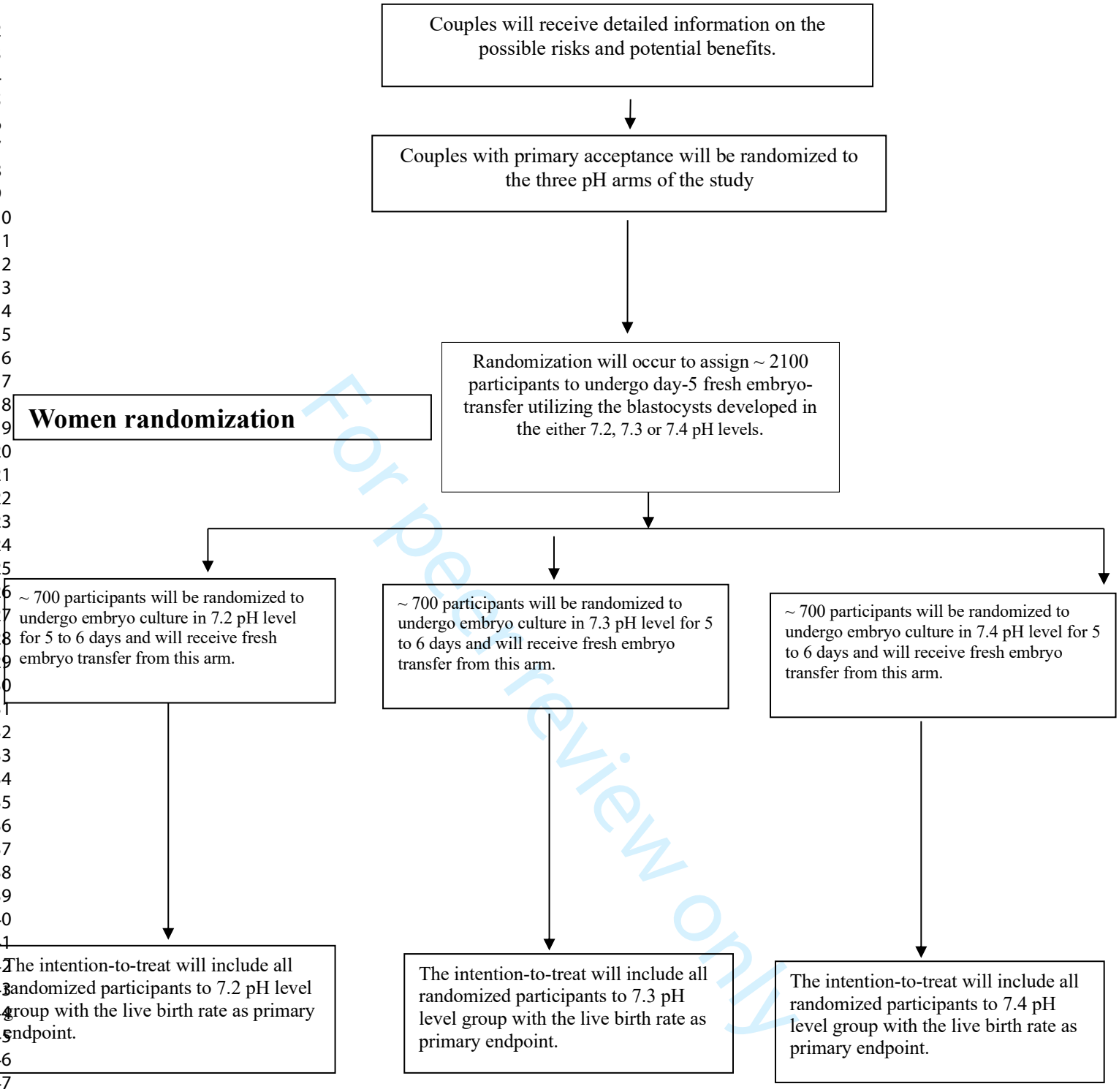
References

1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi: 10.1001/jama.2015.17296 [published Online First: 2015/12/31]

2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update* 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First: 2012/07/26]
4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First: 2012/02/09]
5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation embryo culture media: effects of culture and batches. *Reprod Biomed Online* 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First: 2018/09/20]
6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi: 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development* 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First: 1998/06/10]
10. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi: 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009;18(6):799-810. [published Online First: 2009/06/06]
12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online First: 2010/11/12]
13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155 [published Online First: 1987/06/01]
14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First: 1999/01/14]
15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J [published Online First: 1998/07/21]
16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ* 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi: 10.1136/bmj.c332 [published Online First: 2010/03/25]

18. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization 2010.
19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-6736(92)92425-f [published Online First: 1992/07/04]
20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory environment and air quality: report of an expert meeting. *Reprod Biomed Online* 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First: 2018/04/17]
21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi: 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and implementing a core outcome set for infertility. *Hum Reprod Open* 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First: 2019/03/22]

Figure Legend: Trial plan for enrolment



pH trial plan for enrollment and outcomes – 7.2, 7.3 and 7.4 pH levels

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	4
Protocol version	#3	Date and version identifier	4
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#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	13
Roles and responsibilities: committees	#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)	4
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	2 & 3
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	2 & 3
Objectives	#7	Specific objectives or hypotheses	3 & 4
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, non-inferiority, exploratory)	4
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	4

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)	5 & 6
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
Interventions: modifications	#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)	4 & 5
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	10 & 11
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)	8
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	11 & 12
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation: 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	5

		provided in a separate document that is unavailable to those who enrol participants or assign interventions	
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	5
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	5
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	5
Methods: Data collection, management, and analysis			
Data collection plan	#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	11
Data collection plan: retention	#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	11
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	11
Statistics: outcomes	#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	11 & 12

Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11 & 12
Statistics: analysis population and missing data	#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)	11 & 12
Methods: Monitoring			
Data monitoring: formal committee	#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	4
Data monitoring: interim analysis	#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	4, 11 & 12
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	4
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	4
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	4
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)	4
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	4

Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	4
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	4
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	4
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	4
Dissemination policy: trial results	#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	4
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	1
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	4
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	n/a

BMJ Open

Triple-arm Trial of pH (Tri-pH) Effect on Live birth After ICSI: Protocol of a randomised controlled trial

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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

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pH-Study Protocol

Title: Triple-arm Trial of pH (Tri-pH) Effect on Live birth After ICSI: Protocol of A Randomised Controlled Trial

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Abstract

Introduction

The pH of culture media for human in vitro fertilization (IVF) is a potential stressor that can affect pre- and post-implantation embryonic growth. There has been no clear evidence about the level that can support in vitro human embryo development optimally. Most manufactures of culture media have specified a range of 7.2 to 7.4, and routine practice is to use a level of 7.25 to 7.3 pH. However, these recommendations resulted from designers' opinions or experiments on mice models. There has been no randomised trial to search for the effect of pH level on live birth rate after IVF. The aim of this trial is to examine if there is an effect on live birth rate using three different levels of pH.

Methods and analysis

This multicentre randomised trial will involve centres specialised in IVF in Egypt. Eligible women amenable for intracytoplasmic sperm injection (ICSI) will be randomized to undergo in vitro culture in either 7.2, 7.3 or 7.4 pH level. The study is designed to detect 10% difference in live birth rate with 93% per cent power at 1% significance level.

Ethics and dissemination

Ethics review boards of the participating centres approved the study and eligible women will sign written informed consent before enrolment. The study has established an independent data monitoring and safety committee from international experts in the field and in trial design.

Trial registration number NCT02896777.

Keywords

Embryo culture, pH level, culture media, blastocyst formation

Strengths and limitations of this study

- The study is randomised controlled which reduces the possibility of bias.
- The study has an independent data monitoring committee with access to the data with no involvement in the study conduct.
- Two possible limitations of the study are that it will be conducted on ICSI cycles as ICSI is the preferred insemination method in the participating centres, and formed blastocyst calculation will be based on assumption for any cleavage-stage transfer.
- The embryologists will be aware of the culture arms during the study conduct.

Background

Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six cycles of *in vitro* fertilisation (IVF),[1] which is relatively suboptimal. In addition, IVF results in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared with the *in vivo* conception.[2] These outcomes can rely on factors relating to patients, stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200 variables have been identified as being potentially relevant to the cycle outcome.[3] One element that may influence embryo development *in vitro* is the pH level of a culture medium, which thus far has been determined by manufacturers of culture media without recourse to a well-powered randomized clinical trial (RCT).[4] The pH levels are potential stressors that vary between media brands and from batch-to-batch depending on the bicarbonate level in culture media and on the CO_2 level of incubators.[5] This would suggest that pH level can vary between incubators within the same laboratory if it is not well adjusted. Recommendations for measuring pH for embryo culture are variable between daily to monthly measurement.[4] Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH (pHe).[6] The *in vitro* conditions including concentrations of bicarbonates, proteins, amino

acids in culture media and the CO_2 of incubators affect the pHe, which is a potential stressor.[7] The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell division and differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal regulation, and microtubule dynamics.[7 8] Drifts in pHe translate into changes in pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt to restore pHi to a safe level.[8] The pHi can compensate through an active exchange among Na^+, $HCO3^-/Cl^-$ and Na^+/H^+ to maintain it between 7 to 7.3.[5 8] Denuded oocyte for ICSI through fertilization thereafter and vitrified-warmed embryos lack robust compensatory mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios can significantly perturbate embryo development.[9-11]

That being said, an optimum level of pH for human embryo culture in vitro is still unknown.[4 9 12-15] Most recommendations rely on mice models or manufacturers of culture media. Theoretically, a wide range of pHe levels (7.0–7.5) can support human embryo development *in vitro*. However, a narrower range of pHe levels (7.2 to 7.4) is used in clinical practice. This is because an extreme acidic pHe level (≤ 7) can adversely affect oocyte spindle leading to no further post-fertilization events.[4] This level of acidic pH can delay or block embryo development *in vitro*.[4] Similar harms can theoretically occur for oocyte and embryo, if alkaline levels of pHe (≥ 7.5) are used.[4] Although these potential harms of extreme pHe levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to investigate a safe range of pHe (7.2 to 7.4). Why this range has been chosen depends on the recommendations of media manufactures and the clinical practice within the majority of human embryo culture laboratories. Although this range of 7.2 to 7.4 is used, there is clear evidence about which level of 7.2, 7.3, or 7.4 can support human embryos to result in live birth after transfer. This multicentre, randomized, clinical trial aims to identify whether pHe

levels of 7.2, 7.3, or 7.4 can perform better on live birth rate after ICSI in order to investigate the potential for optimisation.

Methods and Design

This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for human embryo culture in vitro on live birth after ICSI (Figure 1). This partially blind design represents that clinicians, participants and outcome assessor, not including the embryologists, will be unaware of the study arms. This multicentre trial will involve private IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre and Amshaj IVF Centre) with the study protocol in their hand before enrolment of participants. If other IVF facilities join this trial before recruitment, this will be reported in the study.

Ethics and dissemination

This trial obtained the approval from Ethics Review Board of Upper Egypt IVF Network relating to the participating sites (Approval No. 009/2016). An independent safety and monitoring committee formed of five experts in reproductive endocrinology, reproductive biology, embryo culture, biostatistics and trial methodology will oversee this trial. All participants will receive independent counselling from research instructors who are not involved in patient care or laboratory work. Participants who will accept to participate will sign a written informed consent before enrolment. Conducting this study will be in accordance with the Declaration of Helsinki.[16] The trial reporting will be according to the CONSORT statement,[17] unless other guideline will have higher ranking at that time. No plan exists to amend this protocol and any amendments will be responsibility for the safety committee and will undergo detailed reporting on the trial registry and in the final manuscript.

Intervention

Oocytes and embryos in the three arms will undergo continuous culture from day 0 through day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after ICSI in pHe of 7.2±0.02. Arm II The “Arm II” is to culture oocytes and resulting embryos after ICSI in pHe of 7.3±0.02. “Arm III” is to culture oocytes and resulting embryos after ICSI in pHe of 7.4±0.02. This trial will include intracytoplasmic sperm injection (ICSI) cycles.

Patient and Public Involvement

No patient involved

Randomization and Masking

Using an online tool, participants will be randomised to the experimental arms with a 1:1:1 allocation ratio. The allocation sequence of participants will be generated using a permuted block randomization of 3, 6 and 9 block sizes with unique identifiers in random order, stratified by trial site. Randomization of participants and its storage in sequentially numbered, opaque, sealed envelopes will occur by a secretary with no involvement in patient care and will be provided to trial sites before enrolment of first participant. Eligible participants will be allocated to the relevant arms on the day of maturation trigger and allocation result will be communicated to the laboratory team. Participants, clinicians and outcome assessors for the clinical outcomes will be unaware of the allocation, while embryologists who will assess embryo development will be aware of the allocation.

Participants

The inclusion criteria include:

1) Women age of ≥ 18 to ≤ 40;

2) BMI of ≤ 31;

3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);

4) Women who have ≥ 1 year of primary or secondary infertility;

5) Fresh ejaculate sperm of any count provided they have ≥ 1% normal forms and a motile fraction;

6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous successful one;

7) Women with > 7 mm endometrial thickness at day of trigger;

and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g. submucosal myomas, polyps or septa).

Women will be excluded if they have:

1) Unilateral oophorectomy;

2) Abnormal karyotyping for them or their male partners;

3) History of repeated abortions or implantation failure;

4) Uncontrolled diabetes;

5) Liver or renal disease;

6) History of severe ovarian hyperstimulation;

7) History of malignancy or borderline pathology;

8) Endometriosis;

9) Plan for PGD-A;

10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;

and 11) PCOS, women with history of severe OHSS, and cycles with agonist trigger or any patient with a plan for a "freeze-all".

Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support

Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg, Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will

continue to the day of maturation trigger. For Antagonist group, women will start the antagonist on day 6 of treatment cycle. All women will receive follicular stimulating hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG; Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger shot (Choriomon, IBSA) for final oocyte maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily, starting on day 1 after retrieval ("day 1") to 12 weeks of gestation, unless negative pregnancy.

Sperm Preparation, Oocyte Denudation and ICSI

Semen samples will be processed through density gradient,[18] using Puresperm (Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in Global HEPES medium under inverted microscope as previously described.[19]

Incubator Management and pH Adjustment

Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to

account for incubator as variable. If another brand of incubators will be used, we will ensure they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be used at some centres; however, we will adjust the analysis by trial site to account for differences between centres. Incubators will undergo stringent control of temperature (36.9±0.1˚C). The temperature will be validated daily using a certified thermometer. Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2 and a proper CO_2 concentration to achieve the required pH. All the three measurements (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with installation of inline filters (Green, Lifeglobal, CooperSurgical).[20]

A minimum of 3 incubators of a single brand within each participating facility with different levels of pH representing the study arms is obligatory: Incubator A of 7.2±0.02 pH, Incubator B of 7.3±0.02 pH, and Incubator C of 7.4±0.02. The three incubators will undergo a strict adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of pH will occur after an overnight incubation of 1mL culture media in a central well dish covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for measuring pH as per the manufacturer protocol. Briefly, after switching on the device, calibration of the device automatically occurs. Next, we adjust the temperature to 37˚C, and select the sample as arterial. Next, we insert the card, which undergoes automatic calibration. Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the first droplet and smoothly inject the sample until the beep. We can see the results of pH,

partial CO_2 and O_2 pressures thereafter. Each laboratory will report the results to also compare the resulting partial pressures of CO_2 and O_2 with the incubator display. pH levels will also be measured every new batch of a culture medium. The measurement of pH and CO_2 across the centres will be performed using a one-brand equipment that will undergo periodic calibration together. To account for errors in measurement, one well-trained personnel will be assigned to measure the pH and double check the CO_2 level across the centres.

Culture Protocol and Embryo Scoring

Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil, Nidacon). If a decision to change culture media at any time point of the study conduct is made, this will be performed at the same time across the study sites. Dishes will undergo overnight incubation in the relevant incubator adjusted to the relevant pH as per the randomization. After ICSI, the injected oocytes will undergo washing in culture medium followed by incubation from day 0 through day 5 or 6 in the relevant arm of pH, except for a small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5/6, with removal of the unfertilized, abnormally fertilized or degenerated oocytes at fertilization check. Two embryologists will perform the fertilization check and embryo grading on day 1, 2 and 3 of culture as per the Istanbul Consensus.[21] All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable for transfer or vitrification on day 5 provided they are graded 311 as per the Istanbul Consensus.[21] Embryos utilized for transfer or cryopreservation will be pictured and recorded in the patient file. All the recorded pictures from all centres will undergo blind grading by two independent experienced embryologists.

Embryo Transfer

Women will undergo fresh embryo transfer by replacing one to two embryos on day 5 with those who replaced embryos on day 3 will be reported as per each centre protocol, except for women with reduced uterine cavity or previous preterm birth, they will replace only one embryo. One participating centre will transfer majority of its cases on day 3. This issue will be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre standardized protocol. The rest of the utilizable embryos will undergo vitrification for transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted from fresh and vitrified-warmed transfer within one year of randomization. Women will test for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will confirm pregnancy at ≥ week 7 of gestation by detection of intrauterine sac with a heartbeat on ultrasound.

Outcome Measures

Each outcome will be calculated including all randomised participants in the arms to which they were allocated, with the exception of implantation rate, which will be interpreted cautiously due to concerns over its validity as a measure of treatment effect, and perinatal outcomes, which by definition are only available in the subset of participants achieving live birth. This study will adopt the COMMIT definitions of outcomes,[22] where appropriate.

Primary outcome

Live birth (delivery of one or more viable infants > 20th weeks of gestation).

Secondary outcomes

1) Biochemical pregnancy (positive βhCG ≥ 10 IU/L at 14 days after egg retrieval).

2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th weeks of gestation).

3) Ongoing pregnancy (continued viable pregnancy > 20th weeks of gestation).

4) Miscarriage (loss of a clinical pregnancy ≤ 20th weeks of gestation).

5) Term live-birth (i.e. delivery of one or more viable infants ≥37 weeks of gestation).

6) Preterm Birth (delivery of one or more viable infants < 37th weeks of gestation).

7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).

8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)

9) Congenital malformation (delivery of congenitally malformed babies).

10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).

11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-warmed within one year of randomization).

12) Fertilization (presence of 2 pronuclei 17±1 hr after ICSI).

13) Embryo cleavage (cleaved embryos per fertilized oocyte).

14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than 10% fragmentation by volume).

15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).

16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume; ≥ 311 grade per fertilized oocyte).

17) Cryopreservation (cryopreserved embryos per fertilized oocyte).

18) Live-birth-implantation rate (live birth per embryo transferred).

19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized oocyte).

20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst cryopreserved of 311 grade per fertilized oocyte).

Statistical Analysis

Sample size estimation

This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested is that adjusting the pH value to the edges of this range might result in improvements to the live birth rate, although we remain in equipoise as to whether higher or lower values will be optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and our goal is to investigate whether this is associated with varying pH.

The study has been powered for a global test of the effect of pH, calculated using plausible birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yields 98% power in this scenario, using a 5% significance level. This test makes no assumption about the ordering of the live birth rates in relation to the ordering of the pH values. The high power level has been adopted to allow for some leeway in relation to the minimum effect size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just seven percentage points) this sample size yields 85% power against a 5% significance level, and 65% at a 1% significance level. We have also been conservative in our inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to 680. In reality, we will conduct analysis on an intention to treat basis, including all randomised women. Women who do not complete treatment (for example, they do not undergo embryo transfer) will be counted as not having a live birth. The only exceptions to this will be participants who withdraw consent for their data to be used in the study. Our inflation for loss to follow up reflects this possibility.

We also note that adjustment for site and age in the analysis will increase power further.

Analytical methods

The study conduct will be according to the intention-to-treat approach, where each participant randomised will be included in the analysis, regardless of protocol deviation. The primary analysis of live birth will be conducted using logistic regression, with live birth event

regressed on pH group, adjusted for study site and participant age, which will be standardised before being entered as a covariate. pH will be entered as a categorical covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate across the three groups to be performed. Secondary supportive analyses will be conducted to try to characterise the nature of any association. This will include a test of linear trend in live birth rates across pH groups, which would imply an optimal pH level for the lowest or highest value, as well as pairwise comparisons between each group (again, these analyses will be adjusted for site and age). The pairwise comparisons will focus on size and precision of the odds ratios. Although it would be desirable to power the study for all pairwise comparisons as the primary outcome, this yields impracticable sample sizes (> 4000 participants) against realistic effects. The study has therefore been designed to represent the most informative test of the hypothesis that pH level affects live birth, that is practicable.

For secondary outcomes, binary variables will be analysed in an analogous fashion to the primary analysis. Count variables will be analysed using Poisson regression, with zero-inflated models wherever the outcome is structurally undefined for some participants. Again, these will be adjusted for site and age. In the analysis of number of usable embryos, implanted embryos arising from the day 3 transfer will be included as formed and good quality blastocysts, while those that do not implant in this portion will be considered blocked at day 3. The total of the number of embryos transferred and the formed blastocysts will be used to calculate number of utilizable embryos. A 1% significance level will be employed. Due to the short treatment duration, it is anticipated that loss to follow up will be minimal, but if any loss does occur then these participants will be analysed as having negative status for the primary outcome, unless consent to use data is withdrawn. The follow-up period is

identified as one year from randomization of the last participant provided that all pregnant women have given birth.

Discussion

Given the lack of evidence for a superior pH level for human embryo culture and whether the pH level could make a difference in live birth after IVF, this trial is performed. This trial is expected to fill the gap in this area leaving the recommendations of manufactures of culture media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1% significance level) to minimize the risk for uninformative results. In any occasion of cleavage-stage transfer, the calculation of the blastocyst formation will be based on the assumption that embryos that will implant will be calculated as a formed blastocyst, while the failure of implantation of an embryo will be considered as embryo block at the cleavage stage. Although this is not the ideal track to calculate blastocyst formation, we find this assumption is the closest one to reflect the blastocyst formation. This will be further discussed when this trial is reported.

Funding and conflict of interest

The study receives no fund and the authors have no conflict of interest to declare.

Authors' contributions

Mohamed is the creator of the concept and design of the study, and is the principal investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across the sites and will make sure that data is periodically sent to for storage in independent database. Jack Wilkinson is the statistician of the study who revised the study design and calculated the sample size and power of the study and he will be responsible for the data analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a primary investigator at Banon IVF centre and a sub-investigator at Ibnsina Centre, and she participated in revising the trial protocol and will participate in trial reporting thereafter.

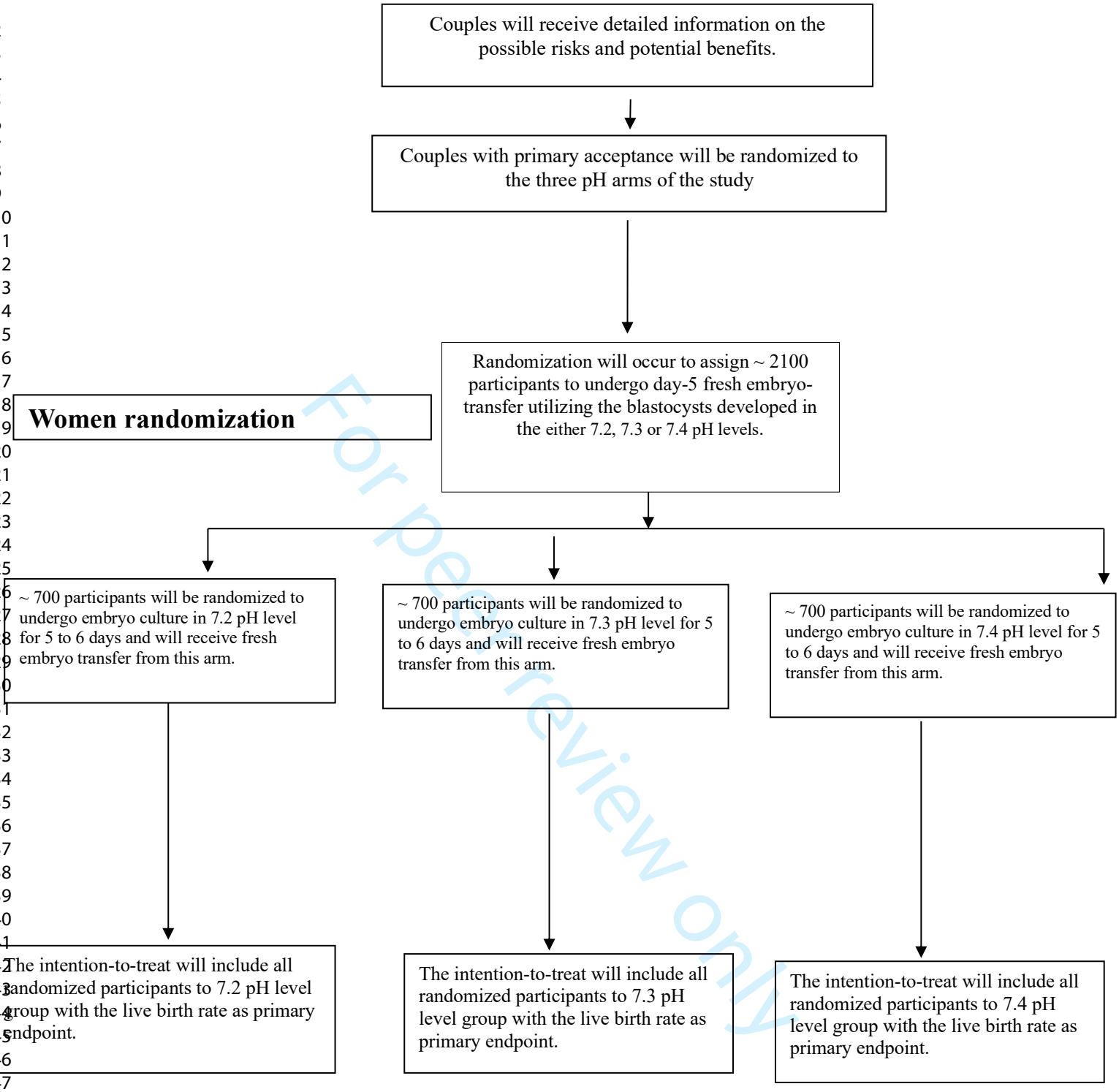
Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the study that will make sure all male partners are in line with the inclusion criteria, and revised the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF Centre and participated revising the protocol and provided comments. Mohamed AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF Centre. Hazem Abdelghafar is a primary investigator at Ibnsina IVF Centre and participated in the trial design. All authors provided comments and agreed on the study design and protocol, and will participate in reporting this trial thereafter.

References

1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi: 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update* 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First: 2012/07/26]
4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First: 2012/02/09]
5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation embryo culture media: effects of culture and batches. *Reprod Biomed Online* 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First: 2018/09/20]
6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi: 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development* 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First: 1998/06/10]
10. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi: 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]

11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009;18(6):799-810. [published Online First: 2009/06/06]
12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online First: 2010/11/12]
13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155 [published Online First: 1987/06/01]
14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First: 1999/01/14]
15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J [published Online First: 1998/07/21]
16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ* 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi: 10.1136/bmj.c332 [published Online First: 2010/03/25]
18. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization 2010.
19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-6736(92)92425-f [published Online First: 1992/07/04]
20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory environment and air quality: report of an expert meeting. *Reprod Biomed Online* 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First: 2018/04/17]
21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi: 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and implementing a core outcome set for infertility. *Hum Reprod Open* 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First: 2019/03/22]

Figure Legend: Trial plan for enrolment



pH trial plan for enrollment and outcomes – 7.2, 7.3 and 7.4 pH levels

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Altman DG, Laupacis A, Gøtzsche PC, Krleža-Jerić K, Hróbjartsson A, Mann H, Dickersin K, Berlin J, Doré C, Parulekar W, Summerskill W, Groves T, Schulz K, Sox H, Rockhold FW, Rennie D, Moher D. SPIRIT 2013 Statement: Defining standard protocol items for clinical trials. Ann Intern Med. 2013;158(3):200-207

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	4
Protocol version	#3	Date and version identifier	4
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#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	13
Roles and responsibilities: committees	#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)	4
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	2 & 3
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	2 & 3
Objectives	#7	Specific objectives or hypotheses	3 & 4
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, non-inferiority, exploratory)	4
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	4

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)	5 & 6
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
Interventions: modifications	#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)	4 & 5
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	10 & 11
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)	8
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	11 & 12
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation: 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	5

		provided in a separate document that is unavailable to those who enrol participants or assign interventions	
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	5
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	5
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	5
Methods: Data collection, management, and analysis			
Data collection plan	#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	11
Data collection plan: retention	#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	11
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	11
Statistics: outcomes	#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	11 & 12

Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11 & 12
Statistics: analysis population and missing data	#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)	11 & 12
Methods: Monitoring			
Data monitoring: formal committee	#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	4
Data monitoring: interim analysis	#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	4, 11 & 12
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	4
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	4
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	4
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)	4
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	4

Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	4
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	4
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	4
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	4
Dissemination policy: trial results	#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	4
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	1
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	4
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	n/a

BMJ Open

Triple-arm trial of pH (Tri-pH) effect on live birth after ICSI in Egyptian IVF facilities: Protocol of a randomised controlled trial

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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

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pH-Study Protocol

Title: Triple-arm trial of pH (Tri-pH) effect on live birth after ICSI in Egyptian IVF facilities: Protocol of a randomised controlled trial

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Abstract

Introduction

The pH of culture media for human in vitro fertilization (IVF) is a potential stressor that can affect pre- and post-implantation embryonic growth. There has been no clear evidence about the level that can support in vitro human embryo development optimally. Most manufactures of culture media have specified a range of 7.2 to 7.4, and routine practice is to use a level of 7.25 to 7.3 pH. However, these recommendations resulted from designers' opinions or experiments on mice models. There has been no randomised trial to search for the effect of pH level on live birth rate after IVF. The aim of this trial is to examine if there is an effect on live birth rate using three different levels of pH.

Methods and analysis

This multicentre randomised trial will involve centres specialised in IVF in Egypt. Eligible women amenable for intracytoplasmic sperm injection (ICSI) will be randomized to undergo in vitro culture in either 7.2, 7.3 or 7.4 pH level. The study is designed to detect 10% difference in live birth rate with 93% per cent power at 1% significance level.

Ethics and dissemination

Ethics review boards of the participating centres approved the study and eligible women will sign written informed consent before enrolment. The study has established an independent data monitoring and safety committee from international experts in the field and in trial design. We have no plan to disseminate the results to participants or any health communities except for the independent monitoring and safety committee established for this trial.

Trial registration number NCT02896777.

Keywords

Embryo culture, pH level, culture media, blastocyst formation

Strengths and limitations of this study

- The study is randomised controlled which reduces the possibility of bias.
- The study has an independent data monitoring committee with access to the data with no involvement in the study conduct.
- Two possible limitations of the study are that it will be conducted on ICSI cycles as ICSI is the preferred insemination method in the participating centres, and formed blastocyst calculation will be based on assumption for any cleavage-stage transfer.
- The embryologists will be aware of the culture arms during the study conduct.

Background

Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six cycles of *in vitro* fertilisation (IVF),[1] which is relatively suboptimal. In addition, IVF results in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared with the *in vivo* conception.[2] These outcomes can rely on factors relating to patients, stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200 variables have been identified as being potentially relevant to the cycle outcome.[3] One element that may influence embryo development *in vitro* is the pH level of a culture medium, which thus far has been determined by manufacturers of culture media without recourse to a well-powered randomized clinical trial (RCT).[4] The pH levels are potential stressors that vary between media brands and from batch-to-batch depending on the bicarbonate level in culture media and on the CO_2 level of incubators.[5] This would suggest that pH level can vary between incubators within the same laboratory if it is not well adjusted. Recommendations for measuring pH for embryo culture are variable between daily to monthly measurement.[4]

Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH (pHe).[6] The *in vitro* conditions including concentrations of bicarbonates, proteins, amino acids in culture media and the CO_2 of incubators affect the pHe, which is a potential stressor.[7] The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell division and differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal regulation, and microtubule dynamics.[7 8] Drifts in pHe translate into changes in pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt to restore pHi to a safe level.[8] The pHi can compensate through an active exchange among Na^+, $HCO3^-/Cl^-$ and Na^+/H^+ to maintain it between 7 to 7.3.[5 8] Denuded oocyte for ICSI through fertilization thereafter and vitrified-warmed embryos lack robust compensatory mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios can significantly perturbate embryo development.[9-11]

That being said, an optimum level of pH for human embryo culture in vitro is still unknown.[4 9 12-15] Most recommendations rely on mice models or manufacturers of culture media. Theoretically, a wide range of pHe levels (7.0–7.5) can support human embryo development *in vitro*. However, a narrower range of pHe levels (7.2 to 7.4) is used in clinical practice. This is because an extreme acidic pHe level (≤ 7) can adversely affect oocyte spindle leading to no further post-fertilization events.[4] This level of acidic pH can delay or block embryo development *in vitro*.[4] Similar harms can theoretically occur for oocyte and embryo, if alkaline levels of pHe (≥ 7.5) are used.[4] Although these potential harms of extreme pHe levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to investigate a safe range of pHe (7.2 to 7.4). Why this range has been chosen depends on the recommendations of media manufactures and the clinical practice within the majority of human embryo culture laboratories. Although this range of 7.2 to 7.4 is used, there is clear evidence about which level of 7.2, 7.3, or 7.4 can support human embryos to result in live

birth after transfer. This multicentre, randomized, clinical trial aims to identify whether pHe levels of 7.2, 7.3, or 7.4 can perform better on live birth rate after ICSI in order to investigate the potential for optimisation.

Methods and Design

This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for human embryo culture in vitro on live birth after ICSI (Figure 1). This partially blind design represents that clinicians, participants and outcome assessor, not including the embryologists, will be unaware of the study arms. This multicentre trial will involve private IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre and Amshaj IVF Centre) with the study protocol in their hand before enrolment of participants. If other IVF facilities join this trial before recruitment, this will be reported in the study.

Ethics and dissemination

This trial obtained the approval from Ethics Review Board of Upper Egypt IVF Network relating to the participating sites (Approval No. 009/2016). An independent safety and monitoring committee formed of five experts in reproductive endocrinology, reproductive biology, embryo culture, biostatistics and trial methodology will oversee this trial. All participants will receive independent counselling from research instructors who are not involved in patient care or laboratory work. Participants who will accept to participate will sign a written informed consent before enrolment. Conducting this study will be in accordance with the Declaration of Helsinki.[16] The trial reporting will be according to the CONSORT statement,[17] unless other guideline will have higher ranking at that time. No plan exists to amend this protocol and any amendments will be responsibility for the safety

committee and will undergo detailed reporting on the trial registry and in the final manuscript.

Intervention

Oocytes and embryos in the three arms will undergo continuous culture from day 0 through day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after ICSI in pHe of 7.2±0.02. Arm II The “Arm II” is to culture oocytes and resulting embryos after ICSI in pHe of 7.3±0.02. “Arm III” is to culture oocytes and resulting embryos after ICSI in pHe of 7.4±0.02. This trial will include intracytoplasmic sperm injection (ICSI) cycles.

Patient and Public Involvement

No patient involved

Randomization and Masking

Using an online tool, participants will be randomised to the experimental arms with a 1:1:1 allocation ratio. The allocation sequence of participants will be generated using a permuted block randomization of 3, 6 and 9 block sizes with unique identifiers in random order, stratified by trial site. Randomization of participants and its storage in sequentially numbered, opaque, sealed envelopes will occur by a secretary with no involvement in patient care and will be provided to trial sites before enrolment of first participant. Eligible participants will be allocated to the relevant arms on the day of maturation trigger and allocation result will be communicated to the laboratory team. Participants, clinicians and outcome assessors for the clinical outcomes will be unaware of the allocation, while embryologists who will assess embryo development will be aware of the allocation.

Participants

The inclusion criteria include:

1) Women age of ≥ 18 to ≤ 40;

2) BMI of ≤ 31;

3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);

4) Women who have ≥ 1 year of primary or secondary infertility;

5) Fresh ejaculate sperm of any count provided they have ≥ 1% normal forms and a motile fraction;

6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous successful one;

7) Women with > 7 mm endometrial thickness at day of trigger;

and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g. submucosal myomas, polyps or septa).

Women will be excluded if they have:

1) Unilateral oophorectomy;

2) Abnormal karyotyping for them or their male partners;

3) History of repeated abortions or implantation failure;

4) Uncontrolled diabetes;

5) Liver or renal disease;

6) History of severe ovarian hyperstimulation;

7) History of malignancy or borderline pathology;

8) Endometriosis;

9) Plan for PGD-A;

10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;

and 11) PCOS, women with history of severe OHSS, and cycles with agonist trigger or any patient with a plan for a "freeze-all".

Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support

Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg, Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will continue to the day of maturation trigger. For Antagonist group, women will start the antagonist on day 6 of treatment cycle. All women will receive follicular stimulating hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG; Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger shot (Choriomon, IBSA) for final oocyte maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily, starting on day 1 after retrieval ("day 1") to 12 weeks of gestation, unless negative pregnancy.

Sperm Preparation, Oocyte Denudation and ICSI

Semen samples will be processed through density gradient,[18] using Puresperm (Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in Global HEPES medium under inverted microscope as previously described.[19]

Incubator Management and pH Adjustment

Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to account for incubator as variable. If another brand of incubators will be used, we will ensure they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be used at some centres; however, we will adjust the analysis by trial site to account for differences between centres. Incubators will undergo stringent control of temperature (36.9±0.1˚C). The temperature will be validated daily using a certified thermometer. Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2 and a proper CO_2 concentration to achieve the required pH. All the three measurements (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with installation of inline filters (Green, Lifeglobal, CooperSurgical).[20]

A minimum of 3 incubators of a single brand within each participating facility with different levels of pH representing the study arms is obligatory: Incubator A of 7.2±0.02 pH, Incubator B of 7.3±0.02 pH, and Incubator C of 7.4±0.02. The three incubators will undergo a strict adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of pH will occur after an overnight incubation of 1mL culture media in a central well dish covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for measuring pH as per the manufacturer protocol. Briefly, after switching on the device, calibration of the device automatically occurs. Next, we adjust the temperature to 37˚C, and select the sample as arterial. Next, we insert the card, which undergoes automatic calibration.

Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the first droplet and smoothly inject the sample until the beep. We can see the results of pH, partial CO_2 and O_2 pressures thereafter. Each laboratory will report the results to also compare the resulting partial pressures of CO_2 and O_2 with the incubator display. pH levels will also be measured every new batch of a culture medium. The measurement of pH and CO_2 across the centres will be performed using a one-brand equipment that will undergo periodic calibration together. To account for errors in measurement, one well-trained personnel will be assigned to measure the pH and double check the CO_2 level across the centres.

Culture Protocol and Embryo Scoring

Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil, Nidacon). If a decision to change culture media at any time point of the study conduct is made, this will be performed at the same time across the study sites. Dishes will undergo overnight incubation in the relevant incubator adjusted to the relevant pH as per the randomization. After ICSI, the injected oocytes will undergo washing in culture medium followed by incubation from day 0 through day 5 or 6 in the relevant arm of pH, except for a small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5/6, with removal of the unfertilized, abnormally fertilized or degenerated oocytes at fertilization check. Two embryologists will perform the fertilization check and embryo grading on day 1, 2 and 3 of culture as per the Istanbul Consensus.[21] All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable for transfer or vitrification on day 5 provided they are graded 311 as per the Istanbul Consensus.[21] Embryos utilized for transfer or cryopreservation will be pictured and recorded

in the patient file. All the recorded pictures from all centres will undergo blind grading by two independent experienced embryologists.

Embryo Transfer

Women will undergo fresh embryo transfer by replacing one to two embryos on day 5 with those who replaced embryos on day 3 will be reported as per each centre protocol, except for women with reduced uterine cavity or previous preterm birth, they will replace only one embryo. One participating centre will transfer majority of its cases on day 3. This issue will be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre standardized protocol. The rest of the utilizable embryos will undergo vitrification for transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted from fresh and vitrified-warmed transfer within one year of randomization. Women will test for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will confirm pregnancy at ≥ week 7 of gestation by detection of intrauterine sac with a heartbeat on ultrasound.

Outcome Measures

Each outcome will be calculated including all randomised participants in the arms to which they were allocated, with the exception of implantation rate, which will be interpreted cautiously due to concerns over its validity as a measure of treatment effect, and perinatal outcomes, which by definition are only available in the subset of participants achieving live birth. This study will adopt the COMMIT definitions of outcomes,[22] where appropriate.

Primary outcome

Live birth (delivery of one or more viable infants > 20th weeks of gestation).

Secondary outcomes

1) Biochemical pregnancy (positive βhCG ≥ 10 IU/L at 14 days after egg retrieval).

2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th weeks of gestation).

3) Ongoing pregnancy (continued viable pregnancy > 20th weeks of gestation).

4) Miscarriage (loss of a clinical pregnancy ≤ 20th weeks of gestation).

5) Term live-birth (i.e. delivery of one or more viable infants ≥37 weeks of gestation).

6) Preterm Birth (delivery of one or more viable infants < 37th weeks of gestation).

7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).

8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)

9) Congenital malformation (delivery of congenitally malformed babies).

10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).

11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-warmed within one year of randomization).

12) Fertilization (presence of 2 pronuclei 17±1 hr after ICSI).

13) Embryo cleavage (cleaved embryos per fertilized oocyte).

14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than 10% fragmentation by volume).

15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).

16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume; ≥ 311 grade per fertilized oocyte).

17) Cryopreservation (cryopreserved embryos per fertilized oocyte).

18) Live-birth-implantation rate (live birth per embryo transferred).

19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized oocyte).

20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst cryopreserved of 311 grade per fertilized oocyte).

Statistical Analysis

Sample size estimation

This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested is that adjusting the pH value to the edges of this range might result in improvements to the live birth rate, although we remain in equipoise as to whether higher or lower values will be optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and our goal is to investigate whether this is associated with varying pH.

The study has been powered for a global test of the effect of pH, calculated using plausible birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yields 98% power in this scenario, using a 5% significance level. This test makes no assumption about the ordering of the live birth rates in relation to the ordering of the pH values. The high power level has been adopted to allow for some leeway in relation to the minimum effect size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just seven percentage points) this sample size yields 85% power against a 5% significance level, and 65% at a 1% significance level. We have also been conservative in our inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to 680. In reality, we will conduct analysis on an intention to treat basis, including all randomised women. Women who do not complete treatment (for example, they do not undergo embryo transfer) will be counted as not having a live birth. The only exceptions to this will be participants who withdraw consent for their data to be used in the study. Our inflation for loss to follow up reflects this possibility.

We also note that adjustment for site and age in the analysis will increase power further.

Analytical methods

The study conduct will be according to the intention-to-treat approach, where each participant randomised will be included in the analysis, regardless of protocol deviation. The primary analysis of live birth will be conducted using logistic regression, with live birth event regressed on pH group, adjusted for study site and participant age, which will be standardised before being entered as a covariate. pH will be entered as a categorical covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate across the three groups to be performed. Secondary supportive analyses will be conducted to try to characterise the nature of any association. This will include a test of linear trend in live birth rates across pH groups, which would imply an optimal pH level for the lowest or highest value, as well as pairwise comparisons between each group (again, these analyses will be adjusted for site and age). The pairwise comparisons will focus on size and precision of the odds ratios. Although it would be desirable to power the study for all pairwise comparisons as the primary outcome, this yields impracticable sample sizes (> 4000 participants) against realistic effects. The study has therefore been designed to represent the most informative test of the hypothesis that pH level affects live birth, that is practicable.

For secondary outcomes, binary variables will be analysed in an analogous fashion to the primary analysis. Count variables will be analysed using Poisson regression, with zero-inflated models wherever the outcome is structurally undefined for some participants. Again, these will be adjusted for site and age. In the analysis of number of usable embryos, implanted embryos arising from the day 3 transfer will be included as formed and good quality blastocysts, while those that do not implant in this portion will be considered blocked at day 3. The total of the number of embryos transferred and the formed blastocysts will be used to calculate number of utilizable embryos. A 1% significance level will be employed.

Due to the short treatment duration, it is anticipated that loss to follow up will be minimal, but if any loss does occur then these participants will be analysed as having negative status for the primary outcome, unless consent to use data is withdrawn. The follow-up period is identified as one year from randomization of the last participant provided that all pregnant women have given birth.

Discussion

Given the lack of evidence for a superior pH level for human embryo culture and whether the pH level could make a difference in live birth after IVF, this trial is performed. This trial is expected to fill the gap in this area leaving the recommendations of manufactures of culture media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1% significance level) to minimize the risk for uninformative results. In any occasion of cleavage-stage transfer, the calculation of the blastocyst formation will be based on the assumption that embryos that will implant will be calculated as a formed blastocyst, while the failure of implantation of an embryo will be considered as embryo block at the cleavage stage. Although this is not the ideal track to calculate blastocyst formation, we find this assumption is the closest one to reflect the blastocyst formation. This will be further discussed when this trial is reported.

Funding and conflict of interest

The study receives no fund and the authors have no conflict of interest to declare.

Authors' contributions

Mohamed is the creator of the concept and design of the study, and is the principal investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across the sites and will make sure that data is periodically sent to for storage in independent database. Jack Wilkinson is the statistician of the study who revised the study design and calculated the sample size and power of the study and he will be responsible for the data

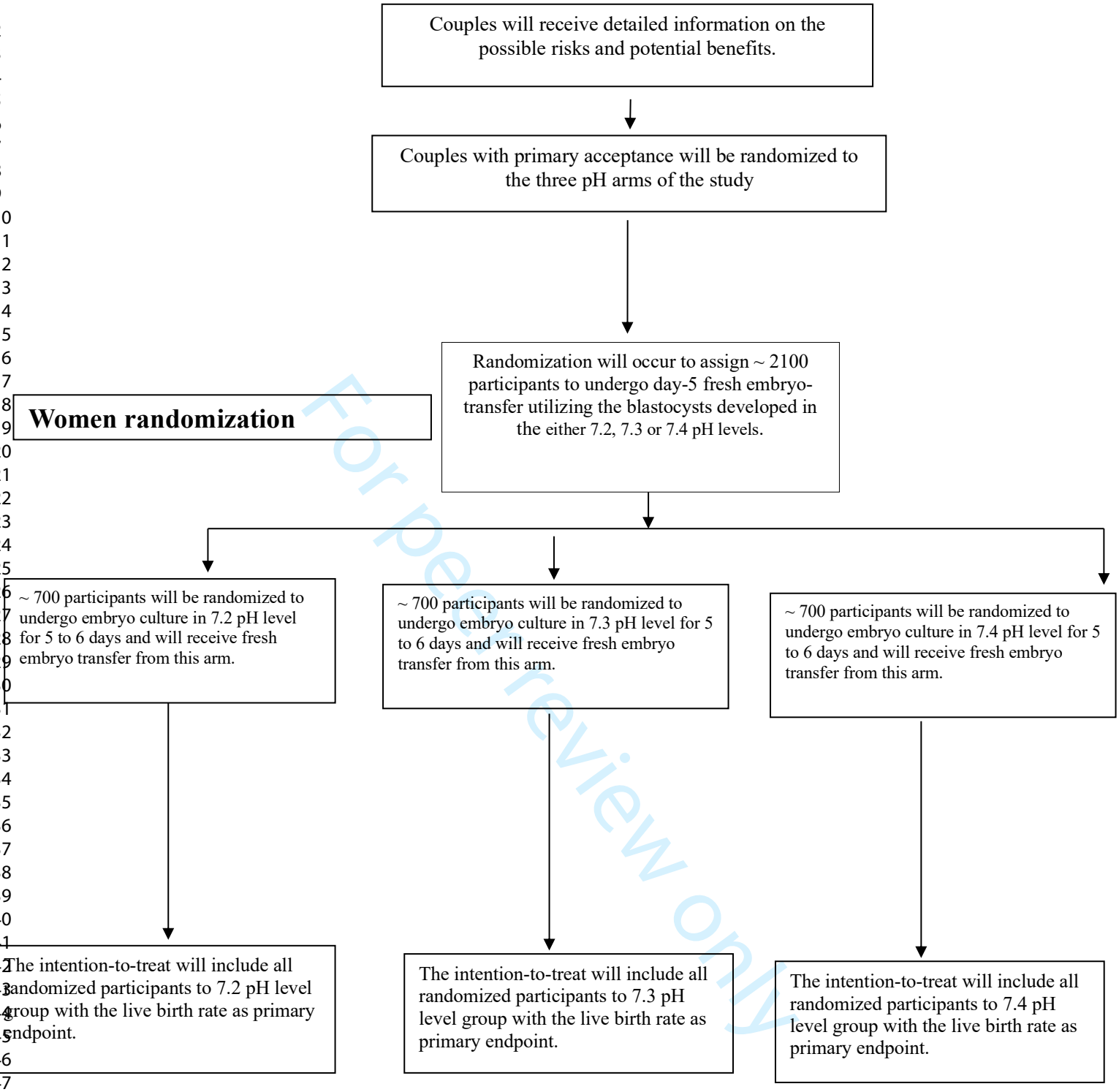
analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a primary investigator at Banon IVF centre and a sub-investigator at Ibnsina Centre, and she participated in revising the trial protocol and will participate in trial reporting thereafter. Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the study that will make sure all male partners are in line with the inclusion criteria, and revised the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF Centre and participated revising the protocol and provided comments. Mohamed AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF Centre. Hazem Abdelghafar is a primary investigator at Ibnsina IVF Centre and participated in the trial design. All authors provided comments and agreed on the study design and protocol, and will participate in reporting this trial thereafter.

References

1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi: 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update* 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First: 2012/07/26]
4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First: 2012/02/09]
5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation embryo culture media: effects of culture and batches. *Reprod Biomed Online* 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First: 2018/09/20]
6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi: 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development* 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]

9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First: 1998/06/10]
10. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi: 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009;18(6):799-810. [published Online First: 2009/06/06]
12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online First: 2010/11/12]
13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155 [published Online First: 1987/06/01]
14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First: 1999/01/14]
15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J [published Online First: 1998/07/21]
16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ* 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi: 10.1136/bmj.c332 [published Online First: 2010/03/25]
18. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization 2010.
19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-6736(92)92425-f [published Online First: 1992/07/04]
20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory environment and air quality: report of an expert meeting. *Reprod Biomed Online* 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First: 2018/04/17]
21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi: 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and implementing a core outcome set for infertility. *Hum Reprod Open* 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First: 2019/03/22]

Figure Legend: Trial plan for enrolment



pH trial plan for enrollment and outcomes – 7.2, 7.3 and 7.4 pH levels

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Altman DG, Laupacis A, Gøtzsche PC, Krleža-Jerić K, Hróbjartsson A, Mann H, Dickersin K, Berlin J, Doré C, Parulekar W, Summerskill W, Groves T, Schulz K, Sox H, Rockhold FW, Rennie D, Moher D. SPIRIT 2013 Statement: Defining standard protocol items for clinical trials. Ann Intern Med. 2013;158(3):200-207

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	4
Protocol version	#3	Date and version identifier	4
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#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	13
Roles and responsibilities: committees	#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)	4
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	2 & 3
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	2 & 3
Objectives	#7	Specific objectives or hypotheses	3 & 4
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, non-inferiority, exploratory)	4
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	4

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)	5 & 6
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
Interventions: modifications	#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)	4 & 5
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	10 & 11
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)	8
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	11 & 12
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation: 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	5

		provided in a separate document that is unavailable to those who enrol participants or assign interventions	
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	5
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	5
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	5
Methods: Data collection, management, and analysis			
Data collection plan	#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	11
Data collection plan: retention	#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	11
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	11
Statistics: outcomes	#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	11 & 12

Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11 & 12
Statistics: analysis population and missing data	#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)	11 & 12
Methods: Monitoring			
Data monitoring: formal committee	#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	4
Data monitoring: interim analysis	#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	4, 11 & 12
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	4
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	4
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	4
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)	4
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	4

Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	4
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	4
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	4
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	4
Dissemination policy: trial results	#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	4
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	1
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	4
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	n/a

BMJ Open

Triple-arm trial of pH (Tri-pH) effect on live birth after ICSI in Egyptian IVF facilities: Protocol of a randomised controlled trial

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Keywords:	Embryo culture, pH level, culture media, blastocyst formation

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pH-Study Protocol

Title: Triple-arm trial of pH (Tri-pH) effect on live birth after ICSI in Egyptian IVF facilities: Protocol of a randomised controlled trial

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Abstract

Introduction

One potential stressor that can affect pre- and post-implantation embryonic growth after in vitro fertilisation (IVF) is the pH of human embryo culture media. No evidence exists regarding a superior pH level to optimally support human embryo development *in vitro*. Manufactures of culture media recommend a pH range of 7.2 to 7.4, and IVF laboratories routinely use a pH range of 7.25 to 7.3. Both resulted from designers' opinions or experiments on mice models. Given the lack of randomised trials searching for pH effect on live birth rate after IVF, this trial examines the effect of three different levels of pH on the live birth rate.

Methods and analysis

This multicentre randomised trial will involve centres specialised in IVF in Egypt. Eligible couples for intracytoplasmic sperm injection (ICSI) will be randomised for embryo culture in either 7.2, 7.3, or 7.4 pH levels. The study is designed to detect 10 percentage points difference in live birth rate with 93% per cent power at 1% significance level. The primary outcome is the rate of live birth (delivery of one or more viable infants > 20th weeks of gestation) after ICSI. Secondary clinical outcomes include biochemical pregnancy, clinical pregnancy, ongoing pregnancy, miscarriage, preterm births, live birth weight babies, stillbirth, congenital malformation, and cumulative live birth (within one year from randomisation). Embryo development outcomes include fertilisation, blastocyst formation and quality, and embryo cryopreservation and utilisation.

Ethics and dissemination

Ethics review boards of the involved centres revised and approved this study. Eligible women will sign written informed consent before enrolment. This study has independent data monitoring and safety committee of international experts in trial design and *in vitro* culture.

No plan exists to disseminate results to participants or health communities, except for the independent monitoring and safety committee of the trial.

Trial registration number NCT02896777.

Keywords

Embryo culture, pH level, culture media, blastocyst formation

Strengths and limitations of this study

- The study is a randomised controlled trial, which reduces the possibility of bias.
- The study has independent, non-involved in the conduct, data monitoring committee with anytime access to the data.
- Limitations of this study include the inclusion of only ICSI cycles because ICSI is the preferred method of insemination in the participating centres, and the calculation of formed blastocyst is based on an assumption for cleavage-stage transfer cycles.
- The embryologists will be aware of the pH arms during the study conduct.

Background

Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six cycles of *in vitro* fertilisation (IVF),[1] which is relatively suboptimal. In addition, IVF results in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared with the *in vivo* conception.[2] These adverse consequences can rely on factors relating to patients, stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200 variables have been identified as having effects on cycle outcome.[3] One element that may influence embryo development *in vitro* is the pH level of a culture medium, which thus far has been determined by manufacturers of culture media without recourse to a well-powered randomised clinical trial (RCT).[4] pH levels are potential stressors that vary between media brands and from batch-to-batch depending on levels of bicarbonate in culture media and CO_2 in incubators.[5] Hence, pH levels can vary between incubators within a laboratory if

it is not well adjusted. Recommendations to measure pH for embryo culture vary from daily to monthly measurement.[4]

Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH (pHe).[6] The *in vitro* conditions including concentrations of bicarbonates, proteins, amino acids in culture media and the CO_2 of incubators also affect the pHe.[7] The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell division and differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal regulation, and microtubule dynamics.[7 8] Drifts in pHe translate into changes in pHi, which can adversely affect cell function if compensatory mechanisms failed to adapt to restore pHi to a safe level.[8] The pHi can compensate through an active exchange among Na^+, $HCO3^-/Cl^-$ and Na^+/H^+ to maintain it between 7 to 7.3.[5 8] Denuded oocyte for ICSI through fertilisation as well as vitrified-warmed embryos lack robust compensatory mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios can disrupt embryo development.[9-11]

That being said, the pH level that can optimise human embryo development *in vitro* is still unknown.[4 9 12-15] Current recommendations rely on mice models or manufactures of culture media. Theoretically, a wide range of pHe levels (7.0–7.5) can support human embryo development *in vitro*. However, a narrower range of pHe levels (7.2 to 7.4) is used in clinical practice. This is because an extreme acidic pHe level (≤ 7) can adversely affect oocyte spindle leading to no further post-fertilisation events.[4] This level of acidic pHe can delay or block embryo development *in vitro*.[4] Similar harms can occur for oocyte and embryo, if alkaline levels of pHe (≥ 7.5) are used.[4] Although these potential harms of extreme pHe levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to investigate a safe range of pHe (7.2 to 7.4). Why this range has been chosen depends on the recommendations of media manufactures and the clinical practice in laboratories of human

embryo culture. Despite the routine use of the range of 7.2 to 7.4 pHe in clinical practice, no clear evidence exists on which level within this range can better support human embryos to result in a live birth. This multicentre, randomised, clinical trial aims to identify whether pHe levels of 7.2, 7.3, or 7.4 can perform better on live birth rate after ICSI in order to investigate the potential for optimisation.

Methods and Design

This is a protocol of a multicentre, randomised, triple-arm, triple-blind clinical trial (NCT02896777, registered at www.ClinicalTrials.gov) that will compare three levels of pH for human embryo culture *in vitro* on live birth after ICSI (Figure 1). This partially blind design represents that clinicians, participants and outcome assessor, not including the embryologists, will be unaware of the study arms. This multicentre trial will involve private IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre and Amshaj IVF Centre) with the study protocol in their hand before enrolment of participants. If other IVF facilities join this trial before recruitment, this will be reported in the study.

Intervention

Oocytes and embryos in the three arms will undergo continuous culture from day 0 through day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after ICSI in pHe of 7.2±0.02. “Arm II” is to culture oocytes and resulting embryos after ICSI in pHe of 7.3±0.02. “Arm III” is to culture oocytes and resulting embryos after ICSI in pHe of 7.4±0.02. This trial will only include intracytoplasmic sperm injection (ICSI) cycles.

Patient and Public Involvement

Patients have not been directly involved in the design, planning, and conception of this trial.

Randomisation and Masking

Using an online tool, participants will be randomised to the experimental arms with a 1:1:1 allocation ratio. The allocation sequence of participants will be generated using a permuted block randomisation of 3, 6 and 9 block sizes with unique identifiers in random order, stratified by trial site. Randomisation of participants and storage of its results in sequentially numbered, opaque, sealed envelopes will occur by a secretary with no involvement in patient care, and the sealed envelopes will be provided to trial sites before enrolment of first participant. Eligible participants will be allocated to the relevant arms on the day of maturation trigger and allocation result will be communicated to the laboratory team. Participants, clinicians and outcome assessors for the clinical outcomes will be unaware of the allocation, while embryologists who will assess embryo development will be aware of the allocation.

Participants

The inclusion criteria include:

1) Women age of ≥ 18 to ≤ 40;

2) BMI of ≤ 31;

3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);

4) Women with ≥ 1 year of primary or secondary infertility;

5) Fresh ejaculate sperm of any count provided that there is ≥ 1% normal forms with any motile fraction;

6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous successful one;

7) Women with > 7 mm endometrial thickness at day of maturation trigger;

and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g. submucosal myomas, polyps or septa).

Women will be excluded if they have:

1) Unilateral oophorectomy;

2) Abnormal karyotyping for them or their male partners;

3) History of repeated abortions or implantation failure;

4) Uncontrolled diabetes;

5) Liver or renal disease;

6) History of severe ovarian hyperstimulation;

7) History of malignancy or borderline pathology;

8) Endometriosis;

9) Plan for PGD-A;

10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;

and 11) PCOS, women with history of severe OHSS, and cycles with agonist trigger or any patient with a plan for a "freeze-all".

Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support

Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg, Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will continue through the day of maturation trigger. In the antagonist protocol, women will start the antagonist on stimulation day 6 of the treatment cycle. Women will begin follicular stimulating hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG; Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through maturation, with dosage adjustment according to ovarian response. When ≥ 3 follicles measure ≥ 18 mm mean diameter on ultrasound, women will be given a 10,000 IU hCG trigger shot (Choriomon, IBSA) for oocyte maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be

handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily, starting on day 1 after retrieval ("day 1") to 12 weeks of gestation, unless negative pregnancy.

Sperm Preparation, Oocyte Denudation and ICSI

Semen samples will be processed using density gradient[16] (Puresperm, Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after collection in 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES using a 170-micrometre stripper (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in Global HEPES medium under an inverted microscope as previously described.[17]

Incubator Management and pH Adjustment

We will use only humidified benchtop incubators for this study, involving Labo C-Top (Labotect, Germany), Minc-1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will use only one brand to account for incubators as a variable. If dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) are used in some centres, we will account for differences between centres by adjusting for trial sites. Incubators will undergo stringent control of temperature (36.9±0.1˚C) by daily validation using certified thermometers. Incubator's CO_2 and O_2 levels will be measured daily using a certified gas analyser to ensure that O_2 measures 5%, and CO_2 concentration is at the prespecified level to achieve the required pH. Well-trained persons across the sites will verify all measurements (temperature, CO_2, O_2, and pH levels). Incubators will be sterilised by 6% H_2O_2 every four weeks, with an installation of a new set of inline filters (Green, Lifeglobal, CooperSurgical).[18]

A minimum of 3 incubators of a single brand within each facility is obligatory to represent the three pH arms: 7.2±0.02 pH level (Incubator A), 7.3±0.02 pH level (Incubator B), and 7.4±0.02 level (Incubator C). The three incubators will undergo strict adjustments to maintain the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card US). Constant pH levels will be ensured by a twice-weekly measurement of pH using the blood gas analyser along with daily measurement of CO_2 levels of incubators. Measurement of pH will occur following overnight incubation of 1 mL culture media in a central well dish covered with 0.4 mL of oil. Before the opening of incubators, the handheld blood gas analyser (Epoc® Reader and Host; BGEM card US) will be prepared for pH measurement as per the manufacturer protocol. Briefly, after switching on the device, the device automatically starts to calibrate. Next, we set the temperature at 37˚C, and sample type as arterial. Next, we insert the card to undergo automatic calibration. Next, when the device is ready, we will aspirate 0.5 mL of calibrated culture media using 1 mL syringe attached to wide needle calibre. Next, we discard the first droplet and smoothly inject the sample until hearing a beep. The results of pH, partial CO_2 and O_2 pressures will be ready thereafter. Results will be reported in each laboratory to compare partial pressures of CO_2 and O_2 with incubator display. pH levels will be measured every new batch of culture media. Measurements of pH and CO_2 across centres will be performed using one-brand equipment with periodic calibration. To account for errors in measurement, one well-trained personnel will be assigned to measure the pH and double-check the CO_2 level across trial sites.

Culture Protocol and Embryo Scoring

Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil, Nidacon). If a decision to change culture media at any time point of the study conduct is made, this will be performed at the same time across study sites. Dishes will undergo

overnight incubation in the relevant incubator adjusted to the proper pH as per randomisation. After ICSI, the injected oocytes will undergo washing in culture medium followed by incubation from day 0 through day 5 or 6 in the relevant pH arm, except for a small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5 or 6, with the removal of the unfertilised, abnormally fertilised or degenerated oocytes at fertilisation check. Two embryologists will perform the fertilisation check and embryo grading on day 1, 2, 3, and 5 of culture as per the Istanbul Consensus.[19] All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable for transfer or vitrification on day 5 provided they are graded 3 1 1 as per the Istanbul Consensus.[19] Embryos utilised for transfer or cryopreservation will be pictured and recorded in the patient file. All the recorded pictures from all centres will undergo blind grading by two independent, experienced embryologists.

Embryo Transfer

Women will receive one to two embryos. Women with a reduced uterine cavity or previous preterm birth will transfer only one embryo. Trial sites will transfer blastocysts on day 5 except for one participating site will transfer embryos on day 3. This issue will be accounted for by adjusting the analysis by trial site. Embryos will be transferred under sonographic guidance using the Sydney IVF Transfer Set (Cook, US) as per the standard transfer protocol in each site. Any remaining utilisable embryos will be vitrified for transfer in subsequent cycles as we plan to monitor the cumulative live birth resulted from fresh and vitrified-warmed transfer within one year from randomisation. Women will test the level of serum hCG for biochemical pregnancy 14 days after oocyte retrieval and will confirm clinical pregnancy at ≥ week 7 of gestation by detection of intrauterine sacs with a heartbeat on the ultrasound.

Outcome Measures

Each outcome will be calculated including all randomised participants in the arms to which they were allocated, with the exception of implantation rate, which will be interpreted cautiously due to concerns over its validity as a measure of treatment effect, and perinatal outcomes, which by definition are only available in the subset of participants achieving live birth. This study will adopt the COMMIT definitions of outcomes,[20] where appropriate.

Primary outcome

Live birth (delivery of one or more viable infants > 20th weeks of gestation).

Secondary outcomes

All secondary outcomes will be cautiously reported, since statistical significance has limited meaning in the context of a plurality of tests.

1) Biochemical pregnancy (positive βhCG ≥ 10 IU/L at 14 days after egg retrieval).

2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th weeks of gestation).

3) Ongoing pregnancy (continued viable pregnancy > 20th weeks of gestation).

4) Miscarriage (loss of a clinical pregnancy ≤ 20th weeks of gestation).

5) Term live-birth (i.e. delivery of one or more viable infants ≥37 weeks of gestation).

6) Preterm birth (delivery of one or more viable infants < 37th weeks of gestation).

7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).

8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)

9) Congenital malformation (delivery of congenitally malformed babies).

10) Stillbirth (delivery of nonviable babies > 20 weeks of gestation).

11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-warmed within one year of randomisation).

12) Fertilisation (presence of 2 pronuclei 17±1 hr after ICSI).

13) Embryo cleavage (cleaved embryos per fertilised oocyte).

14) Top-quality embryos on day 3 (7-8 cells with appropriate sizes blastomeres and less than 10% fragmentation by volume).

15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilised oocyte).

16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume; ≥ 3 1 1 grade per fertilised oocyte).

17) Cryopreservation (cryopreserved embryos per fertilised oocyte).

18) Live-birth-implantation rate (live birth per embryo transferred).

19) Utilised embryos (number of cryopreserved plus transferred embryos per fertilised oocyte).

20) Top-quality utilised embryos (number of high-quality embryos transferred plus blastocyst cryopreserved of 3 1 1 grade per fertilised oocyte).

Statistical Analysis

Sample size estimation

This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested is that adjusting the pH value to the edges of this range might result in improvements to the live birth rate, although we remain in equipoise as to whether higher or lower values will be optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and our goal is to investigate whether this is associated with varying pH levels.

The study has been powered for a global test of the effect of pH, calculated using plausible birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yield 98% power in this scenario, using a 5% significance level. This test makes no assumption about the ordering of the live birth rates in relation to the ordering of the pH values. The high-power level has been adopted to allow for some leeway in relation to the minimum effect

size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just seven percentage points) this sample size yields 85% power against a 5% significance level, and 65% at a 1% significance level. We have also been conservative in our inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to 680. In reality, we will conduct the analysis on an intention to treat basis, including all randomised women. Women who do not complete treatment (for example, they do not undergo embryo transfer) will be counted as not having a live birth. The only exceptions to this will be participants who withdraw consent for their data to be used in the study. Our inflation for loss to follow up reflects this possibility. We also note that adjustment for site and age in the analysis will increase power further.

Analytical methods

The study conduct will be according to the intention-to-treat approach, where each participant randomised will be included in the analysis, regardless of protocol deviation. The primary analysis of live birth will be conducted using logistic regression, with live birth event regressed on pH groups, adjusted for study site and participant age, which will be standardised before being entered as a covariate. pH will be entered as a categorical covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate across the three groups to be performed. Secondary supportive analyses will be conducted to try to characterise the nature of any association. This will include a test of a linear trend in live birth rates across pH groups, which would imply an optimal pH level for the lowest or highest value, as well as pairwise comparisons between each group (again, these analyses will be adjusted for site and age). The pairwise comparisons will focus on size and precision of the odds ratios. Although it would be desirable to power the study for all pairwise comparisons as the primary outcome, this yields impracticable sample sizes (> 4000

participants) against realistic effects. The study has therefore been designed to represent the most informative test of the hypothesis that pH level affects live birth, that is practicable. For secondary outcomes, binary variables will be analysed in an analogous fashion to the primary analysis. Count variables will be analysed using Poisson regression, with zero-inflated models wherever the outcome is structurally undefined for some participants. Again, these will be adjusted for site and age. In the analysis of the number of usable embryos, implanted embryos arising from the day 3 transfer will be included as formed and good quality blastocysts, while those that do not implant in this portion will be considered blocked at day 3. Utilisable embryos will be calculated as the total number of transferred embryos and formed blastocysts. A 1% significance level will be employed. Due to the short treatment duration, it is anticipated that loss to follow up will be minimal (it is unusual for patients not to return to clinic to have their embryos transferred, for example). However, if any loss does occur, these participants will be analysed as having a negative status for the primary outcome, unless consent to use data is withdrawn. The follow-up period is identified as one year from randomisation of the last participant provided that all pregnant women have given birth.

Ethics and dissemination

Ethics Review Board of Upper Egypt IVF Network relating to the participating sites approved this trial (Approval No. 009/2016). An independent safety and monitoring committee formed of five experts in reproductive endocrinology, reproductive biology, embryo culture, biostatistics and trial methodology will oversee the trial conduct. All participants will receive independent counselling from research instructors who are not involved in patient care or laboratory work. Participants who will accept to participate will sign a written informed consent before enrolment. Conducting this study will be in accordance with the Declaration of Helsinki.[21] The trial reporting will be according to the

CONSORT statement.[22] No plan exists to amend this protocol and any amendments will require approval of the safety committee, and will undergo detailed reporting on the trial registry and in the final manuscript.

Discussion

Given the lack of evidence for a superior pH level for human embryo culture and whether the pH level could make a difference in live birth after IVF, a trial is warranted. This trial is expected to fill the gap in this area, since at present embryologists must rely upon the recommendations of manufactures of culture media rather than on a robust evidence base. This trial is powered to a high level (>90%, with a 1% significance level) against clinically important differences to minimize the risk for uninformative results. In the case of cleavage-stage transfer, the calculation of formed blastocysts will be based on the assumption that implanted embryos represent formed blastocysts, while any transferred embryos which fail to implant will be counted as blocked at the cleavage stage. Although this definition will be subject to some error, we believe it is a reasonable way to assess blastocyst formation without excluding a portion of the data. This point will be further discussed when this trial is reported. A large number of secondary outcomes will be measured and reported. This is partially driven by adherence to a recently developed core outcome set for infertility trials,[22] as well as by the inclusion of some embryological variables which might shed light on the mechanism of any effects of pH levels. As is usual in clinical trials, Type 1 error is controlled by the fact that the study inference will be based on the primary outcome variable, live birth. Accordingly, we will interpret the results of secondary endpoints cautiously in the final report, since these endpoints are not subject to Type 1 error control.

Funding and conflict of interest

The study receives no fund and the authors have no conflict of interest to declare.

Authors' contributions

Mohamed is the creator of the concept and design of the study, and is the principal investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across the sites and will make sure that data is periodically sent to for storage in independent database. Jack Wilkinson is the statistician of the study who revised the study design and calculated the sample size and power of the study and he will be responsible for the data analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a primary investigator at Banon IVF centre and a sub-investigator at Ibnsina Centre, and she participated in revising the trial protocol and will participate in trial reporting thereafter. Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the study that will make sure all male partners are in line with the inclusion criteria, and revised the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF Centre and participated revising the protocol and provided comments. Mohamed AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF Centre. Hazem Abdelghafar is a primary investigator at Ibnsina IVF Centre and participated in the trial design. All authors provided comments and agreed on the study design and protocol, and will participate in reporting this trial thereafter.

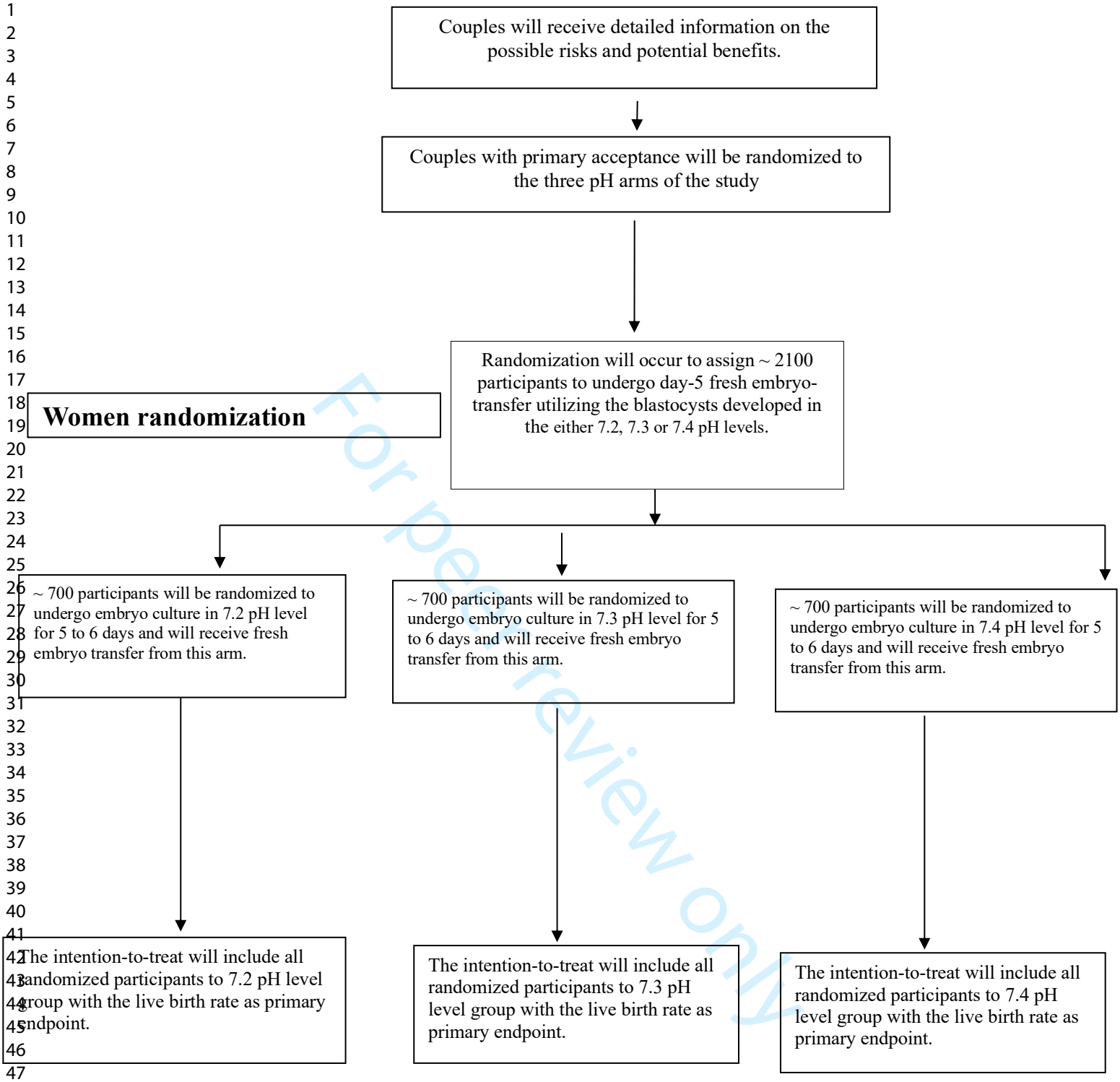
References

1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi: 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update* 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First: 2012/07/26]
4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First: 2012/02/09]

5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation embryo culture media: effects of culture and batches. *Reprod Biomed Online* 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First: 2018/09/20]
6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi: 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development* 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First: 1998/06/10]
10. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi: 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009;18(6):799-810. [published Online First: 2009/06/06]
12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online First: 2010/11/12]
13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155 [published Online First: 1987/06/01]
14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First: 1999/01/14]
15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J [published Online First: 1998/07/21]
16. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization 2010.
17. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-6736(92)92425-f [published Online First: 1992/07/04]
18. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory environment and air quality: report of an expert meeting. *Reprod Biomed Online* 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First: 2018/04/17]
19. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi: 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
20. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and implementing a core outcome set for infertility. *Hum Reprod Open*

2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First: 2019/03/22]
21. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ* 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
22. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi: 10.1136/bmj.c332 [published Online First: 2010/03/25]

Figure Legend: Trial plan for enrolment



pH trial plan for enrollment and outcomes – 7.2, 7.3 and 7.4 pH levels

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	4
Protocol version	#3	Date and version identifier	4
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#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	13
Roles and responsibilities: committees	#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)	4
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	2 & 3
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	2 & 3
Objectives	#7	Specific objectives or hypotheses	3 & 4
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, non-inferiority, exploratory)	4
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	4

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)	5 & 6
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
Interventions: modifications	#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)	4 & 5
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	10 & 11
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)	8
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	11 & 12
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation: 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	5

		provided in a separate document that is unavailable to those who enrol participants or assign interventions	
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	5
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	5
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	5
Methods: Data collection, management, and analysis			
Data collection plan	#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	11
Data collection plan: retention	#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	11
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	11
Statistics: outcomes	#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	11 & 12

Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11 & 12
Statistics: analysis population and missing data	#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)	11 & 12
Methods: Monitoring			
Data monitoring: formal committee	#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	4
Data monitoring: interim analysis	#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	4, 11 & 12
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	4
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	4
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	4
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)	4
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	4

Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	4
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	4
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	4
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	4
Dissemination policy: trial results	#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	4
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	1
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	4
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	n/a

BMJ Open

Triple-arm trial of pH (Tri-pH) effect on live birth after ICSI in Egyptian IVF facilities: Protocol of a randomised controlled trial

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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

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pH-Study Protocol

Title: Triple-arm trial of pH (Tri-pH) effect on live birth after ICSI in Egyptian IVF facilities: Protocol of a randomised controlled trial

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Abstract

Introduction

One potential stressor that can affect pre- and post-implantation embryonic growth after in vitro fertilisation (IVF) is the pH of the human embryo culture medium, but no evidence exists to indicate which pH level is optimal for IVF. Based on anecdotal evidence or mouse models, culture media manufacturers recommend a pH range of 7.2 to 7.4, and IVF laboratories routinely use a pH range of 7.25 to 7.3. Given the lack of randomised trials evaluating the effect of pH on live birth rate after IVF, this trial examines the effect of three different pH levels on the live birth rate.

Methods and analysis

This multicentre randomised trial will involve centres specialised in IVF in Egypt. Eligible couples for intracytoplasmic sperm injection (ICSI) will be randomised for embryo culture at pH 7.2, 7.3, or 7.4. The study is designed to detect 10 percentage points difference in live birth rate between the best and worst performing media with 93% power at a 1% significance level. The primary outcome is the rate of live birth (delivery of one or more viable infants beyond the 20th week of gestation) after ICSI. Secondary clinical outcomes include biochemical pregnancy, clinical pregnancy, ongoing pregnancy, miscarriage, preterm births, birth weight, stillbirth, congenital malformation, and cumulative live birth (within one year from randomisation). Embryo development outcomes include fertilisation, blastocyst formation and quality, and embryo cryopreservation and utilisation.

Ethics and dissemination

The study was reviewed and approved by the Ethics Review Boards of the participating centres. Eligible women will sign a written informed consent before enrolment. This study has an independent data monitoring and safety committee comprised of international experts

in trial design and *in vitro* culture. No plan exists to disseminate results to participants or health communities, except for the independent monitoring and safety committee of the trial.

Trial registration number NCT02896777.

Keywords

Embryo culture, pH level, culture media, blastocyst formation

Strengths and limitations of this study

- The study is a randomised controlled trial, which reduces the possibility of bias.
- The study has an independent data monitoring committee with full access to the data.
- Limitations of this study include the inclusion of only ICSI cycles because ICSI is the preferred method of insemination in the participating centres, and that the calculation of blastocyst formation rate is based on an assumption for cleavage-stage transfer cycles.
- The embryologists will be aware of the pH level as the study is being conducted.

Background

Assisted reproductive techniques (ART) result in a cumulative live birth rate of around 65% within six cycles of *in vitro* fertilisation (IVF),[1] which is relatively suboptimal. In addition, IVF has been associated with adverse perinatal outcomes, such as preterm birth and low birth weight babies compared with the *in vivo* conception.[2] These adverse consequences can be due to a range of factors including patient demographics, ovarian stimulation, and the culture system. In relation to embryo culture conditions, over 200 variables have been identified as having effects on cycle outcome.[3] One element that may influence embryo development *in vitro* is the culture medium pH, which thus far has been determined by manufacturers of culture media without recourse to a well-powered randomised clinical trial (RCT).[4] pH levels are potential stressors that vary between media brands and from batch-to-batch depending on the levels of bicarbonate in the culture media and of CO_2 in incubators.[5] Hence, pH levels can

vary between incubators within a laboratory. Recommendations for the frequency of pH measurement for embryo culture vary from daily to monthly.[4]

The intracellular pH (pHi) of oocytes and embryos is modulated by the extracellular pH (pHe),[6] which is affected by the culture conditions, including the concentrations of bicarbonate, protein, and amino acids in culture media, and of the CO_2 in incubators.[7] The mechanism of pHi in oocytes and embryos is complex, regulating enzymatic activity, cell division and differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal regulation, and microtubule dynamics.[7 8] Drifts in pHe translate into changes in pHi, which can adversely affect cell function if compensatory mechanisms fail to restore pHi to a safe level.[8] These compensatory mechanisms include an active exchange among Na^+, HCO_3^-/Cl^- and Na^+/H^+ to maintain pHi between 7 to 7.3.[5 8] Oocytes that have been denuded of their surrounding corona and cumulus cells prior to insemination via ICSI, as well as vitrified-warmed embryos, lack robust compensatory mechanisms for maintenance of pHi; therefore, drastic differences between pHe and pHi in these cases can disrupt embryo development.[9-11]

That being said, the optimal pH level to support human embryo development *in vitro* is still undefined.[4 9 12-15] Current recommendations are based on results derived using mouse models and/or literature from culture medium manufacturers. Theoretically, a wide range of pHe levels (7.0–7.5) could support human embryo development *in vitro*, but a narrower range of pHe levels (7.2 to 7.4) is used in clinical practice. This is because a more acidic pHe (≤ 7) can adversely affect the oocyte spindle, delaying or even blocking embryo development *in vitro*.[4] Alkaline levels of pHe (≥ 7.5) can similarly harm oocytes and embryos.[4] Although these reports of the effects of extreme pHe levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to limit our investigation to the range of pHe used in clinical practice (7.2 to 7.4) as the safer alternative. Despite the routine use of pHe 7.2

to 7.4 in clinical practice, there is no clear evidence as to whether there is a level within this range that could better support human embryo development to result in a live birth. The aim of this multicentre, randomised, clinical trial is to identify whether pHe 7.2, 7.3, or 7.4 results in an improved live birth rate after ICSI in order to investigate the potential for optimisation.

Methods and Design

This is a protocol of a multicentre, randomised, triple-arm, triple-blind clinical trial (NCT02896777, registered at www.ClinicalTrials.gov) that will compare the effect of three pH levels for human embryo culture *in vitro* on live birth after ICSI (Figure 1). In this partially blind design, the clinicians, participants and outcome assessor will be unaware of the study arms, but the embryologists will be aware. This multicentre trial will involve private IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre and Amshaj IVF Centre) which will all have the study protocol prior to enrolment of participants. If other IVF facilities join this trial before recruitment, this will be reported in the study.

Intervention

In each arm of the trial, which will only include intracytoplasmic sperm injection (ICSI) cycles, oocytes and embryos will undergo continuous culture from day 0 through day 5 or 6 without medium renewal. The oocytes and resulting embryos after ICSI will be cultured in either pHe 7.2±0.02 (“Arm I”), pHe 7.3±0.02 (“Arm II”), or pHe 7.4±0.02 (“Arm III”).

Patient and Public Involvement

Patients have not been directly involved in the design, planning, and conception of this trial.

Randomisation and Masking

Using an online tool, participants will be randomised to the experimental arms with a 1:1:1 allocation ratio. The allocation sequence of participants will be generated using a permuted block randomisation of 3, 6 and 9 block sizes with unique identifiers in random order,

stratified by trial site. Randomisation of participants and storage of the results in sequentially numbered, opaque, sealed envelopes will be performed by a secretary with no involvement in patient care, and the sealed envelopes will be provided to trial sites before enrolment of the first participant. Eligible participants will be allocated to the relevant arms on the day of maturation trigger and the allocation result will be communicated to the laboratory team. Participants, clinicians and outcome assessors for the clinical outcomes will be unaware of the allocation, while embryologists who will assess embryo development will be aware of the allocation.

Participants

The inclusion criteria include:

1) Women age of ≥ 18 to ≤ 40;

2) BMI of ≤ 31;

3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);

4) Women with ≥ 1 year of primary or secondary infertility;

5) Fresh ejaculate sperm of any count provided that there is ≥ 1% normal forms with any motile fraction;

6) Women undergoing their first ICSI cycle or their second ICSI cycle after a previously successful treatment;

7) Women with > 7 mm endometrial thickness at day of maturation trigger; and

8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g. submucosal myomas, polyps or septa).

Women will be excluded if they have:

1) Unilateral oophorectomy;

2) Abnormal karyotyping for them or their male partners;

3) History of repeated abortions or implantation failure;

4) Uncontrolled diabetes;

5) Liver or renal disease;

6) History of severe ovarian hyperstimulation;

7) History of malignancy or borderline pathology;

8) Endometriosis;

9) Plan for PGD-A;

10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm; or

11) PCOS, women with history of severe OHSS, and cycles with agonist trigger or any patient with a plan for a "freeze-all".

Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support

Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg, Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will continue through the day of maturation trigger. In the antagonist protocol, women will start the antagonist on stimulation day 6 of the treatment cycle. Women will begin follicular stimulating hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG; Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through maturation, with dosage adjustment according to ovarian response. When ≥ 3 follicles measure ≥ 18 mm mean diameter on ultrasound, women will be given a 10,000 IU hCG trigger shot (Choriomon, IBSA) for oocyte maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg Prontogest) twice daily,

starting on day 1 after retrieval ("day 1") to 12 weeks of gestation, if a pregnancy is established.

Sperm Preparation, Oocyte Denudation and ICSI

Semen samples will be processed using density gradient centrifugation[16] (Puresperm, Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after collection in 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES using a 170-micrometre stripper (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in Global HEPES medium under an inverted microscope as previously described.[17]

Incubator Management and pH Adjustment

We will use only humidified benchtop incubators for this study, involving Labo C-Top (Labotect, Germany), Minc-1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will use only one brand to account for incubators as a variable. It is possible that dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) are used in some centres, but we note that our analysis will account for differences between centres by adjusting for trial sites. Incubators will undergo stringent control of temperature (36.9±0.1˚C) by daily validation using certified thermometers. Incubator CO_2 and O_2 levels will be measured daily using a certified gas analyser to ensure that O_2 measures 5%, and CO_2 concentration is at the prespecified level to achieve the required pH. Well-trained persons across the sites will verify all measurements (temperature, CO_2, O_2, and pH levels). Incubators will be sterilised by 6% H_2O_2 every four weeks, along with the installation of a new set of inline filters (Green, Lifeglobal, CooperSurgical).[18]

A minimum of 3 incubators of a single brand within each facility is obligatory to represent the three pH arms: 7.2±0.02 pH level (Incubator A), 7.3±0.02 pH level (Incubator B), and

7.4±0.02 level (Incubator C). The three incubators will undergo strict adjustments to maintain the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card US). Constant pH levels will be ensured by a twice-weekly measurement of pH using the blood gas analyser along with daily measurement of CO_2 levels of incubators. Measurement of pH will occur following overnight incubation of 1 mL culture media in a central well dish covered with 0.4 mL oil. Before the opening of incubators, the handheld blood gas analyser (Epoc® Reader and Host; BGEM card US) will be prepared for pH measurement as per the manufacturer's protocol. Briefly, after switching on the device, the temperature will be set at 37˚C, sample type set as arterial, and automatic calibration will be initiated. When the device is ready, 0.5 mL calibrated culture media will be aspirated using a 1 mL syringe attached to a wide calibre needle. The first droplet will be discarded and the remainder of the sample injected into the device, for the assessment of pH, and partial pressures of CO_2 and O_2. In each laboratory, the partial pressures of CO_2 and O_2 will be compared with those on the incubator display. The pH levels will be measured with every new batch of culture media. Measurements of pH and CO_2 across centres will be performed using the same brand of equipment with periodic calibration. To account for errors in measurement, one well-trained staff member will be assigned to measure the pH and double-check the CO_2 level across trial sites.

Culture Protocol and Embryo Scoring

Each culture dish (micro-droplet, Vitrolife) will hold 12 × 20 µl droplets of Global Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml oil (NidOil, Nidacon). If there is a decision to change culture media at any time during the study, this will be performed at the same time across study sites. Dishes will be incubated overnight in the relevant incubator adjusted to the proper pH as per randomisation. After ICSI, the injected oocytes will undergo washing in culture medium followed by incubation from day 0 through

day 5 or 6 in the relevant pH arm, except for a small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5 or 6, with the removal of the unfertilised, abnormally fertilised or degenerated oocytes at fertilisation check. Two embryologists will perform the fertilisation check and embryo grading on day 1, 2, 3, and 5 of culture as per the Istanbul Consensus.[19] All laboratories will vitrify embryos no earlier than day 5. Embryos will be suitable for transfer or vitrification on day 5 provided they are graded 3 1 1 as per the Istanbul Consensus.[19] Images of embryos utilised for transfer or cryopreservation will be recorded in the patient file. All the images from all centres will undergo blind grading by two independent, experienced embryologists.

Embryo Transfer

One to two embryos will be transferred per cycle. Women with a reduced uterine cavity or previous preterm birth will receive only one embryo. Trial sites will transfer blastocysts on day 5 except for one participating site that will transfer embryos on day 3. This issue will be accounted for by adjusting the analysis by trial site. Embryos will be transferred under sonographic guidance using the Sydney IVF Transfer Set (Cook, US) as per the standard transfer protocol in each site. Any remaining utilisable embryos will be vitrified for transfer in subsequent cycles as we plan to monitor the cumulative live birth rate from fresh and vitrified-warmed transfers within one year from randomisation. Women will test the level of serum hCG for biochemical pregnancy 14 days after oocyte retrieval and will confirm clinical pregnancy at ≥ week 7 of gestation by detection of intrauterine sacs with a heartbeat on the ultrasound.

Outcome Measures

Each outcome will be calculated including all randomised participants in the arms to which they were allocated, with the exception of implantation rate, which will be interpreted cautiously due to concerns over its validity as a measure of treatment effect, and perinatal

outcomes, which by definition are only available in the subset of participants achieving live birth. This study will adopt the definitions of outcomes included in a forthcoming core outcome set for infertility trials [20] where appropriate. This outcome set was developed by means of an international consensus process involving clinicians, clinical scientists, patients and researchers.

Primary outcome

Live birth (delivery of one or more viable infants beyond the 20th week of gestation).

Secondary outcomes

All secondary outcomes will be cautiously reported, since statistical significance has limited meaning in the context of a plurality of tests.

1) Biochemical pregnancy (positive βhCG ≥ 10 IU/L at 14 days after egg retrieval).

2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th week of gestation).

3) Ongoing pregnancy (continued viable pregnancy > 20th week of gestation).

4) Miscarriage (loss of a clinical pregnancy ≤ 20th week of gestation).

5) Term live-birth (i.e. delivery of one or more viable infants ≥37 weeks of gestation).

6) Preterm birth (delivery of one or more viable infants < 37th week of gestation).

7) Very preterm birth (delivery of one or more viable infants < 32nd week of gestation).

8) Low birth weight babies (babies weighing < 2500 gm within 24 hours of delivery)

9) Congenital malformation (delivery of congenitally malformed babies).

10) Stillbirth (delivery of nonviable babies > 20 weeks of gestation).

11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-warmed embryo transfer within one year of randomisation).

12) Fertilisation (presence of 2 pronuclei 17±1 hr after ICSI).

13) Embryo cleavage (cleaved embryos per fertilised oocyte).

14) Top-quality embryos on day 3 (7-8 cells with appropriate sizes blastomeres and less than 10% fragmentation by volume).

15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilised oocyte).

16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume; ≥ 3 1 1 grade per fertilised oocyte).

17) Cryopreservation (cryopreserved embryos per fertilised oocyte).

18) Live-birth-implantation rate (live birth per embryo transferred).

19) Utilised embryos (number of cryopreserved plus transferred embryos per fertilised oocyte).

20) Top-quality utilised embryos (number of high-quality embryos transferred plus blastocyst cryopreserved of 3 1 1 grade per fertilised oocyte).

Statistical Analysis

Sample size estimation

This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested is that adjusting the pH value to the edges of this range might result in improvements to the live birth rate, although we remain in equipoise as to whether higher or lower values will be optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and our goal is to investigate whether this is associated with varying pH levels.

The study has been powered for a global test of the effect of pH, calculated using plausible birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yield 98% power in this scenario, using a 5% significance level. This test makes no assumption about the ordering of the live birth rates in relation to the ordering of the pH values. The high-power level has been adopted to allow for some leeway in relation to the minimum effect size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a

spread of just seven percentage points) this sample size yields 85% power against a 5% significance level, and 65% at a 1% significance level. We have also been conservative in our inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to 680. In reality, we will conduct the analysis on an intention to treat basis, including all randomised women. Women who do not complete treatment (for example, they do not undergo embryo transfer) will be counted as not having a live birth, and so do not represent a reduction in sample size. The only exceptions to this will be participants who withdraw consent for their data to be used in the study. Our inflation for loss to follow up reflects this possibility. We also note that adjustment for site and age in the analysis will increase power further.

Analytical methods

The study will be conducted according to the intention-to-treat approach, where each participant randomised will be included in the analysis, regardless of protocol deviation. The primary analysis of live birth will be conducted using logistic regression, with live birth event regressed on pH groups, adjusted for study site and participant age, which will be standardised before being entered as a covariate. pH will be entered as a categorical covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate across the three groups to be performed. Secondary supportive analyses will be conducted to try to characterise the nature of any association. This will include a test of a linear trend in live birth rates across pH groups, which would imply an optimal pH level for the lowest or highest value, as well as pairwise comparisons between each group (again, these analyses will be adjusted for site and age). The pairwise comparisons will focus on size and precision of the odds ratios. Although it would be desirable to power the study for all pairwise comparisons as the primary outcome, this yields impracticable sample sizes (> 4000

participants) against realistic effects. The study has therefore been designed to represent the most informative test of the hypothesis that pH level affects live birth, that is practicable. For secondary outcomes, binary variables will be analysed in an analogous fashion to the primary analysis. Count variables will be analysed using Poisson regression, with zero-inflated models wherever the outcome is structurally undefined for some participants. Again, these will be adjusted for site and age. In the analysis of the number of usable embryos, implanted embryos arising from the day 3 transfer will be included as formed and good quality blastocysts, while those that do not implant in this portion will be considered blocked at day 3. Utilisable embryos will be calculated as the total number of transferred embryos and formed blastocysts. A 1% significance level will be employed. Due to the short treatment duration, it is anticipated that loss to follow up will be minimal (it is unusual for patients not to return to clinic to have their embryos transferred, for example). However, if any loss does occur, these participants will be analysed as having a negative status for the primary outcome, unless consent to use data is withdrawn. The follow-up period is identified as one year from randomisation of the last participant provided that all pregnant women have given birth.

Ethics and dissemination

The Ethics Review Board of the Upper Egypt IVF Network relating to the participating sites approved this trial (Approval No. 009/2016). An independent safety and monitoring committee formed of five experts in reproductive endocrinology, reproductive biology, embryo culture, biostatistics and trial methodology will oversee the conduct of trial. All participants will receive independent counselling from research instructors who are not involved in patient care or laboratory work. Participants who agree to participate will sign a written informed consent before enrolment. This study will be conducted in accordance with the Declaration of Helsinki.[21] The trial reporting will be according to the CONSORT

statement.[22] No plan exists to amend this protocol and any amendments will require approval of the safety committee, and will undergo detailed reporting on the trial registry and in the final manuscript.

Discussion

Given the lack of evidence for a superior pH level for human embryo culture and whether the pH level could make a difference in live birth after IVF, a trial is warranted. This trial is expected to fill the gap in this area, since at present embryologists must rely upon the recommendations of culture media manufacturers rather than on a robust evidence base. This trial is powered to a high level (>90%, with a 1% significance level) against clinically important differences to minimize the risk for uninformative results. In the case of cleavage-stage transfer, the calculation of formed blastocysts will be based on the assumption that implanted embryos represent formed blastocysts, while any transferred embryos which fail to implant will be counted as blocked at the cleavage stage. Although this definition will be subject to some error, we believe it is a reasonable way to assess blastocyst formation without excluding a portion of the data. This point will be further discussed when this trial is reported. A large number of secondary outcomes will be measured and reported. This is partially driven by adherence to a recently developed core outcome set for infertility trials,[20] as well as by the inclusion of some embryological variables which might shed light on the mechanism of any effects of pH levels. As is usual in clinical trials, Type 1 error is controlled by the fact that the study inference will be based on the primary outcome variable, live birth. Accordingly, we will interpret the results of secondary endpoints cautiously in the final report, since these endpoints are not subject to Type 1 error control.

Funding and conflict of interest

The study receives no funding and the authors have no conflict of interest to declare.

Authors' contributions

Mohamed Fawzy is the creator of the concept and design of the study, and is the principal investigator of the study. Mohamed Fawzy is also a supervisor for the conduct of the study across the sites and will ensure that data is periodically sent for storage in an independent database. Jack Wilkinson is the statistician of the study who revised the study design and calculated the sample size and power of the study and he will be responsible for the data analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a primary investigator at Banon IVF centre and a sub-investigator at Ibnsina Centre, and she participated in revising the trial protocol and will participate in trial reporting thereafter. Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the study who will ensure all male partners meet the inclusion criteria, and revised the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF Centre and participated revising the protocol and provided comments. Mohamed AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF Centre. Hazem Abdelghafar is a primary investigator at Ibnsina IVF Centre and participated in the trial design. All authors provided comments and agreed on the study design and protocol, and will participate in reporting this trial hereafter.

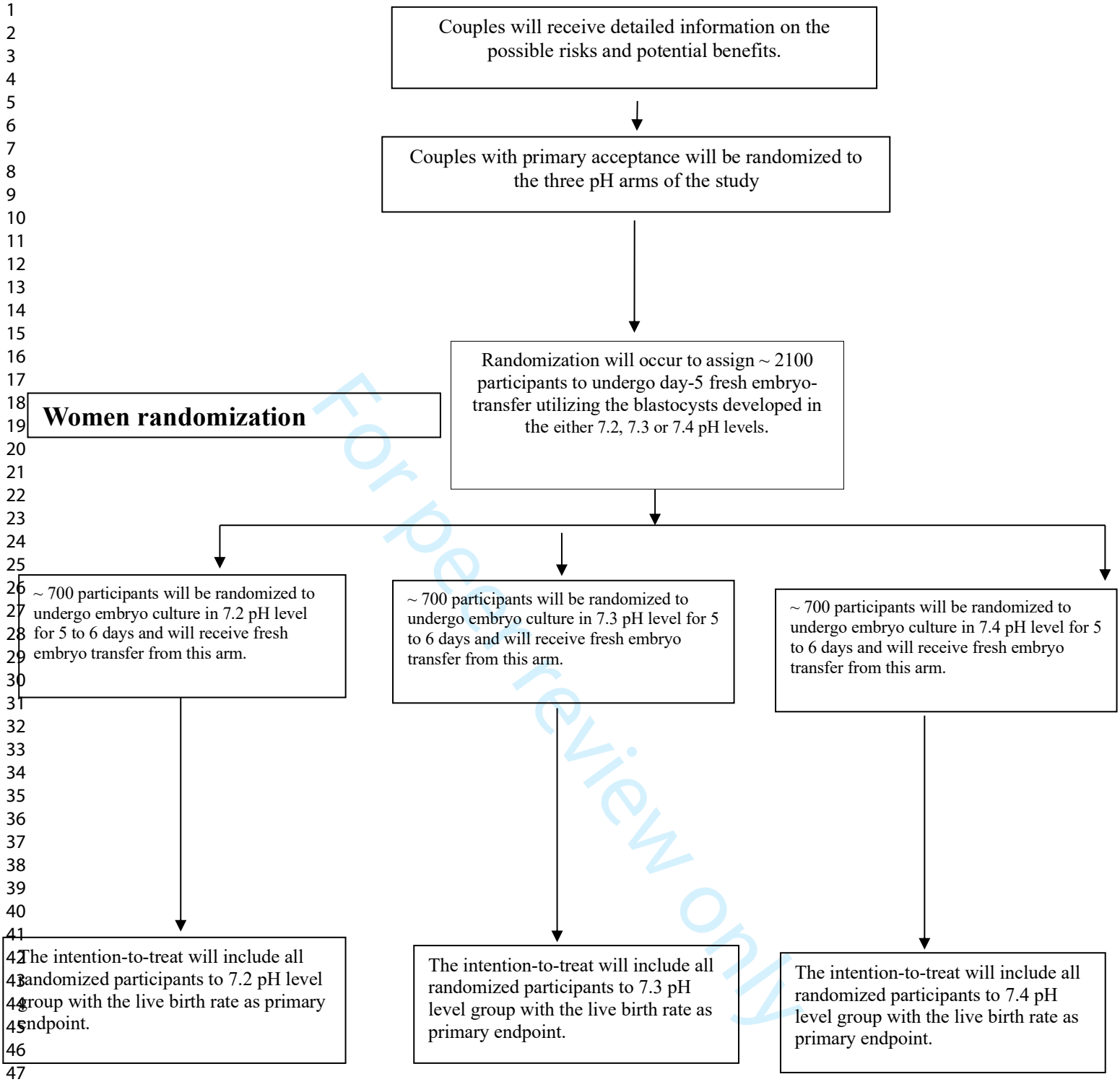
References

1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi: 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update* 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First: 2012/07/26]
4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First: 2012/02/09]

5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation embryo culture media: effects of culture and batches. *Reprod Biomed Online* 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First: 2018/09/20]
6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi: 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development* 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First: 1998/06/10]
10. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi: 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod Biomed Online* 2009;18(6):799-810. [published Online First: 2009/06/06]
12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online First: 2010/11/12]
13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155 [published Online First: 1987/06/01]
14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First: 1999/01/14]
15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J [published Online First: 1998/07/21]
16. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization 2010.
17. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-6736(92)92425-f [published Online First: 1992/07/04]
18. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory environment and air quality: report of an expert meeting. *Reprod Biomed Online* 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First: 2018/04/17]
19. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi: 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
20. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and implementing a core outcome set for infertility. *Hum Reprod Open*

2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First: 2019/03/22]
21. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ* 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
22. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi: 10.1136/bmj.c332 [published Online First: 2010/03/25]

Figure Legend: Trial plan for enrolment



pH trial plan for enrollment and outcomes – 7.2, 7.3 and 7.4 pH levels

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	4
Protocol version	#3	Date and version identifier	4
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#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	13
Roles and responsibilities: committees	#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)	4
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	2 & 3
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	2 & 3
Objectives	#7	Specific objectives or hypotheses	3 & 4
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, non-inferiority, exploratory)	4
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	4

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)	5 & 6
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
Interventions: modifications	#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)	4 & 5
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	10 & 11
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)	8
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	11 & 12
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
Methods: Assignment of interventions (for controlled trials)			
Allocation: 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	5

		provided in a separate document that is unavailable to those who enrol participants or assign interventions	
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	5
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	5
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	5
Methods: Data collection, management, and analysis			
Data collection plan	#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	11
Data collection plan: retention	#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	11
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	11
Statistics: outcomes	#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	11 & 12

Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11 & 12
Statistics: analysis population and missing data	#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)	11 & 12
Methods: Monitoring			
Data monitoring: formal committee	#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	4
Data monitoring: interim analysis	#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	4, 11 & 12
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	4
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	4
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	4
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)	4
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	4

Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	4
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	4
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	4
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	4
Dissemination policy: trial results	#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	4
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	1
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	4
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	n/a