

BMJ Open

BMJ Open is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers' comments and the authors' responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

BMJ Open is an open access journal and the full, final, typeset and author-corrected version of record of the manuscript is available on our site with no access controls, subscription charges or pay-per-view fees (<http://bmjopen.bmj.com>).

If you have any questions on BMJ Open's open peer review process please email info.bmjopen@bmj.com

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2019-034194
Article Type:	Protocol
Date Submitted by the Author:	09-Sep-2019
Complete List of Authors:	Fawzy, Mohamed; IbnSina Hospital, Ibsina IVF Centre Emad, Mai Wilkinson, Jack; University of Manchester, Centre for Biostatistics; Salford Royal NHS Foundation Trust, Research and Development Mansour, Ragaa; Egyptian IVF-ET Center Mahran, Ali Fetih, Ahmed Abdelrahman , Mohamed AbdelGhafar, Hazem
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

pH-Study Protocol

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

Mohamed Fawzy,^a Mai Emad,^a Jack Wilkinson,^b Ragaa Mansour,^c Ali Mahran,^d Ahmed N. Fetih,^e Mohamed Y. AbdelRahman,^f Hazem Abdelghafar,^f

IbnSina IVF Centre, Sohag and Banon IVF Centre, Assuit, Egypt

^aIbnSina IVF Centre, IbnSina Hospital, Sohag, Egypt; ^bCentre for Biostatistics, University of Manchester, UK; ^cEgyptian IVF-ET Centre, Cairo, Egypt; ^dDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine, Assiut University, Egypt; ^eDepartment of Obstetrics and Gynecology, Faculty of Medicine, Assiut University, Egypt; ^fDepartment of Obstetrics and Gynecology, Sohag University, Egypt

Corresponding Author: Dr. Mohamed Fawzy, IVF Laboratory Director (IbnSina and Banon IVF Centres), IbnSina Hospital, 146 El Aref Square, Sohag, Egypt; Cell: +201011122286; E-mail: drfawzy001@me.com

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

57 **Background**

58
59 Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six
60 cycles of *in vitro* fertilisation (IVF),¹ which is relatively suboptimal. In addition, IVF results
61 in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared
62 with the *in vivo* conception.² These outcomes can rely on factors relating to patients,
63 stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200
64 variables have been identified as being potentially relevant to the cycle outcome.³ One
65 element that may influence embryo development *in vitro* is the pH level of a culture medium,
66 which thus far has been determined by manufacturers of culture media without recourse to a
67 well-powered randomized clinical trial (RCT).⁴ The pH levels are potential stressors that vary
68 between media brands and from batch-to-batch depending on the bicarbonate level in culture
69 media and on the CO₂ level of incubators.⁵ This would suggest that pH level can vary
70 between incubators within the same laboratory if it is not well adjusted. Recommendations
71 for measuring pH for embryo culture are variable between daily to monthly measurement.⁴
72 Oocytes and embryos have intercellular (pHi), which is modulated by the extracellular pH
73 (pHe).⁶ The *in vitro* conditions including concentrations of bicarbonates, proteins, amino
74 acids in culture media and the CO₂ of incubators affect the pHe, which is a potential stressor.⁷
75 The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell
76 division and differentiation, protein synthesis, metabolism, mitochondrial function,
77 cytoskeletal regulation, and microtubule dynamics.^{7,8} Drifts in pHe translate into changes in
78 pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt
79 to restore pHi to a safe level.⁸ The pHi can compensate through an active exchange among
80 Na⁺, HCO₃⁻/Cl⁻ and Na⁺/H⁺ to maintain it between 7 to 7.3.^{5,8} Denuded oocyte for ICSI
81 through fertilization thereafter and vitrified-warmed embryos lack robust compensatory

1
2
3 82 mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios
4
5 83 can significantly perturbate embryo development.⁹⁻¹¹
6
7
8 84 That being said, an optimum level of pH for human embryo culture in vitro is still unknown.⁴
9
10 85 ^{9 12-15} Most recommendations rely on mice models or manufacturers of culture media.
11
12 86 Theoretically, a wide range of pHe levels (7.0–7.5) are believed to support human embryo
13
14 87 development *in vitro*. This multicentre, randomized, clinical trial aims to compare the
15
16 88 influence of three commonly used levels of pH on term live birth rate after ICSI, in order to
17
18 89 investigate the potential for optimisation.
19
20

21 90 **Methods and Design**

22
23
24 91 This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial
25
26 92 (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for
27
28 93 human embryo culture in vitro on term livebirth after ICSI. This partially blind design
29
30 94 represents that clinicians, participants and outcome assessor, not including the embryologists,
31
32 95 will be unaware of the study arms. This multicentre trial will involve private IVF facilities in
33
34 96 Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre
35
36 97 and Amshaj IVF Centre and others) with the study protocol in their hand before enrolment of
37
38 98 participants. This trial obtained the approval from Ethics Review Board of Upper Egypt IVF
39
40 99 Network relating to the participating sites (Approval No. 009/2016). An independent safety
41
42 100 and monitoring committee formed of five experts in reproductive endocrinology,
43
44 101 reproductive biology, embryo culture, biostatistics and trial methodology will oversee this
45
46 102 trial. All participants will receive independent counselling from research instructors who are
47
48 103 not involved in patient care or laboratory work. Participants who will accept to participate
49
50 104 will sign a written informed consent before enrolment. Conducting this study will be in
51
52 105 accordance with the Declaration of Helsinki.¹⁶ The trial reporting will be according to the
53
54 106 CONSORT statement,¹⁷ unless other guideline will have higher ranking at that time. No plan
55
56
57
58
59
60

1
2
3 107 exists to amend this protocol and any amendments will be responsibility for the safety
4
5 108 committee and will undergo detailed reporting on the trial registry and in the final
6
7
8 109 manuscript.
9
10 110 Oocytes and embryos in the three arms will undergo continuous culture from day 0 through
11
12 111 day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after
13
14 112 ICSI in pHe of 7.2 ± 0.02 . Arm II The “Arm II” is to culture oocytes and resulting embryos
15
16 113 after ICSI in pHe of 7.3 ± 0.02 . “Arm III” is to culture oocytes and resulting embryos after
17
18 114 ICSI in pHe of 7.4 ± 0.02 . This trial will include intracytoplasmic sperm injection (ICSI)
19
20 115 cycles.
21
22

23 116 **Randomization and Masking**

24
25
26 117 Using an online tool, participants will be randomised to the experimental arms with a 1:1:1
27
28 118 allocation ratio. The allocation sequence of participants will be generated using a permuted
29
30 119 block randomization of 3, 6 and 9 block sizes with unique identifiers in random order,
31
32
33 120 stratified by trial site. Randomization of participants and its storage in sequentially numbered,
34
35 121 opaque, sealed envelopes will occur by a secretary with no involvement in patient care and
36
37 122 will be provided to trial sites before enrolment of first participant. Eligible participants will
38
39 123 be allocated to the relevant arms on the day of maturation trigger and allocation result will be
40
41 124 communicated to the laboratory team. Participants, clinicians and outcome assessors for the
42
43 125 clinical outcomes will be unaware of the allocation, while embryologists who will assess
44
45 126 embryo development will be aware of the allocation.
46
47
48

49 127 **Participants**

50
51 128 The inclusion criteria include:

- 52
53 129 1) Women age of ≥ 18 to ≤ 40 ;
54
55 130 2) BMI of ≤ 31 ;
56
57 131 3) Anticipated normal responder by 10 basal ultrasound examination or AMH measurement);
58
59
60

- 1
2
3 132 4) Women who have ≥ 1 year of primary or secondary infertility;
4
5 133 5) Fresh ejaculate sperm of any count provided they have $\geq 1\%$ normal forms and a motile
6
7 fraction;
8 134
9
10 135 6) Women undergoing their first ICSI cycle or following a previous successful attempt;
11
12 136 7) Women with > 7 mm endometrial thickness at day of trigger;
13
14 and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g.
15 137
16 submucosal myomas, polyps or septa).
17 138

18
19 139 ***Women will be excluded if they have:***

- 20
21 140 1) Unilateral oophorectomy;
22
23 141 2) Abnormal karyotyping for them or their male partners;
24
25 142 3) History of repeated abortions or implantation failure;
26
27 143 4) Uncontrolled diabetes;
28
29 144 5) Liver or renal disease;
30
31 145 6) History of severe ovarian hyperstimulation;
32
33 146 7) History of malignancy or borderline pathology;
34
35 147 8) Endometriosis;
36
37 148 9) Plan for PGD-A;
38
39 149 10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;
40
41 and 11) A plan for a “freeze-all”.
42
43
44
45
46

47 151 **Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support**

48
49 152 Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-
50
51 153 regulation (Decapeptyl[®] 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide[®] 0.25 mg,
52
53 Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will
54 154
55 continue to the day of maturation trigger. For Antagonist group, women will start the
56 155
57 antagonist on day 6 of treatment cycle. All women will receive follicular stimulating
58 156
59
60

1
2
3 157 hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG;
4
5 158 Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular
6
7 159 maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles
8
9 160 measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger
10
11 161 shot (Choriomon, IBSA) or 250 μ g rhCG (Ovitrelle, Merck Serono) for final oocyte
12
13 162 maturation. Oocyte retrieval will be performed 37 hours after hCG trigger under transvaginal
14
15 163 sonographic guidance. Follicular aspirates will be handled in HEPES-buffered medium
16
17 164 (global® HEPES, LifeGlobal, Canada) at 37°C using tube warmers. Luteal-phase support
18
19 165 will be achieved with intramuscular progesterone (100 mg/mL [Prontogest, IBSA]) once
20
21 166 daily or vaginal pessaries (400 mg prontosgest) twice daily, starting on day 1 after retrieval
22
23 167 (“day 1”) to 12 weeks of gestation, unless negative pregnancy.
24
25
26
27

28 168 **Sperm Preparation, Oocyte Denudation and ICSI**

29
30 169 Semen samples will be processed through density gradient,¹⁸ using Puresperm (Nidacon,
31
32 170 Sweden). The pellet will undergo once washing and incubation at room temperature in
33
34 171 HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur
35
36 172 immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in
37
38 173 Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes
39
40 174 will undergo ICSI in Global HEPES medium under inverted microscope as previously
41
42 175 described.¹⁹
43
44
45
46

47 176 **Incubator Management and pH Adjustment**

48
49 177 Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US),
50
51 178 and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to
52
53 179 account for incubator as variable. If another brand of incubators will be used, we will ensure
54
55 180 they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be
56
57 181 used at some centres; however, we will adjust the analysis by trial site to account for
58
59
60

1
2
3 182 differences between centres. Incubators will undergo stringent control of temperature
4
5 183 ($36.9\pm 0.1^{\circ}\text{C}$). The temperature will be validated daily using a certified thermometer.
6
7
8 184 Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2
9
10 185 and a proper CO_2 concentration to achieve the required pH. All the three measurements
11
12 186 (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling
13
14 187 across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with
15
16 188 installation of inline filters (Green, Lifeglobal, CooperSurgical).²⁰
17
18
19 189 A minimum of 3 incubators of a single brand within each participating facility with different
20
21 190 levels of pH representing the study arms is obligatory: Incubator A of 7.2 ± 0.02 pH, Incubator
22
23 191 B of 7.3 ± 0.02 pH, and Incubator C of 7.4 ± 0.02 . The three incubators will undergo a strict
24
25 192 adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host;
26
27 193 BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH
28
29 194 with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of
30
31 195 pH will occur after an overnight incubation of 1mL culture media in a central well dish
32
33 196 covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld
34
35 197 blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for
36
37 198 measuring pH as per the manufacturer protocol. Briefly, after switching on the device,
38
39 199 calibration of the device automatically occurs. Next, we adjust the temperature to 37°C , and
40
41 200 select the sample as arterial. Next, we insert the card, which undergoes automatic calibration.
42
43 201 Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to
44
45 202 wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the
46
47 203 first droplet and smoothly inject the sample until the beep. We can see the results of pH,
48
49 204 partial CO_2 and O_2 pressures thereafter. Each laboratory will report the results to also
50
51 205 compare the resulting partial pressures of CO_2 and O_2 with the incubator display. pH will also
52
53 206 be measured every new batch of a culture medium. The measurement of pH and CO_2 across
54
55
56
57
58
59
60

1
2
3 207 the centres will be performed using a one-brand equipment that will undergo periodic
4
5 208 calibration together. To account for errors in measurement, one well-trained personnel will be
6
7
8 209 assigned to measure the pH and double check the CO₂ level across the centres.
9

210

211

212 **Culture Protocol and Embryo Scoring**

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

229 **Embryo Transfer**

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

1
2
3 232 women with reduced uterine cavity or previous preterm birth, they will replace only one
4
5 233 embryo. One participating centre will transfer majority of its cases on day 3. This issue will
6
7
8 234 be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under
9
10 235 sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre
11
12 236 standardized protocol. The rest of the utilizable embryos will undergo vitrification for
13
14 237 transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted
15
16 238 from fresh and vitrified-warmed transfer within one year of randomization. Women will test
17
18 239 for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will
19
20 240 confirm pregnancy at \geq week 7 of gestation by detection of intrauterine sac with a heartbeat
21
22 241 on ultrasound.

26 242 **Outcome Measures**

28 243 Each outcome will be calculated including all randomised participants in the arms to which
29
30 244 they were allocated, with the exception of implantation rate, which will be interpreted
31
32 245 cautiously due to concerns over its validity as a measure of treatment effect, and perinatal
33
34 246 outcomes, which by definition are only available in the subset of participants achieving live
35
36 247 birth. This study will adopt the COMMIT definitions of outcomes,²² where appropriate.

40 248 ***Primary outcome***

42 249 Live birth (delivery of one or more viable infants $> 20^{\text{th}}$ weeks of gestation).

44 250 ***Secondary outcomes***

- 47 251 1) Biochemical pregnancy (positive $\beta\text{hCG} \geq 10$ IU/L at 14 days after egg retrieval).
- 48 252 2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound $> 7^{\text{th}}$ weeks of
51 253 gestation).
- 53 254 3) Ongoing pregnancy (continued viable pregnancy $> 20^{\text{th}}$ weeks of gestation).
- 55 255 4) Miscarriage (loss of a clinical pregnancy $\leq 20^{\text{th}}$ weeks of gestation).
- 57 256 5) Term live-birth (i.e. delivery of one or more viable infants ≥ 37 weeks of gestation).

- 1
2
3 257 6) Preterm Birth (delivery of one or more viable infants < 37th weeks of gestation).
4
5 258 7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).
6
7
8 259 8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)
9
10 260 9) Congenital malformation (delivery of congenitally malformed babies).
11
12 261 10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).
13
14 262 11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-
15
16 263 warmed within one year of randomization).
17
18 264 12) Fertilization (presence of 2 pronuclei 17±1 hr after ICSI).
19
20 265 13) Embryo cleavage (cleaved embryos per fertilized oocyte).
21
22 266 14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than
23
24 267 10% fragmentation by volume).
25
26 268 15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).
27
28 269 16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many
29
30 270 trophoctodermal cells creating a connected zone and a blastocoel more than 100% by volume;
31
32 271 ≥ 311 grade per fertilized oocyte).
33
34 272 17) Cryopreservation (cryopreserved embryos per fertilized oocyte).
35
36 273 18) Live-birth-implantation rate (number of viable neonates per number of embryos
37
38 274 transferred).
39
40 275 19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized
41
42 276 oocyte).
43
44 277 20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst
45
46 278 cryopreserved of 311 grade per fertilized oocyte).
47
48
49
50
51
52
53

279 **Statistical Analysis**

280 ***Sample size estimation***

1
2
3 281 The primary analysis will test whether there is an increase or decrease in live birth rate as pH
4
5 282 changes, using logistic regression. Supposing an increase from 25% to 35% live birth rate
6
7 283 across the tested pH range, a sample size of 680 per group would give 93% per cent power to
8
9 284 reject the null at a 1% significance level. If, on the other hand, live birth decreased (e.g. from
10
11 285 25% to 15%), then 680 participants per arm would grant more than 99% power at a 5%
12
13 286 significance level and 98% power at a 1% level. This is before adjustment for prognostic
14
15 287 covariates. This sample size is robust to 5% dropout, yielding > 90% for all scenarios
16
17 288 discussed above. Therefore, we anticipate recruiting 2100 participants assigned to the groups
18
19 289 at 1:1:1 ratio.
20
21
22

23 290 *Analytical methods*

24
25
26 291 The study conduct will be according to the intention-to-treat approach, where each participant
27
28 292 randomised will be included in the analysis, regardless of protocol deviation. The primary
29
30 293 analysis of term live-birth will be conducted using logistic regression, with term live birth
31
32 294 event regressed on log(pH), adjusted for study site and participant age, which will be
33
34 295 standardised before being entered as a covariate. A secondary analysis of live birth will
35
36 296 compare the outcome between each pH group, by replacing log(pH) in the regression model
37
38 297 with a dummy variable for treatment group. For secondary outcomes, binary variables will be
39
40 298 analysed in an analogous fashion to the primary analysis. Count variables will be analysed
41
42 299 using Poisson regression, with zero-inflated models wherever the outcome is undefined for
43
44 300 some participants. Again, these will be adjusted for site and age. A 1% significance level will
45
46 301 be employed. Due to the short treatment duration, it is anticipated that loss to follow up will
47
48 302 be minimal, but if any loss does occur then these participants will be analysed as having
49
50 303 negative status for the primary outcome.
51
52
53
54

55 304 **Discussion**

56
57
58
59
60

1
2
3 305 Given the lack of evidence for a superior pH level for human embryo culture and whether
4
5 306 the pH level could make a difference in live birth after IVF, this trial is performed. This trial
6
7
8 307 is expected to fill the gap in this area leaving the wishes of manufactures of culture media to
9
10 308 a solid base relying on evidence. The trial power is set to be high (>90%, with a 1%
11
12 309 significance level) to minimize the risk for uninformative results.

14 310 **Strengths and limitations of this study**

16
17 311 The study is randomised controlled which reduces the possibility of bias. The study has an
18
19 312 independent data monitoring committee with access to the data with no involvement in the
20
21 313 study conduct. A possible limitation is that the study will be conducted on ICSI cycles as
22
23 314 ICSI is the preferred insemination method in the participating centres. The embryologists will
24
25
26 315 be aware of the culture arms during the study conduct.

28 316 **Funding and conflict of interest**

29
30 317 The study receives no fund and the authors have no conflict of interest to declare.
31
32
33 318

35 319 **References**

- 37
38 320 1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro
39 321 Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi:
40 322 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
41 323 2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children
42 324 conceived by ART: ‘the chicken or the egg?’. *Human Reproduction Update*
43 325 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
44 326 3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol*
45 327 *Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First:
46 328 2012/07/26]
47 329 4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod*
48 330 *Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First:
49 331 2012/02/09]
50 332 5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation
51 333 embryo culture media: effects of culture and batches. *Reprod Biomed Online*
52 334 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First:
53 335 2018/09/20]
54 336 6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J*
55 337 *Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
56
57
58
59
60

- 1
2
3 338 7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human
4 339 preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi:
5 340 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
6
7 341 8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH
8 342 against acidosis in preantral follicles by multiple mechanisms. *Development*
9 343 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
10 344 9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum*
11 345 *Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First:
12 346 1998/06/10]
13 347 10. Lane M, Baltz JM, Bavister BD. Na⁺/H⁺ antiporter activity in hamster embryos is
14 348 activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi:
15 349 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
16 350 11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and
17 351 embryo manipulations for assisted reproduction. *Reprod Biomed Online*
18 352 2009;18(6):799-810. [published Online First: 2009/06/06]
19 353 12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil*
20 354 *Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online
21 355 First: 2010/11/12]
22 356 13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon
23 357 dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155
24 358 [published Online First: 1987/06/01]
25 359 14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation
26 360 embryo: amino acids act as buffers of intracellular pH. *Hum Reprod*
27 361 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First:
28 362 1999/01/14]
29 363 15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse
30 364 embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-
31 365 42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J
32 366 [published Online First: 1998/07/21]
33 367 16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ*
34 368 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
35 369 17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines
36 370 for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi:
37 371 10.1136/bmj.c332 [published Online First: 2010/03/25]
38 372 18. World Health Organization. WHO laboratory manual for the examination and processing
39 373 of human semen. 5th ed. Geneva: World Health Organization 2010.
40 374 19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of
41 375 single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-
42 376 6736(92)92425-f [published Online First: 1992/07/04]
43 377 20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory
44 378 environment and air quality: report of an expert meeting. *Reprod Biomed Online*
45 379 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First:
46 380 2018/04/17]
47 381 21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment:
48 382 proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi:
49 383 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
50 384 22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and
51 385 implementing a core outcome set for infertility. *Hum Reprod Open*
52 386 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First:
53 387 2019/03/22]

388

For peer review only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 SPIRIT reporting 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

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
14				
15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	4
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	2 & 3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2 & 3
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	3 & 4
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	4
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
51				
52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	4
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
57				
58				
59				
60				

1	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
2				
3				
4				
5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
7	description			
8				
9				
10	Interventions:	#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
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
16	adherence			
17				
18				
19				
20				
21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
22	concomitant care			
23				
24				
25	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
26				
27				
28				
29				
30				
31				
32				
33				
34	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
35				
36				
37				
38				
39				
40	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
41				
42				
43				
44				
45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
46				
47				
48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#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
55	generation			
56				
57				
58				
59				
60				

provided in a separate document that is unavailable to those who enrol participants or assign interventions

1			
2			
3			
4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
6			describing any steps to conceal the sequence until interventions
7	mechanism		are assigned
8			
9			
10			
11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol
12	implementation		participants, and who will assign participants to interventions
13			
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial
15			participants, care providers, outcome assessors, data analysts),
16			and how
17			
18			
19			
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,
21	emergency unblinding		and procedure for revealing a participant's allocated intervention
22			during the trial
23			
24			
25	Methods: Data		
26	collection,		
27	management, and		
28	analysis		
29			
30			
31			
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and
33			other trial data, including any related processes to promote data
34			quality (eg, duplicate measurements, training of assessors) and a
35			description of study instruments (eg, questionnaires, laboratory
36			tests) along with their reliability and validity, if known.
37			Reference to where data collection forms can be found, if not in
38			the protocol
39			
40			
41			
42			
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,
44	retention		including list of any outcome data to be collected for participants
45			who discontinue or deviate from intervention protocols
46			
47			
48	Data management	#19	Plans for data entry, coding, security, and storage, including any
49			related processes to promote data quality (eg, double data entry;
50			range checks for data values). Reference to where details of data
51			management procedures can be found, if not in the protocol
52			
53			
54			
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary
56			outcomes. Reference to where other details of the statistical
57			analysis plan can be found, if not in the protocol
58			
59			
60			

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	11 & 12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	11 & 12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
8				
9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	4
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
19				
20				
21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	4, 11 &
23	interim analysis		including who will have access to these interim results and make	12
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	4
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
31				
32				
33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	4
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	4
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	4
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	4
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
57				
58				
59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	4
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	4
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	4
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	4
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	4
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	1
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	4
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	n/a
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 09. September 2019 using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2019-034194.R1
Article Type:	Protocol
Date Submitted by the Author:	19-Nov-2019
Complete List of Authors:	Fawzy, Mohamed; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Emad, Mai; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Wilkinson, Jack; University of Manchester, Centre for Biostatistics; Salford Royal NHS Foundation Trust, Research and Development Mansour, Ragaa; Egyptian IVF-ET Center Mahran, Ali; Assiut University Faculty of Medicine, Department of Dermatology, Venereology and Andrology Fetih, Ahmed; Assiut University Faculty of Medicine, Department of Obstetrics and Gynecology Abdelrahman , Mohamed; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology AbdelGhafar, Hazem; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

pH-Study Protocol

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

Mohamed Fawzy,^{ab} Mai Emad,^{ab} Jack Wilkinson,^c Ragaa Mansour,^d Ali Mahran,^d Ahmed
N. Fetih,^f Mohamed Y. AbdelRahman,^g Hazem Abdelghafar,^g

IbnSina IVF Centre, Sohag and Banon IVF Centre, Assuit, Egypt

^aIbnSina IVF Centre, IbnSina Hospital, Sohag, Egypt; ^bBanon IVF Centre, Assiut, Egypt;
^cCentre for Biostatistics, University of Manchester, UK; ^dEgyptian IVF-ET Centre, Cairo,
Egypt; ^eDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine,
Assiut University, Egypt; ^fDepartment of Obstetrics and Gynecology, Faculty of Medicine,
Assiut University, Egypt; ^gDepartment of Obstetrics and Gynecology, Faculty of Medicine,
Sohag University, Egypt

Corresponding Author: Dr. Mohamed Fawzy, IVF Laboratory Director (IbnSina and Banon
IVF Centres), IbnSina Hospital, 146 El Aref Square, Sohag, Egypt; Cell: +201011122286; E-
mail: drfawzy001@me.com

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

1
2
3
4 55
5 56
6 57

Strengths and limitations of this study

- 8 58
- The study is randomised controlled which reduces the possibility of bias.
- 10 59
- The study has an independent data monitoring committee with access to the data with
- 12 60
- no involvement in the study conduct.
- 14 61
- A possible limitation is that the study will be conducted on ICSI cycles as ICSI is the
- 16 62
- preferred insemination method in the participating centres.
- 18 63
- The embryologists will be aware of the culture arms during the study conduct.
- 20 64

Background

22 64

23 65

24 66

25 66

26 67

27 67

28 68

29 68

30 69

31 69

32 69

33 69

34 70

35 70

36 71

37 71

38 72

39 72

40 73

41 73

42 74

43 74

44 75

45 75

46 76

47 76

48 77

49 77

50 78

51 78

52 79

53 79

54 80

55 80

56 81

57 81

58 81

59 81

81

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 CO₂ 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 CO₂ of incubators affect the pHe, which is a potential stressor.⁷

1
2
3 82 The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell
4
5 83 division and differentiation, protein synthesis, metabolism, mitochondrial function,
6
7 84 cytoskeletal regulation, and microtubule dynamics.^{7,8} Drifts in pHe translate into changes in
8
9 85 pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt
10
11 86 to restore pHi to a safe level.⁸ The pHi can compensate through an active exchange among
12
13 87 Na⁺, HCO₃⁻/Cl⁻ and Na⁺/H⁺ to maintain it between 7 to 7.3.^{5,8} Denuded oocyte for ICSI
14
15 88 through fertilization thereafter and vitrified-warmed embryos lack robust compensatory
16
17 89 mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios
18
19 90 can significantly perturbate embryo development.⁹⁻¹¹
20
21 91 That being said, an optimum level of pH for human embryo culture *in vitro* is still unknown.⁴
22
23 92 ^{9,12-15} Most recommendations rely on mice models or manufacturers of culture media.
24
25 93 Theoretically, a wide range of pHe levels (7.0–7.5) are believed to support human embryo
26
27 94 development *in vitro*. However, extreme pHe levels can adversely affect oocyte and embryo
28
29 95 development.⁴ An acidic range of pHe can negative affect the oocyte spindle leading to no
30
31 96 fertilization, degeneration or blocking embryo development.⁴ An alkaline shift in pHe has the
32
33 97 similar adverse effects but lesser than the acidic shift.⁴ Recently, there has been anecdotal
34
35 98 evidence that culturing embryos at pHe away from 7.2 to 7.4 can lead to mitotic errors.
36
37 99 Therefore, the optimal level of pHe for human embryo development is still to be determined.
38
39 100 Given the anecdotal evidence that levels between 7.2 to 7.4 pHe can support embryo
40
41 101 development with variable results, this multicentre, randomized, clinical trial aims to
42
43 102 compare the influence of three commonly used levels of pH (7.2, 7.3 and 7.4) on live birth
44
45 103 rate after ICSI, in order to investigate the potential for optimisation.
46
47
48
49
50
51
52
53

104 **Methods and Design**

54
55 105 This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial
56
57 106 (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for
58
59
60

1
2
3 107 human embryo culture in vitro on live birth after ICSI (Figure 1). This partially blind design
4
5 108 represents that clinicians, participants and outcome assessor, not including the embryologists,
6
7
8 109 will be unaware of the study arms. This multicentre trial will involve private IVF facilities in
9
10 110 Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre
11
12 111 and Amshaj IVF Centre) with the study protocol in their hand before enrolment of
13
14
15 112 participants. If other IVF facilities join this trial before recruitment, this will be reported in
16
17 113 the study.

19 114 **Ethics and dissemination**

21 115 This trial obtained the approval from Ethics Review Board of Upper Egypt IVF Network
22
23 116 relating to the participating sites (Approval No. 009/2016). An independent safety and
24
25
26 117 monitoring committee formed of five experts in reproductive endocrinology, reproductive
27
28 118 biology, embryo culture, biostatistics and trial methodology will oversee this trial. All
29
30 119 participants will receive independent counselling from research instructors who are not
31
32
33 120 involved in patient care or laboratory work. Participants who will accept to participate will
34
35 121 sign a written informed consent before enrolment. Conducting this study will be in
36
37 122 accordance with the Declaration of Helsinki.¹⁶ The trial reporting will be according to the
38
39 123 CONSORT statement,¹⁷ unless other guideline will have higher ranking at that time. No plan
40
41 124 exists to amend this protocol and any amendments will be responsibility for the safety
42
43 125 committee and will undergo detailed reporting on the trial registry and in the final
44
45
46 126 manuscript.

49 127 **Intervention**

51 128 Oocytes and embryos in the three arms will undergo continuous culture from day 0 through
52
53 129 day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after
54
55 130 ICSI in pHe of 7.2 ± 0.02 . Arm II The “Arm II” is to culture oocytes and resulting embryos
56
57 131 after ICSI in pHe of 7.3 ± 0.02 . “Arm III” is to culture oocytes and resulting embryos after
58
59
60

1
2
3 132 ICSI in pHe of 7.4 ± 0.02 . This trial will include intracytoplasmic sperm injection (ICSI)
4
5 133 cycles.

7
8 **134 Patient and Public Involvement**

9
10 135 No patient involved

11
12 **136 Randomization and Masking**

13
14 137 Using an online tool, participants will be randomised to the experimental arms with a 1:1:1
15
16 138 allocation ratio. The allocation sequence of participants will be generated using a permuted
17
18 139 block randomization of 3, 6 and 9 block sizes with unique identifiers in random order,
19
20 140 stratified by trial site. Randomization of participants and its storage in sequentially numbered,
21
22 141 opaque, sealed envelopes will occur by a secretary with no involvement in patient care and
23
24 142 will be provided to trial sites before enrolment of first participant. Eligible participants will
25
26 143 be allocated to the relevant arms on the day of maturation trigger and allocation result will be
27
28 144 communicated to the laboratory team. Participants, clinicians and outcome assessors for the
29
30 145 clinical outcomes will be unaware of the allocation, while embryologists who will assess
31
32 146 embryo development will be aware of the allocation.

33
34
35
36
37 **147 Participants**

38
39 148 The inclusion criteria include:

- 40
41 149 1) Women age of ≥ 18 to ≤ 40 ;
42
43 150 2) BMI of ≤ 31 ;
44
45 151 3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);
46
47 152 4) Women who have ≥ 1 year of primary or secondary infertility;
48
49 153 5) Fresh ejaculate sperm of any count provided they have $\geq 1\%$ normal forms and a motile
50
51 154 fraction;
52
53 155 6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous
54
55 156 successful one;

1
2
3 157 7) Women with > 7 mm endometrial thickness at day of trigger;
4
5 158 and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g.
6
7 159 submucosal myomas, polyps or septa).

8
9
10 160 ***Women will be excluded if they have:***

- 11
12 161 1) Unilateral oophorectomy;
13
14 162 2) Abnormal karyotyping for them or their male partners;
15
16 163 3) History of repeated abortions or implantation failure;
17
18 164 4) Uncontrolled diabetes;
19
20 165 5) Liver or renal disease;
21
22 166 6) History of severe ovarian hyperstimulation;
23
24 167 7) History of malignancy or borderline pathology;
25
26 168 8) Endometriosis;
27
28 169 9) Plan for PGD-A;
29
30 170 10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;
31
32 171 and 11) Severe PCOS, hyper-responder, OHSS patients, and cycles with agonist trigger or
33
34 172 any patient with a plan for a “freeze-all”.

35
36
37
38
39
40 173 **Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support**

41
42 174 Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-
43
44 175 regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg,
45
46 176 Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will
47
48 177 continue to the day of maturation trigger. For Antagonist group, women will start the
49
50 178 antagonist on day 6 of treatment cycle. All women will receive follicular stimulating
51
52 179 hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG;
53
54 180 Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular
55
56 181 maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles
57
58
59
60

1
2
3 182 measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger
4
5 183 shot (Choriomon, IBSA) for final oocyte maturation. Oocyte retrieval will be performed 37
6
7 184 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be
8
9 185 handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using
10
11 186 tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100
12
13 187 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily,
14
15 188 starting on day 1 after retrieval (“day 1”) to 12 weeks of gestation, unless negative
16
17 189 pregnancy.

18 190 **Sperm Preparation, Oocyte Denudation and ICSI**

19
20
21
22
23 191 Semen samples will be processed through density gradient,¹⁸ using Puresperm (Nidacon,
24
25 192 Sweden). The pellet will undergo once washing and incubation at room temperature in
26
27 193 HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur
28
29 194 immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in
30
31 195 Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes
32
33 196 will undergo ICSI in Global HEPES medium under inverted microscope as previously
34
35 197 described.¹⁹

36 198 **Incubator Management and pH Adjustment**

37
38
39
40 199 Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US),
41
42 200 and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to
43
44 201 account for incubator as variable. If another brand of incubators will be used, we will ensure
45
46 202 they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be
47
48 203 used at some centres; however, we will adjust the analysis by trial site to account for
49
50 204 differences between centres. Incubators will undergo stringent control of temperature
51
52 205 (36.9±0.1°C). The temperature will be validated daily using a certified thermometer.
53
54 206 Incubator’s CO₂ and O₂ will be measured daily using a certified gas analyser to ensure 5% O₂
55
56
57
58
59
60

1
2
3 207 and a proper CO₂ concentration to achieve the required pH. All the three measurements
4
5 208 (temperature, CO₂ level, and pH levels) will be verified by well-trained person traveling
6
7 209 across the sites. Incubators will undergo sterilization with 6% H₂O₂ every four weeks, with
8
9 210 installation of inline filters (Green, Lifeglobal, CooperSurgical).²⁰
11
12 211 A minimum of 3 incubators of a single brand within each participating facility with different
13
14 212 levels of pH representing the study arms is obligatory: Incubator A of 7.2±0.02 pH, Incubator
15
16 213 B of 7.3±0.02 pH, and Incubator C of 7.4±0.02. The three incubators will undergo a strict
17
18 214 adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host;
19
20 215 BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH
21
22 216 with blood gas analyser and a daily measurement of CO₂ level of incubators. Measurement of
23
24 217 pH will occur after an overnight incubation of 1mL culture media in a central well dish
25
26 218 covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld
27
28 219 blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for
29
30 220 measuring pH as per the manufacturer protocol. Briefly, after switching on the device,
31
32 221 calibration of the device automatically occurs. Next, we adjust the temperature to 37°C, and
33
34 222 select the sample as arterial. Next, we insert the card, which undergoes automatic calibration.
35
36 223 Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to
37
38 224 wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the
39
40 225 first droplet and smoothly inject the sample until the beep. We can see the results of pH,
41
42 226 partial CO₂ and O₂ pressures thereafter. Each laboratory will report the results to also
43
44 227 compare the resulting partial pressures of CO₂ and O₂ with the incubator display. pH will also
45
46 228 be measured every new batch of a culture medium. The measurement of pH and CO₂ across
47
48 229 the centres will be performed using a one-brand equipment that will undergo periodic
49
50 230 calibration together. To account for errors in measurement, one well-trained personnel will be
51
52 231 assigned to measure the pH and double check the CO₂ level across the centres.
53
54
55
56
57
58
59
60

232

233

234 **Culture Protocol and Embryo Scoring**

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

251 **Embryo Transfer**

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

1
2
3 257 sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre
4
5 258 standardized protocol. The rest of the utilizable embryos will undergo vitrification for
6
7 259 transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted
8
9 260 from fresh and vitrified-warmed transfer within one year of randomization. Women will test
10
11 261 for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will
12
13 262 confirm pregnancy at \geq week 7 of gestation by detection of intrauterine sac with a heartbeat
14
15 263 on ultrasound.

19 264 **Outcome Measures**

21 265 Each outcome will be calculated including all randomised participants in the arms to which
22
23 266 they were allocated, with the exception of implantation rate, which will be interpreted
24
25 267 cautiously due to concerns over its validity as a measure of treatment effect, and perinatal
26
27 268 outcomes, which by definition are only available in the subset of participants achieving live
28
29 269 birth. This study will adopt the COMMIT definitions of outcomes,²² where appropriate.

33 270 **Primary outcome**

35 271 Live birth (delivery of one or more viable infants $>$ 20th weeks of gestation).

38 272 **Secondary outcomes**

- 40 273 1) Biochemical pregnancy (positive β hCG \geq 10 IU/L at 14 days after egg retrieval).
- 42 274 2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound $>$ 7th weeks of
44 275 gestation).
- 46 276 3) Ongoing pregnancy (continued viable pregnancy $>$ 20th weeks of gestation).
- 48 277 4) Miscarriage (loss of a clinical pregnancy \leq 20th weeks of gestation).
- 50 278 5) Term live-birth (i.e. delivery of one or more viable infants \geq 37 weeks of gestation).
- 52 279 6) Preterm Birth (delivery of one or more viable infants $<$ 37th weeks of gestation).
- 54 280 7) Very preterm birth (delivery of one or more viable infants $<$ 32nd weeks of gestation).
- 56 281 8) Low birth weight babies (babies with $<$ 2500 gm within 24 hours of delivery)

- 1
2
3 282 9) Congenital malformation (delivery of congenitally malformed babies).
4
5 283 10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).
6
7 284 11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-
8
9 warmed within one year of randomization).
10
11 285
12 286 12) Fertilization (presence of 2 pronuclei 17±1 hr after ICSI).
13
14 287 13) Embryo cleavage (cleaved embryos per fertilized oocyte).
15
16 288 14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than
17
18 289 10% fragmentation by volume).
19
20 290 15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).
21
22 291 16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many
23
24 292 trophoctodermal cells creating a connected zone and a blastocoel more than 100% by volume;
25
26 293 ≥ 311 grade per fertilized oocyte).
27
28 294 17) Cryopreservation (cryopreserved embryos per fertilized oocyte).
29
30 295 18) Live-birth-implantation rate (live birth per embryo transferred).
31
32 296 19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized
33
34 297 oocyte).
35
36 298 20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst
37
38 299 cryopreserved of 311 grade per fertilized oocyte).
39
40
41
42
43

300 **Statistical Analysis**

301 ***Sample size estimation***

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

1
2
3 307 The study has been powered for a global test of the effect of pH, calculated using plausible
4
5 308 birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yields 98%
6
7 309 power in this scenario, using a 5% significance level. This test makes no assumption about
8
9 310 the ordering of the live birth rates in relation to the ordering of the pH values. The high power
10
11 311 level has been adopted to allow for some leeway in relation to the minimum effect size. For
12
13 312 illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just
14
15 313 seven percentage points) this sample size yields 85% power against a 5% significance level,
16
17 314 and 65% at a 1% significance level. We have also been conservative in our inflation of
18
19 315 numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to
20
21 316 680. In reality, we will conduct analysis on an intention to treat basis, including all
22
23 317 randomised women. Women who do not complete treatment (for example, they do not
24
25 318 undergo embryo transfer) will be counted as not having a live birth. The only exceptions to
26
27 319 this will be participants who withdraw consent for their data to be used in the study. Our
28
29 320 inflation for loss to follow up reflects this possibility.
30
31 321 We also note that adjustment for site and age in the analysis will increase power further.
32
33
34
35
36
37
38
39

40 323 *Analytical methods*

41
42 324 The study conduct will be according to the intention-to-treat approach, where each participant
43
44 325 randomised will be included in the analysis, regardless of protocol deviation. The primary
45
46 326 analysis of live birth will be conducted using logistic regression, with live birth event
47
48 327 regressed on pH group, adjusted for study site and participant age, which will be standardised
49
50 328 before being entered as a covariate. pH will be entered as a categorical covariate, allowing a
51
52 329 Likelihood Ratio test of the association between pH and live birth rate across the three groups
53
54 330 to be performed. Secondary supportive analyses will be conducted to try to characterise the
55
56 331 nature of any association. This will include a test of linear trend in live birth rates across pH
57
58
59
60

1
2
3 332 groups, which would imply an optimal pH level for the lowest or highest value, as well as
4
5 333 pairwise comparisons between each group (again, these analyses will be adjusted for site and
6
7 334 age). The pairwise comparisons will focus on size and precision of the odds ratios. Although
8
9
10 335 it would be desirable to power the study for all pairwise comparisons as the primary outcome,
11
12 336 this yields impracticable sample sizes (> 4000 participants) against realistic effects. The study
13
14 337 has therefore been designed to represent the most informative test of the hypothesis that pH
15
16 338 level affects live birth, that is practicable.

17
18
19 339

20
21 340 For secondary outcomes, binary variables will be analysed in an analogous fashion to the
22
23 341 primary analysis. Count variables will be analysed using Poisson regression, with zero-
24
25 342 inflated models wherever the outcome is structurally undefined for some participants. Again,
26
27 343 these will be adjusted for site and age. In the analysis of number of usable embryos,
28
29 344 implanted embryos arising from the day 3 transfer will be included as formed and good
30
31 345 quality blastocysts, while those that do not implant in this portion will be considered blocked
32
33 346 at day 3. The total of the number of embryos transferred and the formed blastocysts will be
34
35 347 used to calculate number of utilizable embryos. A 1% significance level will be employed.
36
37 348 Due to the short treatment duration, it is anticipated that loss to follow up will be minimal,
38
39 349 but if any loss does occur then these participants will be analysed as having negative status
40
41 350 for the primary outcome, unless consent to use data is withdrawn. The follow-up period is
42
43 351 identified as one year from randomization of the last participant provided that all pregnant
44
45 352 women have given birth.

51 353 **Discussion**

52
53 354 Given the lack of evidence for a superior pH level for human embryo culture and whether
54
55 355 the pH level could make a difference in live birth after IVF, this trial is performed. This trial
56
57 356 is expected to fill the gap in this area leaving the recommendations of manufactures of culture
58
59
60

1
2
3 357 media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1%
4
5 358 significance level) to minimize the risk for uninformative results.
6
7

8 359 **Funding and conflict of interest**

9
10 360 The study receives no fund and the authors have no conflict of interest to declare.
11

12 361 **Authors' contributions**

13
14 362 Mohamed is the creator of the concept and design of the study, and is the principal
15
16 363 investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across
17
18 364 the sites and will make sure that data is periodically sent to for storage in independent
19
20 365 database. Jack Wilkinson is the statistician of the study who revised the study design and
21
22 366 calculated the sample size and power of the study and he will be responsible for the data
23
24 367 analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a
25
26 368 primary investigator at Banon IVF centre and a sub-investigator at Ibsina Centre, and she
27
28 369 participated in revising the trial protocol and will participate in trial reporting thereafter.
29
30
31 370 Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial
32
33 371 protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the
34
35 372 study that will make sure all male partners are in line with the inclusion criteria, and revised
36
37 373 the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF
38
39 374 Centre and participated revising the protocol and provided comments. Mohamed
40
41 375 AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF
42
43 376 Centre. Hazem Abdelghafar is a primary investigator at Ibsina IVF Centre and participated
44
45 377 in the trial design. All authors provided comments and agreed on the study design and
46
47 378 protocol, and will participate in reporting this trial thereafter.
48
49
50
51
52

53 379 **References**

54
55
56 380 1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro
57 381 Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi:
58 382 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
59
60

- 1
2
3 383 2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children
4 384 conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update*
5 385 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
- 6 386 3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol*
7 387 *Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First:
8 388 2012/07/26]
- 9 389 4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod*
10 390 *Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First:
11 391 2012/02/09]
- 12 392 5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation
13 393 embryo culture media: effects of culture and batches. *Reprod Biomed Online*
14 394 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First:
15 395 2018/09/20]
- 16 396 6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J*
17 397 *Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
- 18 398 7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human
19 399 preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi:
20 400 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
- 21 401 8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH
22 402 against acidosis in preantral follicles by multiple mechanisms. *Development*
23 403 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
- 24 404 9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum*
25 405 *Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First:
26 406 1998/06/10]
- 27 407 10. Lane M, Baltz JM, Bavister BD. Na⁺/H⁺ antiporter activity in hamster embryos is
28 408 activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi:
29 409 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
- 30 410 11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and
31 411 embryo manipulations for assisted reproduction. *Reprod Biomed Online*
32 412 2009;18(6):799-810. [published Online First: 2009/06/06]
- 33 413 12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil*
34 414 *Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online
35 415 First: 2010/11/12]
- 36 416 13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon
37 417 dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155
38 418 [published Online First: 1987/06/01]
- 39 419 14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation
40 420 embryo: amino acids act as buffers of intracellular pH. *Hum Reprod*
41 421 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First:
42 422 1999/01/14]
- 43 423 15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse
44 424 embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-
45 425 42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J
46 426 [published Online First: 1998/07/21]
- 47 427 16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ*
48 428 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
- 49 429 17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines
50 430 for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi:
51 431 10.1136/bmj.c332 [published Online First: 2010/03/25]
- 52
53
54
55
56
57
58
59
60

- 1
2
3 432 18. World Health Organization. WHO laboratory manual for the examination and processing
4 433 of human semen. 5th ed. Geneva: World Health Organization 2010.
5 434 19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of
6 435 single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-
7 436 6736(92)92425-f [published Online First: 1992/07/04]
8 437 20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory
9 438 environment and air quality: report of an expert meeting. *Reprod Biomed Online*
10 439 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First:
11 440 2018/04/17]
12 441 21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment:
13 442 proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi:
14 443 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
15 444 22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and
16 445 implementing a core outcome set for infertility. *Hum Reprod Open*
17 446 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First:
18 447 2019/03/22]
19
20
21
22 448

23
24 449 Figure Legend: Trial plan for enrolment
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Couples will receive detailed information on the possible risks and potential benefits.

Couples with primary acceptance will be randomized to the three pH arms of the study

Randomization will occur to assign ~ 2100 participants to undergo day-5 fresh embryo-transfer utilizing the blastocysts developed in the either 7.2, 7.3 or 7.4 pH levels.

Women randomization

~ 700 participants will be randomized to undergo embryo culture in 7.2 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.3 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.4 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

The intention-to-treat will include all randomized participants to 7.2 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.3 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.4 pH level group with the live birth rate as primary endpoint.

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 SPIRIT reporting 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

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
14				
15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	4
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	2 & 3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2 & 3
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	3 & 4
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	4
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
51				
52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	4
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
57				
58				
59				
60				

1	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
2				
3				
4				
5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
7	description			
8				
9				
10	Interventions:	#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
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
16	adherence			
17				
18				
19				
20				
21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
22	concomitant care			
23				
24				
25	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
26				
27				
28				
29				
30				
31				
32				
33				
34	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
35				
36				
37				
38				
39				
40	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
41				
42				
43				
44				
45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
46				
47				
48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#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
55	generation			
56				
57				
58				
59				
60				

provided in a separate document that is unavailable to those who enrol participants or assign interventions

1				
2				
3				
4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central	5
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),	
6			describing any steps to conceal the sequence until interventions	
7	mechanism		are assigned	
8				
9				
10				
11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol	5
12	implementation		participants, and who will assign participants to interventions	
13				
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial	5
15			participants, care providers, outcome assessors, data analysts),	
16			and how	
17				
18				
19				
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,	5
21	emergency unblinding		and procedure for revealing a participant's allocated intervention	
22			during the trial	
23				
24				
25	Methods: Data			
26	collection,			
27	management, and			
28	analysis			
29				
30				
31				
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and	11
33			other trial data, including any related processes to promote data	
34			quality (eg, duplicate measurements, training of assessors) and a	
35			description of study instruments (eg, questionnaires, laboratory	
36			tests) along with their reliability and validity, if known.	
37			Reference to where data collection forms can be found, if not in	
38			the protocol	
39				
40				
41				
42				
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,	11
44	retention		including list of any outcome data to be collected for participants	
45			who discontinue or deviate from intervention protocols	
46				
47				
48	Data management	#19	Plans for data entry, coding, security, and storage, including any	11
49			related processes to promote data quality (eg, double data entry;	
50			range checks for data values). Reference to where details of data	
51			management procedures can be found, if not in the protocol	
52				
53				
54				
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary	11 & 12
56			outcomes. Reference to where other details of the statistical	
57			analysis plan can be found, if not in the protocol	
58				
59				
60				

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	11 & 12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	11 & 12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
8				
9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	4
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
19				
20				
21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	4, 11 &
23	interim analysis		including who will have access to these interim results and make	12
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	4
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
31				
32				
33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	4
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	4
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	4
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	4
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
57				
58				
59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	4
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	4
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	4
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	4
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	4
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	1
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	4
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	n/a
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 09. September 2019 using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)

BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2019-034194.R2
Article Type:	Protocol
Date Submitted by the Author:	05-Dec-2019
Complete List of Authors:	Fawzy, Mohamed; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Emad, Mai; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Wilkinson, Jack; University of Manchester, Centre for Biostatistics; Salford Royal NHS Foundation Trust, Research and Development Mansour, Ragaa; Egyptian IVF-ET Center Mahran, Ali; Assiut University Faculty of Medicine, Department of Dermatology, Venereology and Andrology Fetih, Ahmed; Assiut University Faculty of Medicine, Department of Obstetrics and Gynecology Abdelrahman , Mohamed; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology AbdelGhafar, Hazem; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

pH-Study Protocol

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

Mohamed Fawzy,^{ab} Mai Emad,^{ab} Jack Wilkinson,^c Ragaa Mansour,^d Ali Mahran,^d Ahmed
N. Fetih,^f Mohamed Y. AbdelRahman,^g Hazem Abdelghafar,^g

IbnSina IVF Centre, Sohag and Banon IVF Centre, Assuit, Egypt

^aIbnSina IVF Centre, IbnSina Hospital, Sohag, Egypt; ^bBanon IVF Centre, Assiut, Egypt;
^cCentre for Biostatistics, University of Manchester, UK; ^dEgyptian IVF-ET Centre, Cairo,
Egypt; ^eDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine,
Assiut University, Egypt; ^fDepartment of Obstetrics and Gynecology, Faculty of Medicine,
Assiut University, Egypt; ^gDepartment of Obstetrics and Gynecology, Faculty of Medicine,
Sohag University, Egypt

Corresponding Author: Dr. Mohamed Fawzy, IVF Laboratory Director (IbnSina and Banon
IVF Centres), IbnSina Hospital, 146 El Aref Square, Sohag, Egypt; Cell: +201011122286; E-
mail: drfawzy001@me.com

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

1
2
3
4 55
5 56
6 57

Strengths and limitations of this study

- 8 58
- The study is randomised controlled which reduces the possibility of bias.
- 10 59
- The study has an independent data monitoring committee with access to the data with
- 12 60
- no involvement in the study conduct.
- 14 61
- Two possible limitations of the study are that it will be conducted on ICSI cycles as
- 16 62
- ICSI is the preferred insemination method in the participating centres, and formed
- 18 63
- blastocyst calculation will be based on assumption for any cleavage-stage transfer.
- 20 64
- The embryologists will be aware of the culture arms during the study conduct.

Background

25 65

26 66

27 67 Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six

28 68

29 69 cycles of *in vitro* fertilisation (IVF),¹ which is relatively suboptimal. In addition, IVF results

30 70

31 71 in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared

32 72

33 73 with the *in vivo* conception.² These outcomes can rely on factors relating to patients,

34 74

35 75 stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200

36 76

37 77 variables have been identified as being potentially relevant to the cycle outcome.³ One

38 78

39 79 element that may influence embryo development *in vitro* is the pH level of a culture medium,

40 80

41 81 which thus far has been determined by manufacturers of culture media without recourse to a

42 82

43 83 well-powered randomized clinical trial (RCT).⁴ The pH levels are potential stressors that vary

44 84

45 85 between media brands and from batch-to-batch depending on the bicarbonate level in culture

46 86

47 87 media and on the CO₂ level of incubators.⁵ This would suggest that pH level can vary

48 88

49 89 between incubators within the same laboratory if it is not well adjusted. Recommendations

50 90

51 91 for measuring pH for embryo culture are variable between daily to monthly measurement.⁴

52 92

53 93 Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH

54 94

55 95 (pHe).⁶ The *in vitro* conditions including concentrations of bicarbonates, proteins, amino

56 96

57 97

58 98

59 99

60 100

1
2
3 82 acids in culture media and the CO₂ of incubators affect the pHe, which is a potential stressor.⁷
4
5 83 The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell
6
7 84 division and differentiation, protein synthesis, metabolism, mitochondrial function,
8
9
10 85 cytoskeletal regulation, and microtubule dynamics.^{7,8} Drifts in pHe translate into changes in
11
12 86 pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt
13
14 87 to restore pHi to a safe level.⁸ The pHi can compensate through an active exchange among
15
16 88 Na⁺, HCO₃⁻/Cl⁻ and Na⁺/H⁺ to maintain it between 7 to 7.3.^{5,8} Denuded oocyte for ICSI
17
18 89 through fertilization thereafter and vitrified-warmed embryos lack robust compensatory
19
20 90 mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios
21
22 91 can significantly perturbate embryo development.⁹⁻¹¹
23
24
25 92 That being said, an optimum level of pH for human embryo culture *in vitro* is still unknown.⁴
26
27 93 ^{9,12-15} Most recommendations rely on mice models or manufacturers of culture media.
28
29
30 94 Theoretically, a wide range of pHe levels (7.0–7.5) can support human embryo development
31
32 95 *in vitro*. However, a narrower range of pHe levels (7.2 to 7.4) is used in clinical practice. This
33
34 96 is because an extreme acidic pHe level (≤ 7) can adversely affect oocyte spindle leading to no
35
36 97 further post-fertilization events.⁴ This level of acidic pH can delay or block embryo
37
38 98 development *in vitro*.⁴ Similar harms can theoretically occur for oocyte and embryo, if
39
40 99 alkaline levels of pHe (≥ 7.5) are used.⁴ Although these potential harms of extreme pHe
41
42
43
44 100 levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to
45
46 101 investigate a safe range of pHe (7.2 to 7.4). Why this range has been chosen depends on the
47
48 102 recommendations of media manufactures and the clinical practice within the majority of
49
50 103 human embryo culture laboratories. Although this range of 7.2 to 7.4 is used, there is clear
51
52 104 evidence about which level of 7.2, 7.3, or 7.4 can support human embryos to result in live
53
54 105 birth after transfer. This multicentre, randomized, clinical trial aims to identify whether pHe
55
56
57
58
59
60

1
2
3 106 levels of 7.2, 7.3, or 7.4 can perform better on live birth rate after ICSI in order to investigate
4
5 107 the potential for optimisation.
6
7

8 108 **Methods and Design**

9
10 109 This protocol version one of a multicentre, randomized, triple-arm, triple-blind clinical trial
11
12 110 (NCT02896777, registered at www.ClinicalTrials.gov) will compare three levels of pH for
13
14 111 human embryo culture in vitro on live birth after ICSI (Figure 1). This partially blind design
15
16 112 represents that clinicians, participants and outcome assessor, not including the embryologists,
17
18 113 will be unaware of the study arms. This multicentre trial will involve private IVF facilities in
19
20 114 Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre, Qena IVF Centre
21
22 115 and Amshaj IVF Centre) with the study protocol in their hand before enrolment of
23
24 116 participants. If other IVF facilities join this trial before recruitment, this will be reported in
25
26 117 the study.
27
28
29

30 118 **Ethics and dissemination**

31
32
33 119 This trial obtained the approval from Ethics Review Board of Upper Egypt IVF Network
34
35 120 relating to the participating sites (Approval No. 009/2016). An independent safety and
36
37 121 monitoring committee formed of five experts in reproductive endocrinology, reproductive
38
39 122 biology, embryo culture, biostatistics and trial methodology will oversee this trial. All
40
41 123 participants will receive independent counselling from research instructors who are not
42
43 124 involved in patient care or laboratory work. Participants who will accept to participate will
44
45 125 sign a written informed consent before enrolment. Conducting this study will be in
46
47 126 accordance with the Declaration of Helsinki.¹⁶ The trial reporting will be according to the
48
49 127 CONSORT statement,¹⁷ unless other guideline will have higher ranking at that time. No plan
50
51 128 exists to amend this protocol and any amendments will be responsibility for the safety
52
53 129 committee and will undergo detailed reporting on the trial registry and in the final
54
55
56 130 manuscript.
57
58
59
60

131 Intervention

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

138 Patient and Public Involvement

139 No patient involved

140 Randomization and Masking

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

151 Participants

152 The inclusion criteria include:

- 153 1) Women age of ≥ 18 to ≤ 40 ;
- 154 2) BMI of ≤ 31 ;
- 155 3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);

- 1
2
3 156 4) Women who have ≥ 1 year of primary or secondary infertility;
4
5 157 5) Fresh ejaculate sperm of any count provided they have $\geq 1\%$ normal forms and a motile
6
7 fraction;
8 158
9
10 159 6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous
11
12 160 successful one;
13
14 161 7) Women with > 7 mm endometrial thickness at day of trigger;
15
16 and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g.
17 162
18 submucosal myomas, polyps or septa).
19 163

20
21 164 ***Women will be excluded if they have:***

- 22
23 165 1) Unilateral oophorectomy;
24
25 166 2) Abnormal karyotyping for them or their male partners;
26
27 167 3) History of repeated abortions or implantation failure;
28
29 168 4) Uncontrolled diabetes;
30
31 169 5) Liver or renal disease;
32
33 170 6) History of severe ovarian hyperstimulation;
34
35 171 7) History of malignancy or borderline pathology;
36
37 172 8) Endometriosis;
38
39 173 9) Plan for PGD-A;
40
41 174 10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;
42
43 and 11) PCOS, women with history of severe OHSS, and cycles with agonist trigger or any
44
45 patient with a plan for a “freeze-all”.
46
47
48
49 176

50
51 177 **Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support**

52
53 178 Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-
54
55 regulation (Decapeptyl[®] 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide[®] 0.25 mg,
56 179
57 Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will
58 180
59
60

1
2
3 181 continue to the day of maturation trigger. For Antagonist group, women will start the
4
5 182 antagonist on day 6 of treatment cycle. All women will receive follicular stimulating
6
7 183 hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG;
8
9 184 Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular
10
11 185 maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles
12
13 186 measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger
14
15 187 shot (Choriomon, IBSA) for final oocyte maturation. Oocyte retrieval will be performed 37
16
17 188 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be
18
19 189 handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using
20
21 190 tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100
22
23 191 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily,
24
25 192 starting on day 1 after retrieval (“day 1”) to 12 weeks of gestation, unless negative
26
27 193 pregnancy.

194 **Sperm Preparation, Oocyte Denudation and ICSI.**

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

202

203 **Incubator Management and pH Adjustment**

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

1
2
3 206 account for incubator as variable. If another brand of incubators will be used, we will ensure
4
5 207 they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be
6
7 208 used at some centres; however, we will adjust the analysis by trial site to account for
8
9 209 differences between centres. Incubators will undergo stringent control of temperature
10
11 210 ($36.9\pm 0.1^{\circ}\text{C}$). The temperature will be validated daily using a certified thermometer.
12
13 211 Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2
14
15 212 and a proper CO_2 concentration to achieve the required pH. All the three measurements
16
17 213 (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling
18
19 214 across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with
20
21 215 installation of inline filters (Green, Lifeglobal, CooperSurgical).²⁰
22
23 216 A minimum of 3 incubators of a single brand within each participating facility with different
24
25 217 levels of pH representing the study arms is obligatory: Incubator A of 7.2 ± 0.02 pH, Incubator
26
27 218 B of 7.3 ± 0.02 pH, and Incubator C of 7.4 ± 0.02 . The three incubators will undergo a strict
28
29 219 adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host;
30
31 220 BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH
32
33 221 with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of
34
35 222 pH will occur after an overnight incubation of 1mL culture media in a central well dish
36
37 223 covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld
38
39 224 blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for
40
41 225 measuring pH as per the manufacturer protocol. Briefly, after switching on the device,
42
43 226 calibration of the device automatically occurs. Next, we adjust the temperature to 37°C , and
44
45 227 select the sample as arterial. Next, we insert the card, which undergoes automatic calibration.
46
47 228 Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to
48
49 229 wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the
50
51 230 first droplet and smoothly inject the sample until the beep. We can see the results of pH,
52
53
54
55
56
57
58
59
60

1
2
3 231 partial CO₂ and O₂ pressures thereafter. Each laboratory will report the results to also
4
5 232 compare the resulting partial pressures of CO₂ and O₂ with the incubator display. pH levels
6
7 233 will also be measured every new batch of a culture medium. The measurement of pH and
8
9 234 CO₂ across the centres will be performed using a one-brand equipment that will undergo
10
11 235 periodic calibration together. To account for errors in measurement, one well-trained
12
13 236 personnel will be assigned to measure the pH and double check the CO₂ level across the
14
15 237 centres.
16
17
18

19 238 **Culture Protocol and Embryo Scoring**

20
21 239 Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global
22
23 240 Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil,
24
25 241 Nidacon). If a decision to change culture media at any time point of the study conduct is
26
27 242 made, this will be performed at the same time across the study sites. Dishes will undergo
28
29 243 overnight incubation in the relevant incubator adjusted to the relevant pH as per the
30
31 244 randomization. After ICSI, the injected oocytes will undergo washing in culture medium
32
33 245 followed by incubation from day 0 through day 5 or 6 in the relevant arm of pH, except for a
34
35 246 small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture
36
37 247 in groups of 3 each from days 0 to 5/6, with removal of the unfertilized, abnormally fertilized
38
39 248 or degenerated oocytes at fertilization check. Two embryologists will perform the
40
41 249 fertilization check and embryo grading on day 1, 2 and 3 of culture as per the Istanbul
42
43 250 Consensus.²¹ All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable
44
45 251 for transfer or vitrification on day 5 provided they are graded 311 as per the Istanbul
46
47 252 Consensus.²¹ Embryos utilized for transfer or cryopreservation will be pictured and recorded
48
49 253 in the patient file. All the recorded pictures from all centres will undergo blind grading by
50
51 254 two independent experienced embryologists.
52
53
54
55
56
57

58 255 **Embryo Transfer**

1
2
3 256 Women will undergo fresh embryo transfer by replacing one to two embryos on day 5 with
4
5 257 those who replaced embryos on day 3 will be reported as per each centre protocol, except for
6
7 258 women with reduced uterine cavity or previous preterm birth, they will replace only one
8
9 259 embryo. One participating centre will transfer majority of its cases on day 3. This issue will
10
11 260 be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under
12
13 261 sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre
14
15 262 standardized protocol. The rest of the utilizable embryos will undergo vitrification for
16
17 263 transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted
18
19 264 from fresh and vitrified-warmed transfer within one year of randomization. Women will test
20
21 265 for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will
22
23 266 confirm pregnancy at \geq week 7 of gestation by detection of intrauterine sac with a heartbeat
24
25 267 on ultrasound.

268 **Outcome Measures**

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

274 ***Primary outcome***

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

276 ***Secondary outcomes***

- 277 1) Biochemical pregnancy (positive β hCG \geq 10 IU/L at 14 days after egg retrieval).
278 2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound $>$ 7th weeks of
279 gestation).
280 3) Ongoing pregnancy (continued viable pregnancy $>$ 20th weeks of gestation).

- 1
2
3 281 4) Miscarriage (loss of a clinical pregnancy $\leq 20^{\text{th}}$ weeks of gestation).
4
5 282 5) Term live-birth (i.e. delivery of one or more viable infants ≥ 37 weeks of gestation).
6
7 283 6) Preterm Birth (delivery of one or more viable infants $< 37^{\text{th}}$ weeks of gestation).
8
9
10 284 7) Very preterm birth (delivery of one or more viable infants $< 32^{\text{nd}}$ weeks of gestation).
11
12 285 8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)
13
14 286 9) Congenital malformation (delivery of congenitally malformed babies).
15
16
17 287 10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).
18
19 288 11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-
20
21 289 warmed within one year of randomization).
22
23 290 12) Fertilization (presence of 2 pronuclei 17 ± 1 hr after ICSI).
24
25 291 13) Embryo cleavage (cleaved embryos per fertilized oocyte).
26
27 292 14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than
28
29 293 10% fragmentation by volume).
30
31 294 15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).
32
33 295 16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many
34
35 296 trophectodermal cells creating a connected zone and a blastocoel more than 100% by volume;
36
37 297 ≥ 311 grade per fertilized oocyte).
38
39 298 17) Cryopreservation (cryopreserved embryos per fertilized oocyte).
40
41 299 18) Live-birth-implantation rate (live birth per embryo transferred).
42
43 300 19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized
44
45 301 oocyte).
46
47 302 20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst
48
49 303 cryopreserved of 311 grade per fertilized oocyte).
50
51
52
53
54
55

304 **Statistical Analysis**

305 ***Sample size estimation***

1
2
3 306 This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested
4
5 307 is that adjusting the pH value to the edges of this range might result in improvements to the
6
7 308 live birth rate, although we remain in equipoise as to whether higher or lower values will be
8
9
10 309 optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and
11
12 310 our goal is to investigate whether this is associated with varying pH.

13
14 311 The study has been powered for a global test of the effect of pH, calculated using plausible
15
16 312 birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yields 98%
17
18 313 power in this scenario, using a 5% significance level. This test makes no assumption about
19
20 314 the ordering of the live birth rates in relation to the ordering of the pH values. The high power
21
22 315 level has been adopted to allow for some leeway in relation to the minimum effect size. For
23
24 316 illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just
25
26 317 seven percentage points) this sample size yields 85% power against a 5% significance level,
27
28 318 and 65% at a 1% significance level. We have also been conservative in our inflation of
29
30 319 numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to
31
32 320 680. In reality, we will conduct analysis on an intention to treat basis, including all
33
34 321 randomised women. Women who do not complete treatment (for example, they do not
35
36 322 undergo embryo transfer) will be counted as not having a live birth. The only exceptions to
37
38 323 this will be participants who withdraw consent for their data to be used in the study. Our
39
40 324 inflation for loss to follow up reflects this possibility.

41
42 325 We also note that adjustment for site and age in the analysis will increase power further.

43
44 326

45 327 *Analytical methods*

46
47 328 The study conduct will be according to the intention-to-treat approach, where each participant
48
49 329 randomised will be included in the analysis, regardless of protocol deviation. The primary
50
51 330 analysis of live birth will be conducted using logistic regression, with live birth event
52
53
54
55
56
57
58
59
60

1
2
3 331 regressed on pH group, adjusted for study site and participant age, which will be standardised
4
5 332 before being entered as a covariate. pH will be entered as a categorical covariate, allowing a
6
7
8 333 Likelihood Ratio test of the association between pH and live birth rate across the three groups
9
10 334 to be performed. Secondary supportive analyses will be conducted to try to characterise the
11
12 335 nature of any association. This will include a test of linear trend in live birth rates across pH
13
14 336 groups, which would imply an optimal pH level for the lowest or highest value, as well as
15
16 337 pairwise comparisons between each group (again, these analyses will be adjusted for site and
17
18 338 age). The pairwise comparisons will focus on size and precision of the odds ratios. Although
19
20 339 it would be desirable to power the study for all pairwise comparisons as the primary outcome,
21
22 340 this yields impracticable sample sizes (> 4000 participants) against realistic effects. The study
23
24 341 has therefore been designed to represent the most informative test of the hypothesis that pH
25
26 342 level affects live birth, that is practicable.
27
28
29
30
31 343

32
33 344 For secondary outcomes, binary variables will be analysed in an analogous fashion to the
34
35 345 primary analysis. Count variables will be analysed using Poisson regression, with zero-
36
37 346 inflated models wherever the outcome is structurally undefined for some participants. Again,
38
39 347 these will be adjusted for site and age. In the analysis of number of usable embryos,
40
41 348 implanted embryos arising from the day 3 transfer will be included as formed and good
42
43 349 quality blastocysts, while those that do not implant in this portion will be considered blocked
44
45 350 at day 3. The total of the number of embryos transferred and the formed blastocysts will be
46
47 351 used to calculate number of utilizable embryos. A 1% significance level will be employed.
48
49 352 Due to the short treatment duration, it is anticipated that loss to follow up will be minimal,
50
51 353 but if any loss does occur then these participants will be analysed as having negative status
52
53 354 for the primary outcome, unless consent to use data is withdrawn. The follow-up period is
54
55
56
57
58
59
60

1
2
3 355 identified as one year from randomization of the last participant provided that all pregnant
4
5 356 women have given birth.

8 357 **Discussion**

10 358 Given the lack of evidence for a superior pH level for human embryo culture and whether
11
12 359 the pH level could make a difference in live birth after IVF, this trial is performed. This trial
13
14 360 is expected to fill the gap in this area leaving the recommendations of manufactures of culture
15
16 361 media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1%
17
18 362 significance level) to minimize the risk for uninformative results. In any occasion of
19
20 363 cleavage-stage transfer, the calculation of the blastocyst formation will be based on the
21
22 364 assumption that embryos that will implant will be calculated as a formed blastocyst, while the
23
24 365 failure of implantation of an embryo will be considered as embryo block at the cleavage
25
26 366 stage. Although this is not the ideal track to calculate blastocyst formation, we find this
27
28 367 assumption is the closest one to reflect the blastocyst formation. This will be further
29
30 368 discussed when this trial is reported.

35 369 **Funding and conflict of interest**

37 370 The study receives no fund and the authors have no conflict of interest to declare.

40 371 **Authors' contributions**

42 372 Mohamed is the creator of the concept and design of the study, and is the principal
43
44 373 investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across
45
46 374 the sites and will make sure that data is periodically sent to for storage in independent
47
48 375 database. Jack Wilkinson is the statistician of the study who revised the study design and
49
50 376 calculated the sample size and power of the study and he will be responsible for the data
51
52 377 analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a
53
54 378 primary investigator at Banon IVF centre and a sub-investigator at Ibsina Centre, and she
55
56 379 participated in revising the trial protocol and will participate in trial reporting thereafter.
57
58
59
60

1
2
3 380 Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial
4
5 381 protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the
6
7 382 study that will make sure all male partners are in line with the inclusion criteria, and revised
8
9 383 the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF
10
11 384 Centre and participated revising the protocol and provided comments. Mohamed
12
13 385 AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF
14
15 386 Centre. Hazem Abdelghafar is a primary investigator at Ibsina IVF Centre and participated
16
17 387 in the trial design. All authors provided comments and agreed on the study design and
18
19 388 protocol, and will participate in reporting this trial thereafter.
20
21
22
23

24 389 **References**

- 25
26 390 1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro
27 391 Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi:
28 392 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
29
30 393 2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children
31 394 conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update*
32 395 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
33 396 3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol*
34 397 *Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First:
35 398 2012/07/26]
36 399 4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod*
37 400 *Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First:
38 401 2012/02/09]
39 402 5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation
40 403 embryo culture media: effects of culture and batches. *Reprod Biomed Online*
41 404 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First:
42 405 2018/09/20]
43 406 6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J*
44 407 *Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
45 408 7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human
46 409 preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi:
47 410 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
48 411 8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH
49 412 against acidosis in preantral follicles by multiple mechanisms. *Development*
50 413 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
51 414 9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum*
52 415 *Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First:
53 416 1998/06/10]
54 417 10. Lane M, Baltz JM, Bavister BD. Na⁺/H⁺ antiporter activity in hamster embryos is
55 418 activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi:
56 419 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]

- 1
2
3 420 11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and
4 421 embryo manipulations for assisted reproduction. *Reprod Biomed Online*
5 422 2009;18(6):799-810. [published Online First: 2009/06/06]
- 6 423 12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil*
7 424 *Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online
8 425 First: 2010/11/12]
- 9 426 13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon
10 427 dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155
11 428 [published Online First: 1987/06/01]
- 12 429 14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation
13 430 embryo: amino acids act as buffers of intracellular pH. *Hum Reprod*
14 431 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First:
15 432 1999/01/14]
- 16 433 15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse
17 434 embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-
18 435 42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J
19 436 [published Online First: 1998/07/21]
- 20 437 16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ*
21 438 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
- 22 439 17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines
23 440 for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi:
24 441 10.1136/bmj.c332 [published Online First: 2010/03/25]
- 25 442 18. World Health Organization. WHO laboratory manual for the examination and processing
26 443 of human semen. 5th ed. Geneva: World Health Organization 2010.
- 27 444 19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of
28 445 single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-
29 446 6736(92)92425-f [published Online First: 1992/07/04]
- 30 447 20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory
31 448 environment and air quality: report of an expert meeting. *Reprod Biomed Online*
32 449 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First:
33 450 2018/04/17]
- 34 451 21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment:
35 452 proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi:
36 453 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
- 37 454 22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and
38 455 implementing a core outcome set for infertility. *Hum Reprod Open*
39 456 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First:
40 457 2019/03/22]
- 41 458

42
43
44
45
46
47
48
49 459 Figure Legend: Trial plan for enrolment
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Couples will receive detailed information on the possible risks and potential benefits.

Couples with primary acceptance will be randomized to the three pH arms of the study

Randomization will occur to assign ~ 2100 participants to undergo day-5 fresh embryo-transfer utilizing the blastocysts developed in the either 7.2, 7.3 or 7.4 pH levels.

Women randomization

~ 700 participants will be randomized to undergo embryo culture in 7.2 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.3 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.4 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

The intention-to-treat will include all randomized participants to 7.2 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.3 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.4 pH level group with the live birth rate as primary endpoint.

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 SPIRIT reporting 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

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
14				
15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	4
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	2 & 3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2 & 3
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	3 & 4
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	4
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
51				
52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	4
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
57				
58				
59				
60				

1	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
2				
3				
4				
5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
7	description			
8				
9				
10	Interventions:	#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
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
16	adherence			
17				
18				
19				
20				
21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
22	concomitant care			
23				
24				
25	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
26				
27				
28				
29				
30				
31				
32				
33				
34	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
35				
36				
37				
38				
39				
40	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
41				
42				
43				
44				
45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
46				
47				
48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#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
55	generation			
56				
57				
58				
59				
60				

provided in a separate document that is unavailable to those who enrol participants or assign interventions

1				
2				
3				
4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central	5
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),	
6			describing any steps to conceal the sequence until interventions	
7	mechanism		are assigned	
8				
9				
10				
11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol	5
12	implementation		participants, and who will assign participants to interventions	
13				
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial	5
15			participants, care providers, outcome assessors, data analysts),	
16			and how	
17				
18				
19				
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,	5
21	emergency unblinding		and procedure for revealing a participant's allocated intervention	
22			during the trial	
23				
24				
25	Methods: Data			
26	collection,			
27	management, and			
28	analysis			
29				
30				
31				
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and	11
33			other trial data, including any related processes to promote data	
34			quality (eg, duplicate measurements, training of assessors) and a	
35			description of study instruments (eg, questionnaires, laboratory	
36			tests) along with their reliability and validity, if known.	
37			Reference to where data collection forms can be found, if not in	
38			the protocol	
39				
40				
41				
42				
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,	11
44	retention		including list of any outcome data to be collected for participants	
45			who discontinue or deviate from intervention protocols	
46				
47				
48	Data management	#19	Plans for data entry, coding, security, and storage, including any	11
49			related processes to promote data quality (eg, double data entry;	
50			range checks for data values). Reference to where details of data	
51			management procedures can be found, if not in the protocol	
52				
53				
54				
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary	11 & 12
56			outcomes. Reference to where other details of the statistical	
57			analysis plan can be found, if not in the protocol	
58				
59				
60				

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	11 & 12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	11 & 12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
8				
9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	4
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
19				
20				
21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	4, 11 &
23	interim analysis		including who will have access to these interim results and make	12
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	4
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
31				
32				
33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	4
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	4
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	4
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	4
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
57				
58				
59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	4
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	4
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	4
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	4
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	4
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	1
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	4
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	n/a
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 09. September 2019 using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2019-034194.R3
Article Type:	Protocol
Date Submitted by the Author:	16-Dec-2019
Complete List of Authors:	Fawzy, Mohamed; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Emad, Mai; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Wilkinson, Jack; University of Manchester, Centre for Biostatistics; Salford Royal NHS Foundation Trust, Research and Development Mansour, Ragaa; Egyptian IVF-ET Center Mahran, Ali; Assiut University Faculty of Medicine, Department of Dermatology, Venereology and Andrology Fetih, Ahmed; Assiut University Faculty of Medicine, Department of Obstetrics and Gynecology Abdelrahman , Mohamed; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology AbdelGhafar, Hazem; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

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

Mohamed Fawzy,^{ab} Mai Emad,^{ab} Jack Wilkinson,^c Ragaa Mansour,^d Ali Mahran,^d Ahmed N. Fetih,^f Mohamed Y. AbdelRahman,^g Hazem Abdelghafar,^g

IbnSina IVF Centre, Sohag and Banon IVF Centre, Assuit, Egypt

^aIbnSina IVF Centre, IbnSina Hospital, Sohag, Egypt; ^bBanon IVF Centre, Assiut, Egypt;

^cCentre for Biostatistics, University of Manchester, UK; ^dEgyptian IVF-ET Centre, Cairo,

Egypt; ^eDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine,

Assiut University, Egypt; ^fDepartment of Obstetrics and Gynecology, Faculty of Medicine,

Assiut University, Egypt; ^gDepartment of Obstetrics and Gynecology, Faculty of Medicine,

Sohag University, Egypt

Corresponding Author: Dr. Mohamed Fawzy, IVF Laboratory Director (IbnSina and Banon

IVF Centres), IbnSina Hospital, 146 El Aref Square, Sohag, Egypt; Cell: +201011122286; E-

mail: drfawzy001@me.com

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

1
2
3 55
4
5 56
6 57
7 58

Strengths and limitations of this study

- 9 59
- The study is randomised controlled which reduces the possibility of bias.
- 11 60
- The study has an independent data monitoring committee with access to the data with
- 13 61
- no involvement in the study conduct.
- 15 62
- Two possible limitations of the study are that it will be conducted on ICSI cycles as
- 17 63
- ICSI is the preferred insemination method in the participating centres, and formed
- 19 64
- blastocyst calculation will be based on assumption for any cleavage-stage transfer.
- 21 65
- The embryologists will be aware of the culture arms during the study conduct.

Background

26 66
27 67
28 68 Assisted reproductive techniques (ART) result in around 65% cumulative live birth within six
29 69 cycles of *in vitro* fertilisation (IVF),¹ which is relatively suboptimal. In addition, IVF results
30 70 in adverse perinatal outcomes, such as preterm birth and low birth weight babies compared
31 71 with the *in vivo* conception.² These outcomes can rely on factors relating to patients,
32 72 stimulation, and *in vitro* culture elements. In relation to embryo culture conditions, over 200
33 73 variables have been identified as being potentially relevant to the cycle outcome.³ One
34 74 element that may influence embryo development *in vitro* is the pH level of a culture medium,
35 75 which thus far has been determined by manufacturers of culture media without recourse to a
36 76 well-powered randomized clinical trial (RCT).⁴ The pH levels are potential stressors that vary
37 77 between media brands and from batch-to-batch depending on the bicarbonate level in culture
38 78 media and on the CO₂ level of incubators.⁵ This would suggest that pH level can vary
39 79 between incubators within the same laboratory if it is not well adjusted. Recommendations
40 80 for measuring pH for embryo culture are variable between daily to monthly measurement.⁴

1
2
3 81 Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH
4
5 82 (pHe).⁶ The *in vitro* conditions including concentrations of bicarbonates, proteins, amino
6
7 83 acids in culture media and the CO₂ of incubators affect the pHe, which is a potential stressor.⁷
8
9
10 84 The mechanism of pHi in oocyte and embryo is complex, regulating enzymatic activity, cell
11
12 85 division and differentiation, protein synthesis, metabolism, mitochondrial function,
13
14 86 cytoskeletal regulation, and microtubule dynamics.^{7,8} Drifts in pHe translate into changes in
15
16 87 pHi, which can adversely affect cell function if the compensatory mechanisms failed to adapt
17
18 88 to restore pHi to a safe level.⁸ The pHi can compensate through an active exchange among
19
20 89 Na⁺, HCO₃⁻/Cl⁻ and Na⁺/H⁺ to maintain it between 7 to 7.3.^{5,8} Denuded oocyte for ICSI
21
22 90 through fertilization thereafter and vitrified-warmed embryos lack robust compensatory
23
24 91 mechanisms of pHi; therefore, drastic differences between pHe and pHi in these scenarios
25
26 92 can significantly perturbate embryo development.⁹⁻¹¹
27
28
29
30 93 That being said, an optimum level of pH for human embryo culture *in vitro* is still unknown.⁴
31
32 94 ^{9,12-15} Most recommendations rely on mice models or manufacturers of culture media.
33
34
35 95 Theoretically, a wide range of pHe levels (7.0–7.5) can support human embryo development
36
37 96 *in vitro*. However, a narrower range of pHe levels (7.2 to 7.4) is used in clinical practice. This
38
39 97 is because an extreme acidic pHe level (≤ 7) can adversely affect oocyte spindle leading to no
40
41 98 further post-fertilization events.⁴ This level of acidic pH can delay or block embryo
42
43 99 development *in vitro*.⁴ Similar harms can theoretically occur for oocyte and embryo, if
44
45 100 alkaline levels of pHe (≥ 7.5) are used.⁴ Although these potential harms of extreme pHe
46
47 101 levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to
48
49 102 investigate a safe range of pHe (7.2 to 7.4). Why this range has been chosen depends on the
50
51 103 recommendations of media manufactures and the clinical practice within the majority of
52
53 104 human embryo culture laboratories. Although this range of 7.2 to 7.4 is used, there is clear
54
55 105 evidence about which level of 7.2, 7.3, or 7.4 can support human embryos to result in live
56
57
58
59
60

1
2
3 106 birth after transfer. This multicentre, randomized, clinical trial aims to identify whether pHe
4
5 107 levels of 7.2, 7.3, or 7.4 can perform better on live birth rate after ICSI in order to investigate
6
7
8 108 the potential for optimisation.

109 **Methods and Design**

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

119 **Ethics and dissemination**

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

1
2
3 130 committee and will undergo detailed reporting on the trial registry and in the final
4
5 131 manuscript.

8 132 **Intervention**

9
10 133 Oocytes and embryos in the three arms will undergo continuous culture from day 0 through
11
12 134 day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after
13
14 135 ICSI in pHe of 7.2 ± 0.02 . Arm II The “Arm II” is to culture oocytes and resulting embryos
15
16 136 after ICSI in pHe of 7.3 ± 0.02 . “Arm III” is to culture oocytes and resulting embryos after
17
18 137 ICSI in pHe of 7.4 ± 0.02 . This trial will include intracytoplasmic sperm injection (ICSI)
19
20
21 138 cycles.

24 139 **Patient and Public Involvement**

25
26 140 No patient involved

28 141 **Randomization and Masking**

29
30 142 Using an online tool, participants will be randomised to the experimental arms with a 1:1:1
31
32 143 allocation ratio. The allocation sequence of participants will be generated using a permuted
33
34 144 block randomization of 3, 6 and 9 block sizes with unique identifiers in random order,
35
36 145 stratified by trial site. Randomization of participants and its storage in sequentially numbered,
37
38 146 opaque, sealed envelopes will occur by a secretary with no involvement in patient care and
39
40 147 will be provided to trial sites before enrolment of first participant. Eligible participants will
41
42 148 be allocated to the relevant arms on the day of maturation trigger and allocation result will be
43
44 149 communicated to the laboratory team. Participants, clinicians and outcome assessors for the
45
46 150 clinical outcomes will be unaware of the allocation, while embryologists who will assess
47
48 151 embryo development will be aware of the allocation.

54 152 **Participants**

55
56 153 The inclusion criteria include:

57
58 154 1) Women age of ≥ 18 to ≤ 40 ;

- 1
2
3 155 2) BMI of ≤ 31 ;
4
5 156 3) Anticipated normal responder (≥ 5 antral follicle count or ≥ 5.4 pmol/L AMH);
6
7 157 4) Women who have ≥ 1 year of primary or secondary infertility;
8
9 158 5) Fresh ejaculate sperm of any count provided they have $\geq 1\%$ normal forms and a motile
10
11 fraction;
12 159
13
14 160 6) Women undergoing their first ICSI cycle or their second ICSI cycle after previous
15
16 successful one;
17 161
18 162 7) Women with > 7 mm endometrial thickness at day of trigger;
19
20 and 8) Women with no detected uterine abnormality on transvaginal ultrasound (e.g.
21
22 submucosal myomas, polyps or septa).
23 164

24
25
26 165 ***Women will be excluded if they have:***

- 27
28 166 1) Unilateral oophorectomy;
29
30 167 2) Abnormal karyotyping for them or their male partners;
31
32 168 3) History of repeated abortions or implantation failure;
33
34 169 4) Uncontrolled diabetes;
35
36 170 5) Liver or renal disease;
37
38 171 6) History of severe ovarian hyperstimulation;
39
40 172 7) History of malignancy or borderline pathology;
41
42 173 8) Endometriosis;
43
44 174 9) Plan for PGD-A;
45
46 175 10) Severe male factor includes surgical sperm retrieval or cryopreserved sperm;
47
48 176 and 11) PCOS, women with history of severe OHSS, and cycles with agonist trigger or any
49
50 patient with a plan for a “freeze-all”.
51
52
53
54 177

55
56 178 **Stimulation Protocol, Oocyte retrieval, and Luteal Phase Support**
57
58
59
60

1
2
3 179 Women will undergo controlled ovarian stimulation with agonist mid-luteal pituitary down-
4
5 180 regulation (Decapeptyl® 0.1mg, Ferring, Switzerland) or antagonist (Cetrotide® 0.25 mg,
6
7 181 Merck Serono) protocols. Agonist will start on day 19–21 of the preceding cycle and will
8
9 182 continue to the day of maturation trigger. For Antagonist group, women will start the
10
11 183 antagonist on day 6 of treatment cycle. All women will receive follicular stimulating
12
13 184 hormone (rFSH; Gonal-F, Merck Serono) and human menopausal gonadotropin (HMG;
14
15 185 Menogon, Ferring) at 150-300 IU with a 2:1 ratio from cycle day 2 through follicular
16
17 186 maturation, with adjustment of the dosage according to the response. When ≥ 3 follicles
18
19 187 measure ≥ 18 mm mean diameter on ultrasound, women will receive a 10,000 IU hCG trigger
20
21 188 shot (Choriomon, IBSA) for final oocyte maturation. Oocyte retrieval will be performed 37
22
23 189 hours after hCG trigger under transvaginal sonographic guidance. Follicular aspirates will be
24
25 190 handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using
26
27 191 tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100
28
29 192 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily,
30
31 193 starting on day 1 after retrieval (“day 1”) to 12 weeks of gestation, unless negative
32
33 194 pregnancy.

34 195 **Sperm Preparation, Oocyte Denudation and ICSI**

35 196 Semen samples will be processed through density gradient,¹⁸ using Puresperm (Nidacon,
36
37 197 Sweden). The pellet will undergo once washing and incubation at room temperature in
38
39 198 HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur
40
41 199 immediately after collection using 40 IU hyaluronidase (LifeGlobal, Canada) diluted in
42
43 200 Global HEPES and a stripper of 170 micrometre (Cook, US). Metaphase II (MII) oocytes
44
45 201 will undergo ICSI in Global HEPES medium under inverted microscope as previously
46
47 202 described.¹⁹

48 203

204 **Incubator Management and pH Adjustment**

205 Incubators for this study involve Labo C-Top (Labotect, Germany), Minc 1000 (Cook, US),
206 and AD-3100 (Astec, Japan). Each centre will use no more than a brand of incubator to
207 account for incubator as variable. If another brand of incubators will be used, we will ensure
208 they are humidified. Dry incubators such as ESCO (Esco Micro Pte. Ltd, Singapore) may be
209 used at some centres; however, we will adjust the analysis by trial site to account for
210 differences between centres. Incubators will undergo stringent control of temperature
211 ($36.9\pm 0.1^{\circ}\text{C}$). The temperature will be validated daily using a certified thermometer.
212 Incubator's CO_2 and O_2 will be measured daily using a certified gas analyser to ensure 5% O_2
213 and a proper CO_2 concentration to achieve the required pH. All the three measurements
214 (temperature, CO_2 level, and pH levels) will be verified by well-trained person traveling
215 across the sites. Incubators will undergo sterilization with 6% H_2O_2 every four weeks, with
216 installation of inline filters (Green, Lifeglobal, CooperSurgical).²⁰
217 A minimum of 3 incubators of a single brand within each participating facility with different
218 levels of pH representing the study arms is obligatory: Incubator A of 7.2 ± 0.02 pH, Incubator
219 B of 7.3 ± 0.02 pH, and Incubator C of 7.4 ± 0.02 . The three incubators will undergo a strict
220 adjustment of the required pH using a handheld blood gas analyser (Epoc® Reader and Host;
221 BGEM card US). Constant pH levels will be ensured with twice weekly measurement of pH
222 with blood gas analyser and a daily measurement of CO_2 level of incubators. Measurement of
223 pH will occur after an overnight incubation of 1mL culture media in a central well dish
224 covered with 0.4mL of oil. In the morning and before opening of incubators, the handheld
225 blood gas analyser (Epoc® Reader and Host; BGEM card US) will undergo preparation for
226 measuring pH as per the manufacturer protocol. Briefly, after switching on the device,
227 calibration of the device automatically occurs. Next, we adjust the temperature to 37°C , and
228 select the sample as arterial. Next, we insert the card, which undergoes automatic calibration.

1
2
3 229 Next, when the device is ready, it asks to inject sample. Next, using 1mL syringe attached to
4
5 230 wide needle calibre, we aspirate 0.5mL of the culture medium under oil. Next, we discard the
6
7 231 first droplet and smoothly inject the sample until the beep. We can see the results of pH,
8
9 232 partial CO₂ and O₂ pressures thereafter. Each laboratory will report the results to also
10
11 233 compare the resulting partial pressures of CO₂ and O₂ with the incubator display. pH levels
12
13 234 will also be measured every new batch of a culture medium. The measurement of pH and
14
15 235 CO₂ across the centres will be performed using a one-brand equipment that will undergo
16
17 236 periodic calibration together. To account for errors in measurement, one well-trained
18
19 237 personnel will be assigned to measure the pH and double check the CO₂ level across the
20
21 238 centres.
22
23
24

25 26 239 **Culture Protocol and Embryo Scoring**

27
28 240 Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global
29
30 241 Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil,
31
32 242 Nidacon). If a decision to change culture media at any time point of the study conduct is
33
34 243 made, this will be performed at the same time across the study sites. Dishes will undergo
35
36 244 overnight incubation in the relevant incubator adjusted to the relevant pH as per the
37
38 245 randomization. After ICSI, the injected oocytes will undergo washing in culture medium
39
40 246 followed by incubation from day 0 through day 5 or 6 in the relevant arm of pH, except for a
41
42 247 small portion of embryos transferred on day 3. The inseminated oocytes will undergo culture
43
44 248 in groups of 3 each from days 0 to 5/6, with removal of the unfertilized, abnormally fertilized
45
46 249 or degenerated oocytes at fertilization check. Two embryologists will perform the
47
48 250 fertilization check and embryo grading on day 1, 2 and 3 of culture as per the Istanbul
49
50 251 Consensus.²¹ All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable
51
52 252 for transfer or vitrification on day 5 provided they are graded 311 as per the Istanbul
53
54 253 Consensus.²¹ Embryos utilized for transfer or cryopreservation will be pictured and recorded
55
56
57
58
59
60

1
2
3 254 in the patient file. All the recorded pictures from all centres will undergo blind grading by
4
5 255 two independent experienced embryologists.
6
7

8 256 **Embryo Transfer**

9
10 257 Women will undergo fresh embryo transfer by replacing one to two embryos on day 5 with
11
12 258 those who replaced embryos on day 3 will be reported as per each centre protocol, except for
13
14 259 women with reduced uterine cavity or previous preterm birth, they will replace only one
15
16 260 embryo. One participating centre will transfer majority of its cases on day 3. This issue will
17
18 261 be accounted for by adjusting the analysis by trial site. Embryo transfer will occur under
19
20 262 sonographic guidance using Sydney IVF Transfer Set (Cook, US) as per each centre
21
22 263 standardized protocol. The rest of the utilizable embryos will undergo vitrification for
23
24 264 transfer in subsequent cycles, while we plan to monitor the cumulative live birth resulted
25
26 265 from fresh and vitrified-warmed transfer within one year of randomization. Women will test
27
28 266 for biochemical pregnancy 14 days after oocyte retrieval with serum hCG level, and will
29
30 267 confirm pregnancy at \geq week 7 of gestation by detection of intrauterine sac with a heartbeat
31
32 268 on ultrasound.
33
34
35
36
37

38 269 **Outcome Measures**

39
40 270 Each outcome will be calculated including all randomised participants in the arms to which
41
42 271 they were allocated, with the exception of implantation rate, which will be interpreted
43
44 272 cautiously due to concerns over its validity as a measure of treatment effect, and perinatal
45
46 273 outcomes, which by definition are only available in the subset of participants achieving live
47
48 274 birth. This study will adopt the COMMIT definitions of outcomes,²² where appropriate.
49
50

51 275 ***Primary outcome***

52
53 276 Live birth (delivery of one or more viable infants > 20th weeks of gestation).
54
55

56 277 ***Secondary outcomes***

57
58 278 1) Biochemical pregnancy (positive β hCG \geq 10 IU/L at 14 days after egg retrieval).
59
60

- 279 2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound > 7th weeks of
280 gestation).
- 281 3) Ongoing pregnancy (continued viable pregnancy > 20th weeks of gestation).
- 282 4) Miscarriage (loss of a clinical pregnancy \leq 20th weeks of gestation).
- 283 5) Term live-birth (i.e. delivery of one or more viable infants \geq 37 weeks of gestation).
- 284 6) Preterm Birth (delivery of one or more viable infants < 37th weeks of gestation).
- 285 7) Very preterm birth (delivery of one or more viable infants < 32nd weeks of gestation).
- 286 8) Low birth weight babies (babies with < 2500 gm within 24 hours of delivery)
- 287 9) Congenital malformation (delivery of congenitally malformed babies).
- 288 10) Still Birth (delivery of nonviable babies > 20 weeks of gestation).
- 289 11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-
290 warmed within one year of randomization).
- 291 12) Fertilization (presence of 2 pronuclei 17 \pm 1 hr after ICSI).
- 292 13) Embryo cleavage (cleaved embryos per fertilized oocyte).
- 293 14) Top-quality embryo on day 3 (7-8 cells with appropriate-sizes blastomeres and less than
294 10% fragmentation by volume).
- 295 15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilized oocyte).
- 296 16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many
297 trophoctodermal cells creating a connected zone and a blastocoel more than 100% by volume;
298 \geq 311 grade per fertilized oocyte).
- 299 17) Cryopreservation (cryopreserved embryos per fertilized oocyte).
- 300 18) Live-birth-implantation rate (live birth per embryo transferred).
- 301 19) Utilized embryos (number of cryopreserved plus transferred embryos per fertilized
302 oocyte).

1
2
3 303 20) Top-quality utilized embryos (number of high-quality embryos transferred plus blastocyst
4
5 304 cryopreserved of 311 grade per fertilized oocyte).

8 305 **Statistical Analysis**

10 306 *Sample size estimation*

12 307 This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested
13
14 308 is that adjusting the pH value to the edges of this range might result in improvements to the
15
16 309 live birth rate, although we remain in equipoise as to whether higher or lower values will be
17
18 310 optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and
19
20 311 our goal is to investigate whether this is associated with varying pH.

23 312 The study has been powered for a global test of the effect of pH, calculated using plausible
24
25 313 birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yields 98%
26
27 314 power in this scenario, using a 5% significance level. This test makes no assumption about
28
29 315 the ordering of the live birth rates in relation to the ordering of the pH values. The high power
30
31 316 level has been adopted to allow for some leeway in relation to the minimum effect size. For
32
33 317 illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a spread of just
34
35 318 seven percentage points) this sample size yields 85% power against a 5% significance level,
36
37 319 and 65% at a 1% significance level. We have also been conservative in our inflation of
38
39 320 numbers for dropout. We have allowed for 5% loss to follow up, inflating our group size to
40
41 321 680. In reality, we will conduct analysis on an intention to treat basis, including all
42
43 322 randomised women. Women who do not complete treatment (for example, they do not
44
45 323 undergo embryo transfer) will be counted as not having a live birth. The only exceptions to
46
47 324 this will be participants who withdraw consent for their data to be used in the study. Our
48
49 325 inflation for loss to follow up reflects this possibility.

50
51 326 We also note that adjustment for site and age in the analysis will increase power further.

52
53
54
55
56
57
58 327
59
60

1
2
3 328 *Analytical methods*
4

5 329 The study conduct will be according to the intention-to-treat approach, where each participant
6
7 330 randomised will be included in the analysis, regardless of protocol deviation. The primary
8
9 331 analysis of live birth will be conducted using logistic regression, with live birth event
10
11 332 regressed on pH group, adjusted for study site and participant age, which will be standardised
12
13 333 before being entered as a covariate. pH will be entered as a categorical covariate, allowing a
14
15 334 Likelihood Ratio test of the association between pH and live birth rate across the three groups
16
17 335 to be performed. Secondary supportive analyses will be conducted to try to characterise the
18
19 336 nature of any association. This will include a test of linear trend in live birth rates across pH
20
21 337 groups, which would imply an optimal pH level for the lowest or highest value, as well as
22
23 338 pairwise comparisons between each group (again, these analyses will be adjusted for site and
24
25 339 age). The pairwise comparisons will focus on size and precision of the odds ratios. Although
26
27 340 it would be desirable to power the study for all pairwise comparisons as the primary outcome,
28
29 341 this yields impracticable sample sizes (> 4000 participants) against realistic effects. The study
30
31 342 has therefore been designed to represent the most informative test of the hypothesis that pH
32
33 343 level affects live birth, that is practicable.
34
35
36
37
38
39

40 344
41
42 345 For secondary outcomes, binary variables will be analysed in an analogous fashion to the
43
44 346 primary analysis. Count variables will be analysed using Poisson regression, with zero-
45
46 347 inflated models wherever the outcome is structurally undefined for some participants. Again,
47
48 348 these will be adjusted for site and age. In the analysis of number of usable embryos,
49
50 349 implanted embryos arising from the day 3 transfer will be included as formed and good
51
52 350 quality blastocysts, while those that do not implant in this portion will be considered blocked
53
54 351 at day 3. The total of the number of embryos transferred and the formed blastocysts will be
55
56 352 used to calculate number of utilizable embryos. A 1% significance level will be employed.
57
58
59
60

1
2
3 353 Due to the short treatment duration, it is anticipated that loss to follow up will be minimal,
4
5 354 but if any loss does occur then these participants will be analysed as having negative status
6
7
8 355 for the primary outcome, unless consent to use data is withdrawn. The follow-up period is
9
10 356 identified as one year from randomization of the last participant provided that all pregnant
11
12 357 women have given birth.

14 358 **Discussion**

16
17 359 Given the lack of evidence for a superior pH level for human embryo culture and whether
18
19 360 the pH level could make a difference in live birth after IVF, this trial is performed. This trial
20
21 361 is expected to fill the gap in this area leaving the recommendations of manufactures of culture
22
23 362 media to a solid base relying on evidence. The trial power is set to be high (>90%, with a 1%
24
25 363 significance level) to minimize the risk for uninformative results. In any occasion of
26
27 364 cleavage-stage transfer, the calculation of the blastocyst formation will be based on the
28
29 365 assumption that embryos that will implant will be calculated as a formed blastocyst, while the
30
31 366 failure of implantation of an embryo will be considered as embryo block at the cleavage
32
33 367 stage. Although this is not the ideal track to calculate blastocyst formation, we find this
34
35 368 assumption is the closest one to reflect the blastocyst formation. This will be further
36
37 369 discussed when this trial is reported.

41 370 **Funding and conflict of interest**

42
43 371 The study receives no fund and the authors have no conflict of interest to declare.

44 372 **Authors' contributions**

45
46 373 Mohamed is the creator of the concept and design of the study, and is the principal
47
48 374 investigator of the study. Mohamed Fawzy is also a supervisor for the study conduct across
49
50 375 the sites and will make sure that data is periodically sent to for storage in independent
51
52 376 database. Jack Wilkinson is the statistician of the study who revised the study design and
53
54 377 calculated the sample size and power of the study and he will be responsible for the data
55
56
57
58
59
60

1
2
3 378 analysis and data transfer to the independent safety and monitoring committee. Mai Emad is a
4
5 379 primary investigator at Banon IVF centre and a sub-investigator at Ibsina Centre, and she
6
7
8 380 participated in revising the trial protocol and will participate in trial reporting thereafter.
9
10 381 Ragaa Mansour is a primary investigator at the Egyptian IVF-ET Centre, and revised the trial
11
12 382 protocol and provided advice during the protocol design. Ali Mahran is the andrologist of the
13
14 383 study that will make sure all male partners are in line with the inclusion criteria, and revised
15
16 384 the protocol and provided comments. Ahmed Fetih is a primary investigator at Banon IVF
17
18 385 Centre and participated revising the protocol and provided comments. Mohamed
19
20 386 AbdelRahman participated in the trial design and is a primary investigator at Amshaj IVF
21
22 387 Centre. Hazem Abdelghafar is a primary investigator at Ibsina IVF Centre and participated
23
24 388 in the trial design. All authors provided comments and agreed on the study design and
25
26 389 protocol, and will participate in reporting this trial thereafter.
27
28
29
30

31 390 **References**

- 32
33 391 1. Smith A, Tilling K, Nelson SM, et al. Live-Birth Rate Associated With Repeat In Vitro
34 392 Fertilization Treatment Cycles. *JAMA* 2015;314(24):2654-62. doi:
35 393 10.1001/jama.2015.17296 [published Online First: 2015/12/31]
36 394 2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, et al. The health of children
37 395 conceived by ART: 'the chicken or the egg?'. *Human Reproduction Update*
38 396 2019;25(2):137-58. doi: 10.1093/humupd/dmz001
39 397 3. Pool TB, Schoolfield J, Han D. Human embryo culture media comparisons. *Methods Mol*
40 398 *Biol* 2012;912:367-86. doi: 10.1007/978-1-61779-971-6_21 [published Online First:
41 399 2012/07/26]
42 400 4. Swain JE. Is there an optimal pH for culture media used in clinical IVF? *Hum Reprod*
43 401 *Update* 2012;18(3):333-9. doi: 10.1093/humupd/dmr053 [published Online First:
44 402 2012/02/09]
45 403 5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation
46 404 embryo culture media: effects of culture and batches. *Reprod Biomed Online*
47 405 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First:
48 406 2018/09/20]
49 407 6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J*
50 408 *Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
51 409 7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human
52 410 preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi:
53 411 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
54 412 8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH
55 413 against acidosis in preantral follicles by multiple mechanisms. *Development*
56 414 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]

- 1
2
3 415 9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum*
4 416 *Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First:
5 417 1998/06/10]
- 6 418 10. Lane M, Baltz JM, Bavister BD. Na⁺/H⁺ antiporter activity in hamster embryos is
7 419 activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi:
8 420 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
- 9 421 11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and
10 422 embryo manipulations for assisted reproduction. *Reprod Biomed Online*
11 423 2009;18(6):799-810. [published Online First: 2009/06/06]
- 12 424 12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil*
13 425 *Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online
14 426 First: 2010/11/12]
- 15 427 13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon
16 428 dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155
17 429 [published Online First: 1987/06/01]
- 18 430 14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation
19 431 embryo: amino acids act as buffers of intracellular pH. *Hum Reprod*
20 432 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First:
21 433 1999/01/14]
- 22 434 15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse
23 435 embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-
24 436 42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J
25 437 [published Online First: 1998/07/21]
- 26 438 16. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ*
27 439 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
- 28 440 17. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines
29 441 for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi:
30 442 10.1136/bmj.c332 [published Online First: 2010/03/25]
- 31 443 18. World Health Organization. WHO laboratory manual for the examination and processing
32 444 of human semen. 5th ed. Geneva: World Health Organization 2010.
- 33 445 19. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of
34 446 single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-
35 447 6736(92)92425-f [published Online First: 1992/07/04]
- 36 448 20. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory
37 449 environment and air quality: report of an expert meeting. *Reprod Biomed Online*
38 450 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First:
39 451 2018/04/17]
- 40 452 21. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment:
41 453 proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi:
42 454 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
- 43 455 22. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and
44 456 implementing a core outcome set for infertility. *Hum Reprod Open*
45 457 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First:
46 458 2019/03/22]
- 47 459
- 48 460
- 49
- 50
- 51
- 52
- 53
- 54
- 55
- 56 460 Figure Legend: Trial plan for enrolment
- 57
- 58
- 59
- 60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Couples will receive detailed information on the possible risks and potential benefits.



Couples with primary acceptance will be randomized to the three pH arms of the study



Randomization will occur to assign ~ 2100 participants to undergo day-5 fresh embryo-transfer utilizing the blastocysts developed in the either 7.2, 7.3 or 7.4 pH levels.

Women randomization



~ 700 participants will be randomized to undergo embryo culture in 7.2 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.3 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.4 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.



The intention-to-treat will include all randomized participants to 7.2 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.3 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.4 pH level group with the live birth rate as primary endpoint.

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 SPIRIT reporting 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

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
14				
15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	4
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	2 & 3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2 & 3
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	3 & 4
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	4
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
51				
52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	4
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
57				
58				
59				
60				

1	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
2				
3				
4				
5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
7	description			
8				
9				
10	Interventions:	#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
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
16	adherence			
17				
18				
19				
20				
21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
22	concomitant care			
23				
24				
25	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
26				
27				
28				
29				
30				
31				
32				
33				
34	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
35				
36				
37				
38				
39				
40	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
41				
42				
43				
44				
45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
46				
47				
48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#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
55	generation			
56				
57				
58				
59				
60				

provided in a separate document that is unavailable to those who enrol participants or assign interventions

1				
2				
3				
4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central	5
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),	
6			describing any steps to conceal the sequence until interventions	
7	mechanism		are assigned	
8				
9				
10				
11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol	5
12	implementation		participants, and who will assign participants to interventions	
13				
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial	5
15			participants, care providers, outcome assessors, data analysts),	
16			and how	
17				
18				
19				
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,	5
21	emergency unblinding		and procedure for revealing a participant's allocated intervention	
22			during the trial	
23				
24				
25	Methods: Data			
26	collection,			
27	management, and			
28	analysis			
29				
30				
31				
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and	11
33			other trial data, including any related processes to promote data	
34			quality (eg, duplicate measurements, training of assessors) and a	
35			description of study instruments (eg, questionnaires, laboratory	
36			tests) along with their reliability and validity, if known.	
37			Reference to where data collection forms can be found, if not in	
38			the protocol	
39				
40				
41				
42				
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,	11
44	retention		including list of any outcome data to be collected for participants	
45			who discontinue or deviate from intervention protocols	
46				
47				
48	Data management	#19	Plans for data entry, coding, security, and storage, including any	11
49			related processes to promote data quality (eg, double data entry;	
50			range checks for data values). Reference to where details of data	
51			management procedures can be found, if not in the protocol	
52				
53				
54				
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary	11 & 12
56			outcomes. Reference to where other details of the statistical	
57			analysis plan can be found, if not in the protocol	
58				
59				
60				

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	11 & 12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	11 & 12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
8				
9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	4
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
19				
20				
21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	4, 11 &
23	interim analysis		including who will have access to these interim results and make	12
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	4
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
31				
32				
33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	4
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	4
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	4
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	4
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
57				
58				
59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	4
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	4
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	4
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	4
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	4
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	1
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	4
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	n/a
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 09. September 2019 using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2019-034194.R4
Article Type:	Protocol
Date Submitted by the Author:	20-Dec-2019
Complete List of Authors:	Fawzy, Mohamed; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Emad, Mai; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Wilkinson, Jack; University of Manchester, Centre for Biostatistics; Salford Royal NHS Foundation Trust, Research and Development Mansour, Ragaa; Egyptian IVF-ET Center Mahran, Ali; Assiut University Faculty of Medicine, Department of Dermatology, Venereology and Andrology Fetih, Ahmed; Assiut University Faculty of Medicine, Department of Obstetrics and Gynecology Abdelrahman , Mohamed; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology AbdelGhafar, Hazem; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

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

Mohamed Fawzy,^{ab} Mai Emad,^{ab} Jack Wilkinson,^c Ragaa Mansour,^d Ali Mahran,^d Ahmed N. Fetih,^f Mohamed Y. AbdelRahman,^g Hazem Abdelghafar,^g

IbnSina IVF Centre, Sohag and Banon IVF Centre, Assuit, Egypt

^aIbnSina IVF Centre, IbnSina Hospital, Sohag, Egypt; ^bBanon IVF Centre, Assiut, Egypt;

^cCentre for Biostatistics, University of Manchester, UK; ^dEgyptian IVF-ET Centre, Cairo,

Egypt; ^eDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine,

Assiut University, Egypt; ^fDepartment of Obstetrics and Gynecology, Faculty of Medicine,

Assiut University, Egypt; ^gDepartment of Obstetrics and Gynecology, Faculty of Medicine,

Sohag University, Egypt

Corresponding Author: Dr. Mohamed Fawzy, IVF Laboratory Director (IbnSina and Banon

IVF Centres), IbnSina Hospital, 146 El Aref Square, Sohag, Egypt; Cell: +201011122286; E-

mail: drfawzy001@me.com

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 CO₂ in incubators.⁵ Hence, pH levels can vary between incubators within a laboratory if

1
2
3 it is not well adjusted. Recommendations to measure pH for embryo culture vary from daily
4
5 to monthly measurement.⁴
6

7
8 Oocytes and embryos have intracellular (pHi), which is modulated by the extracellular pH
9
10 (pHe).⁶ The *in vitro* conditions including concentrations of bicarbonates, proteins, amino
11
12 acids in culture media and the CO₂ of incubators also affect the pHe.⁷ The mechanism of pHi
13
14 in oocyte and embryo is complex, regulating enzymatic activity, cell division and
15
16 differentiation, protein synthesis, metabolism, mitochondrial function, cytoskeletal
17
18 regulation, and microtubule dynamics.^{7,8} Drifts in pHe translate into changes in pHi, which
19
20 can adversely affect cell function if compensatory mechanisms failed to adapt to restore pHi
21
22 to a safe level.⁸ The pHi can compensate through an active exchange among Na⁺, HCO₃⁻/Cl⁻
23
24 and Na⁺/H⁺ to maintain it between 7 to 7.3.^{5,8} Denuded oocyte for ICSI through fertilisation
25
26 as well as vitrified-warmed embryos lack robust compensatory mechanisms of pHi; therefore,
27
28 drastic differences between pHe and pHi in these scenarios can disrupt embryo
29
30 development.⁹⁻¹¹
31
32
33
34

35
36 That being said, the pH level that can optimise human embryo development *in vitro* is still
37
38 unknown.^{4,9,12-15} Current recommendations rely on mice models or manufactures of culture
39
40 media. Theoretically, a wide range of pHe levels (7.0–7.5) can support human embryo
41
42 development *in vitro*. However, a narrower range of pHe levels (7.2 to 7.4) is used in clinical
43
44 practice. This is because an extreme acidic pHe level (≤ 7) can adversely affect oocyte
45
46 spindle leading to no further post-fertilisation events.⁴ This level of acidic pHe can delay or
47
48 block embryo development *in vitro*.⁴ Similar harms can occur for oocyte and embryo, if
49
50 alkaline levels of pHe (≥ 7.5) are used.⁴ Although these potential harms of extreme pHe
51
52 levels rely on animal models, underpowered studies, or anecdotal beliefs, we have decided to
53
54 investigate a safe range of pHe (7.2 to 7.4). Why this range has been chosen depends on the
55
56 recommendations of media manufactures and the clinical practice in laboratories of human
57
58
59
60

1
2
3 embryo culture. Despite the routine use of the range of 7.2 to 7.4 pHe in clinical practice, no
4
5 clear evidence exists on which level within this range can better support human embryos to
6
7 result in a live birth. This multicentre, randomised, clinical trial aims to identify whether pHe
8
9 levels of 7.2, 7.3, or 7.4 can perform better on live birth rate after ICSI in order to investigate
10
11 the potential for optimisation.
12
13

14 **Methods and Design**

15
16 This is a protocol of a multicentre, randomised, triple-arm, triple-blind clinical trial
17
18 (NCT02896777, registered at www.ClinicalTrials.gov) that will compare three levels of pH
19
20 for human embryo culture *in vitro* on live birth after ICSI (Figure 1). This partially blind
21
22 design represents that clinicians, participants and outcome assessor, not including the
23
24 embryologists, will be unaware of the study arms. This multicentre trial will involve private
25
26 IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre,
27
28 Qena IVF Centre and Amshaj IVF Centre) with the study protocol in their hand before
29
30 enrolment of participants. If other IVF facilities join this trial before recruitment, this will be
31
32 reported in the study.
33
34
35
36

37 **Intervention**

38
39 Oocytes and embryos in the three arms will undergo continuous culture from day 0 through
40
41 day 5 or 6 without medium renewal. “Arm I” is to culture oocytes and resulting embryos after
42
43 ICSI in pHe of 7.2 ± 0.02 . “Arm II” is to culture oocytes and resulting embryos after ICSI in
44
45 pHe of 7.3 ± 0.02 . “Arm III” is to culture oocytes and resulting embryos after ICSI in pHe of
46
47 7.4 ± 0.02 . This trial will only include intracytoplasmic sperm injection (ICSI) cycles.
48
49
50

51 **Patient and Public Involvement**

52
53 Patients have not been directly involved in the design, planning, and conception of this trial.
54
55
56
57
58
59
60

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 $\geq 1\%$ 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

1
2
3 handled in HEPES-buffered medium (global® HEPES, LifeGlobal, Canada) at 37°C using
4
5 tube warmers. Luteal-phase support will be achieved with intramuscular progesterone (100
6
7 mg/mL [Prontogest, IBSA]) once daily or vaginal pessaries (400 mg prontogest) twice daily,
8
9 starting on day 1 after retrieval (“day 1”) to 12 weeks of gestation, unless negative
10
11 pregnancy.
12
13

14 **Sperm Preparation, Oocyte Denudation and ICSI**

15
16 Semen samples will be processed using density gradient¹⁶ (Puresperm, Nidacon, Sweden).
17
18 The pellet will undergo once washing and incubation at room temperature in HEPES buffered
19
20 medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur immediately after
21
22 collection in 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global HEPES using a
23
24 170-micrometre stripper (Cook, US). Metaphase II (MII) oocytes will undergo ICSI in
25
26 Global HEPES medium under an inverted microscope as previously described.¹⁷
27
28
29

30 **Incubator Management and pH Adjustment**

31
32 We will use only humidified benchtop incubators for this study, involving Labo C-Top
33
34 (Labotect, Germany), Minc-1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will
35
36 use only one brand to account for incubators as a variable. If dry incubators such as ESCO
37
38 (Esco Micro Pte. Ltd, Singapore) are used in some centres, we will account for differences
39
40 between centres by adjusting for trial sites. Incubators will undergo stringent control of
41
42 temperature (36.9±0.1°C) by daily validation using certified thermometers. Incubator’s CO₂
43
44 and O₂ levels will be measured daily using a certified gas analyser to ensure that O₂ measures
45
46 5%, and CO₂ concentration is at the prespecified level to achieve the required pH. Well-
47
48 trained persons across the sites will verify all measurements (temperature, CO₂, O₂, and pH
49
50 levels). Incubators will be sterilised by 6% H₂O₂ every four weeks, with an installation of a
51
52 new set of inline filters (Green, Lifeglobal, CooperSurgical).¹⁸
53
54
55
56
57
58
59
60

1
2
3 A minimum of 3 incubators of a single brand within each facility is obligatory to represent
4 the three pH arms: 7.2 ± 0.02 pH level (Incubator A), 7.3 ± 0.02 pH level (Incubator B), and
5
6 7.4 ± 0.02 level (Incubator C). The three incubators will undergo strict adjustments to maintain
7
8 the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card
9
10 US). Constant pH levels will be ensured by a twice-weekly measurement of pH using the
11
12 blood gas analyser along with daily measurement of CO₂ levels of incubators. Measurement
13
14 of pH will occur following overnight incubation of 1 mL culture media in a central well dish
15
16 covered with 0.4 mL of oil. Before the opening of incubators, the handheld blood gas
17
18 analyser (Epoc® Reader and Host; BGEM card US) will be prepared for pH measurement as
19
20 per the manufacturer protocol. Briefly, after switching on the device, the device automatically
21
22 starts to calibrate. Next, we set the temperature at 37°C, and sample type as arterial. Next, we
23
24 insert the card to undergo automatic calibration. Next, when the device is ready, we will
25
26 aspirate 0.5 mL of calibrated culture media using 1 mL syringe attached to wide needle
27
28 calibre. Next, we discard the first droplet and smoothly inject the sample until hearing a beep.
29
30 The results of pH, partial CO₂ and O₂ pressures will be ready thereafter. Results will be
31
32 reported in each laboratory to compare partial pressures of CO₂ and O₂ with incubator
33
34 display. pH levels will be measured every new batch of culture media. Measurements of pH
35
36 and CO₂ across centres will be performed using one-brand equipment with periodic
37
38 calibration. To account for errors in measurement, one well-trained personnel will be
39
40 assigned to measure the pH and double-check the CO₂ level across trial sites.
41
42
43
44
45
46
47
48

49 **Culture Protocol and Embryo Scoring**

50
51 Each culture dish (micro-droplet, Vitrolife) will hold 12 droplets of 20 µl each from Global
52
53 Total culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml of oil (NidOil,
54
55 Nidacon). If a decision to change culture media at any time point of the study conduct is
56
57 made, this will be performed at the same time across study sites. Dishes will undergo
58
59
60

1
2
3 overnight incubation in the relevant incubator adjusted to the proper pH as per randomisation.
4
5 After ICSI, the injected oocytes will undergo washing in culture medium followed by
6
7 incubation from day 0 through day 5 or 6 in the relevant pH arm, except for a small portion
8
9 of embryos transferred on day 3. The inseminated oocytes will undergo culture in groups of 3
10
11 each from days 0 to 5 or 6, with the removal of the unfertilised, abnormally fertilised or
12
13 degenerated oocytes at fertilisation check. Two embryologists will perform the fertilisation
14
15 check and embryo grading on day 1, 2, 3, and 5 of culture as per the Istanbul Consensus.¹⁹
16
17 All laboratories will vitrify embryos no earlier than day 5. Embryos are suitable for transfer
18
19 or vitrification on day 5 provided they are graded 3 1 1 as per the Istanbul Consensus.¹⁹
20
21 Embryos utilised for transfer or cryopreservation will be pictured and recorded in the patient
22
23 file. All the recorded pictures from all centres will undergo blind grading by two
24
25 independent, experienced embryologists.
26
27
28
29

30 **Embryo Transfer**

31
32
33 Women will receive one to two embryos. Women with a reduced uterine cavity or previous
34
35 preterm birth will transfer only one embryo. Trial sites will transfer blastocysts on day 5
36
37 except for one participating site will transfer embryos on day 3. This issue will be accounted
38
39 for by adjusting the analysis by trial site. Embryos will be transferred under sonographic
40
41 guidance using the Sydney IVF Transfer Set (Cook, US) as per the standard transfer protocol
42
43 in each site. Any remaining utilisable embryos will be vitrified for transfer in subsequent
44
45 cycles as we plan to monitor the cumulative live birth resulted from fresh and vitrified-
46
47 warmed transfer within one year from randomisation. Women will test the level of serum
48
49 hCG for biochemical pregnancy 14 days after oocyte retrieval and will confirm clinical
50
51 pregnancy at \geq week 7 of gestation by detection of intrauterine sacs with a heartbeat on the
52
53 ultrasound.
54
55
56
57
58
59
60

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 β hCG \geq 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 \leq 20th weeks of gestation).
- 5) Term live-birth (i.e. delivery of one or more viable infants \geq 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 trophoctodermal 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

1
2
3 size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a
4 spread of just seven percentage points) this sample size yields 85% power against a 5%
5 significance level, and 65% at a 1% significance level. We have also been conservative in our
6 inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our
7 group size to 680. In reality, we will conduct the analysis on an intention to treat basis,
8 including all randomised women. Women who do not complete treatment (for example, they
9 do not undergo embryo transfer) will be counted as not having a live birth. The only
10 exceptions to this will be participants who withdraw consent for their data to be used in the
11 study. Our inflation for loss to follow up reflects this possibility. We also note that
12 adjustment for site and age in the analysis will increase power further.
13
14
15
16
17
18
19
20
21
22
23
24
25

26 *Analytical methods*

27
28 The study conduct will be according to the intention-to-treat approach, where each participant
29 randomised will be included in the analysis, regardless of protocol deviation. The primary
30 analysis of live birth will be conducted using logistic regression, with live birth event
31 regressed on pH groups, adjusted for study site and participant age, which will be
32 standardised before being entered as a covariate. pH will be entered as a categorical
33 covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate
34 across the three groups to be performed. Secondary supportive analyses will be conducted to
35 try to characterise the nature of any association. This will include a test of a linear trend in
36 live birth rates across pH groups, which would imply an optimal pH level for the lowest or
37 highest value, as well as pairwise comparisons between each group (again, these analyses
38 will be adjusted for site and age). The pairwise comparisons will focus on size and precision
39 of the odds ratios. Although it would be desirable to power the study for all pairwise
40 comparisons as the primary outcome, this yields impracticable sample sizes (> 4000
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 participants) against realistic effects. The study has therefore been designed to represent the
4
5 most informative test of the hypothesis that pH level affects live birth, that is practicable.
6
7 For secondary outcomes, binary variables will be analysed in an analogous fashion to the
8
9 primary analysis. Count variables will be analysed using Poisson regression, with zero-
10
11 inflated models wherever the outcome is structurally undefined for some participants. Again,
12
13 these will be adjusted for site and age. In the analysis of the number of usable embryos,
14
15 implanted embryos arising from the day 3 transfer will be included as formed and good
16
17 quality blastocysts, while those that do not implant in this portion will be considered blocked
18
19 at day 3. Utilisable embryos will be calculated as the total number of transferred embryos and
20
21 formed blastocysts. A 1% significance level will be employed. Due to the short treatment
22
23 duration, it is anticipated that loss to follow up will be minimal (it is unusual for patients not
24
25 to return to clinic to have their embryos transferred, for example). However, if any loss does
26
27 occur, these participants will be analysed as having a negative status for the primary
28
29 outcome, unless consent to use data is withdrawn. The follow-up period is identified as one
30
31 year from randomisation of the last participant provided that all pregnant women have given
32
33 birth.
34
35
36
37
38

39 **Ethics and dissemination**

40
41
42 Ethics Review Board of Upper Egypt IVF Network relating to the participating sites
43
44 approved this trial (Approval No. 009/2016). An independent safety and monitoring
45
46 committee formed of five experts in reproductive endocrinology, reproductive biology,
47
48 embryo culture, biostatistics and trial methodology will oversee the trial conduct. All
49
50 participants will receive independent counselling from research instructors who are not
51
52 involved in patient care or laboratory work. Participants who will accept to participate will
53
54 sign a written informed consent before enrolment. Conducting this study will be in
55
56 accordance with the Declaration of Helsinki.²¹ The trial reporting will be according to the
57
58
59
60

1
2
3 CONSORT statement.²² No plan exists to amend this protocol and any amendments will
4
5 require approval of the safety committee, and will undergo detailed reporting on the trial
6
7 registry and in the final manuscript.
8
9

10 **Discussion**

11
12 Given the lack of evidence for a superior pH level for human embryo culture and whether
13
14 the pH level could make a difference in live birth after IVF, a trial is warranted. This trial is
15
16 expected to fill the gap in this area, since at present embryologists must rely upon the
17
18 recommendations of manufactures of culture media rather than on a robust evidence base.
19
20 This trial is powered to a high level (>90%, with a 1% significance level) against clinically
21
22 important differences to minimize the risk for uninformative results. In the case of cleavage-
23
24 stage transfer, the calculation of formed blastocysts will be based on the assumption that
25
26 implanted embryos represent formed blastocysts, while any transferred embryos which fail to
27
28 implant will be counted as blocked at the cleavage stage. Although this definition will be
29
30 subject to some error, we believe it is a reasonable way to assess blastocyst formation without
31
32 excluding a portion of the data. This point will be further discussed when this trial is
33
34 reported. A large number of secondary outcomes will be measured and reported. This is
35
36 partially driven by adherence to a recently developed core outcome set for infertility trials,²²
37
38 as well as by the inclusion of some embryological variables which might shed light on the
39
40 mechanism of any effects of pH levels. As is usual in clinical trials, Type 1 error is controlled
41
42 by the fact that the study inference will be based on the primary outcome variable, live birth.
43
44 Accordingly, we will interpret the results of secondary endpoints cautiously in the final
45
46 report, since these endpoints are not subject to Type 1 error control.
47
48
49
50
51
52
53

54 **Funding and conflict of interest**

55
56 The study receives no fund and the authors have no conflict of interest to declare.
57
58
59
60

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 Ibsina 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 Ibsina 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]

- 1
2
3 5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation
4 embryo culture media: effects of culture and batches. *Reprod Biomed Online*
5 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First:
6 2018/09/20]
- 7
8 6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J*
9 *Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
- 10
11 7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human
12 preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi:
13 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
- 14
15 8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH
16 against acidosis in preantral follicles by multiple mechanisms. *Development*
17 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
- 18
19 9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum*
20 *Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First:
21 1998/06/10]
- 22
23 10. Lane M, Baltz JM, Bavister BD. Na⁺/H⁺ antiporter activity in hamster embryos is
24 activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi:
25 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
- 26
27 11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and
28 embryo manipulations for assisted reproduction. *Reprod Biomed Online*
29 2009;18(6):799-810. [published Online First: 2009/06/06]
- 30
31 12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil*
32 *Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online
33 First: 2010/11/12]
- 34
35 13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon
36 dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155
37 [published Online First: 1987/06/01]
- 38
39 14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation
40 embryo: amino acids act as buffers of intracellular pH. *Hum Reprod*
41 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First:
42 1999/01/14]
- 43
44 15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse
45 embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-
46 42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J
47 [published Online First: 1998/07/21]
- 48
49 16. World Health Organization. WHO laboratory manual for the examination and processing
50 of human semen. 5th ed. Geneva: World Health Organization 2010.
- 51
52 17. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of
53 single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-
54 6736(92)92425-f [published Online First: 1992/07/04]
- 55
56 18. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory
57 environment and air quality: report of an expert meeting. *Reprod Biomed Online*
58 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First:
59 2018/04/17]
- 60
61 19. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment:
62 proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi:
63 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
- 64
65 20. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and
66 implementing a core outcome set for infertility. *Hum Reprod Open*

- 1
2
3 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First:
4 2019/03/22]
5
6 21. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ*
7 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
8 22. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines
9 for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi:
10 10.1136/bmj.c332 [published Online First: 2010/03/25]
11
12

13 Figure Legend: Trial plan for enrolment
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Couples will receive detailed information on the possible risks and potential benefits.

Couples with primary acceptance will be randomized to the three pH arms of the study

Randomization will occur to assign ~ 2100 participants to undergo day-5 fresh embryo-transfer utilizing the blastocysts developed in the either 7.2, 7.3 or 7.4 pH levels.

Women randomization

~ 700 participants will be randomized to undergo embryo culture in 7.2 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.3 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.4 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

The intention-to-treat will include all randomized participants to 7.2 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.3 pH level group with the live birth rate as primary endpoint.

The intention-to-treat will include all randomized participants to 7.4 pH level group with the live birth rate as primary endpoint.

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 SPIRIT reporting 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

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
14				
15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	4
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	2 & 3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2 & 3
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	3 & 4
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	4
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
51				
52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	4
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
57				
58				
59				
60				

1	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
2				
3				
4				
5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
7	description			
8				
9				
10	Interventions:	#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
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
16	adherence			
17				
18				
19				
20				
21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
22	concomitant care			
23				
24				
25	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
26				
27				
28				
29				
30				
31				
32				
33				
34	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
35				
36				
37				
38				
39				
40	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
41				
42				
43				
44				
45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
46				
47				
48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#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
55	generation			
56				
57				
58				
59				
60				

provided in a separate document that is unavailable to those who enrol participants or assign interventions

1			
2			
3			
4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
6			describing any steps to conceal the sequence until interventions
7	mechanism		are assigned
8			
9			
10			
11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol
12	implementation		participants, and who will assign participants to interventions
13			
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial
15			participants, care providers, outcome assessors, data analysts),
16			and how
17			
18			
19			
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,
21	emergency unblinding		and procedure for revealing a participant's allocated intervention
22			during the trial
23			
24			
25	Methods: Data		
26	collection,		
27	management, and		
28	analysis		
29			
30			
31			
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and
33			other trial data, including any related processes to promote data
34			quality (eg, duplicate measurements, training of assessors) and a
35			description of study instruments (eg, questionnaires, laboratory
36			tests) along with their reliability and validity, if known.
37			Reference to where data collection forms can be found, if not in
38			the protocol
39			
40			
41			
42			
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,
44	retention		including list of any outcome data to be collected for participants
45			who discontinue or deviate from intervention protocols
46			
47			
48	Data management	#19	Plans for data entry, coding, security, and storage, including any
49			related processes to promote data quality (eg, double data entry;
50			range checks for data values). Reference to where details of data
51			management procedures can be found, if not in the protocol
52			
53			
54			
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary
56			outcomes. Reference to where other details of the statistical
57			analysis plan can be found, if not in the protocol
58			
59			
60			

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	11 & 12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	11 & 12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
8				
9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	4
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
19				
20				
21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	4, 11 &
23	interim analysis		including who will have access to these interim results and make	12
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	4
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
31				
32				
33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	4
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	4
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	4
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	4
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
57				
58				
59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	4
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	4
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	4
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	4
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	4
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	1
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	4
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	n/a
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 09. September 2019 using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2019-034194.R5
Article Type:	Protocol
Date Submitted by the Author:	13-Jan-2020
Complete List of Authors:	Fawzy, Mohamed; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Emad, Mai; IbnSina Hospital, Ibsina IVF Centre; Banon IVF Centre Wilkinson, Jack; University of Manchester, Centre for Biostatistics; Salford Royal NHS Foundation Trust, Research and Development Mansour, Ragaa; Egyptian IVF-ET Center Mahran, Ali; Assiut University Faculty of Medicine, Department of Dermatology, Venereology and Andrology Fetih, Ahmed; Assiut University Faculty of Medicine, Department of Obstetrics and Gynecology Abdelrahman , Mohamed; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology AbdelGhafar, Hazem; Sohag University Faculty of Medicine, Department of Obstetrics and Gynecology
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Reproductive medicine, Obstetrics and gynaecology
Keywords:	Embryo culture, pH level, culture media, blastocyst formation

SCHOLARONE™
Manuscripts



I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our [licence](#).

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which [Creative Commons](#) licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

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

Mohamed Fawzy,^{ab} Mai Emad,^{ab} Jack Wilkinson,^c Ragaa Mansour,^d Ali Mahran,^d Ahmed N. Fetih,^f Mohamed Y. AbdelRahman,^g Hazem Abdelghafar,^g

IbnSina IVF Centre, Sohag and Banon IVF Centre, Assuit, Egypt

^aIbnSina IVF Centre, IbnSina Hospital, Sohag, Egypt; ^bBanon IVF Centre, Assiut, Egypt;

^cCentre for Biostatistics, University of Manchester, UK; ^dEgyptian IVF-ET Centre, Cairo,

Egypt; ^eDepartment of Dermatology, Venereology and Andrology, Faculty of Medicine,

Assiut University, Egypt; ^fDepartment of Obstetrics and Gynecology, Faculty of Medicine,

Assiut University, Egypt; ^gDepartment of Obstetrics and Gynecology, Faculty of Medicine,

Sohag University, Egypt

Corresponding Author: Dr. Mohamed Fawzy, IVF Laboratory Director (IbnSina and Banon

IVF Centres), IbnSina Hospital, 146 El Aref Square, Sohag, Egypt; Cell: +201011122286; E-

mail: drfawzy001@me.com

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

1
2
3 in trial design and *in vitro* culture. No plan exists to disseminate results to participants or
4
5 health communities, except for the independent monitoring and safety committee of the trial.

6
7
8 **Trial registration number** NCT02896777.

9
10 **Keywords**

11
12 Embryo culture, pH level, culture media, blastocyst formation

13
14
15 **Strengths and limitations of this study**

- 16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- 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.

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

33
34 **Background**

35
36 Assisted reproductive techniques (ART) result in a cumulative live birth rate of around 65%
37
38 within six cycles of *in vitro* fertilisation (IVF),¹ which is relatively suboptimal. In addition,
39
40 IVF has been associated with adverse perinatal outcomes, such as preterm birth and low birth
41
42 weight babies compared with the *in vivo* conception.² These adverse consequences can be
43
44 due to a range of factors including patient demographics, ovarian stimulation, and the culture
45
46 system. In relation to embryo culture conditions, over 200 variables have been identified as
47
48 having effects on cycle outcome.³ One element that may influence embryo development *in*
49
50 *vitro* is the culture medium pH, which thus far has been determined by manufacturers of
51
52 culture media without recourse to a well-powered randomised clinical trial (RCT).⁴ pH levels
53
54 are potential stressors that vary between media brands and from batch-to-batch depending on
55
56 the levels of bicarbonate in the culture media and of CO₂ in incubators.⁵ Hence, pH levels can
57
58
59
60

1
2
3 vary between incubators within a laboratory. Recommendations for the frequency of pH
4
5 measurement for embryo culture vary from daily to monthly.⁴
6

7
8 The intracellular pH (pHi) of oocytes and embryos is modulated by the extracellular pH
9
10 (pHe),⁶ which is affected by the culture conditions, including the concentrations of
11
12 bicarbonate, protein, and amino acids in culture media, and of the CO₂ in incubators.⁷ The
13
14 mechanism of pHi in oocytes and embryos is complex, regulating enzymatic activity, cell
15
16 division and differentiation, protein synthesis, metabolism, mitochondrial function,
17
18 cytoskeletal regulation, and microtubule dynamics.^{7,8} Drifts in pHe translate into changes in
19
20 pHi, which can adversely affect cell function if compensatory mechanisms fail to restore pHi
21
22 to a safe level.⁸ These compensatory mechanisms include an active exchange among Na⁺,
23
24 HCO₃⁻/Cl⁻ and Na⁺/H⁺ to maintain pHi between 7 to 7.3.^{5,8} Oocytes that have been denuded
25
26 of their surrounding corona and cumulus cells prior to insemination via ICSI, as well as
27
28 vitrified-warmed embryos, lack robust compensatory mechanisms for maintenance of pHi;
29
30 therefore, drastic differences between pHe and pHi in these cases can disrupt embryo
31
32 development.⁹⁻¹¹
33
34
35
36

37
38 That being said, the optimal pH level to support human embryo development *in vitro* is still
39
40 undefined.^{4,9,12-15} Current recommendations are based on results derived using mouse models
41
42 and/or literature from culture medium manufacturers. Theoretically, a wide range of pHe
43
44 levels (7.0–7.5) could support human embryo development *in vitro*, but a narrower range of
45
46 pHe levels (7.2 to 7.4) is used in clinical practice. This is because a more acidic pHe (≤ 7)
47
48 can adversely affect the oocyte spindle, delaying or even blocking embryo development *in*
49
50 *vitro*.⁴ Alkaline levels of pHe (≥ 7.5) can similarly harm oocytes and embryos.⁴ Although
51
52 these reports of the effects of extreme pHe levels rely on animal models, underpowered
53
54 studies, or anecdotal beliefs, we have decided to limit our investigation to the range of pHe
55
56 used in clinical practice (7.2 to 7.4) as the safer alternative. Despite the routine use of pHe 7.2
57
58
59
60

1
2
3 to 7.4 in clinical practice, there is no clear evidence as to whether there is a level within this
4
5 range that could better support human embryo development to result in a live birth. The aim
6
7 of this multicentre, randomised, clinical trial is to identify whether pHe 7.2, 7.3, or 7.4 results
8
9 in an improved live birth rate after ICSI in order to investigate the potential for optimisation.
10
11

12 **Methods and Design**

13
14 This is a protocol of a multicentre, randomised, triple-arm, triple-blind clinical trial
15
16 (NCT02896777, registered at www.ClinicalTrials.gov) that will compare the effect of three
17
18 pH levels for human embryo culture *in vitro* on live birth after ICSI (Figure 1). In this
19
20 partially blind design, the clinicians, participants and outcome assessor will be unaware of the
21
22 study arms, but the embryologists will be aware. This multicentre trial will involve private
23
24 IVF facilities in Egypt (Ibnsina IVF Centre, Egyptian IVF-ET centre, Banon IVF Centre,
25
26 Qena IVF Centre and Amshaj IVF Centre) which will all have the study protocol prior to
27
28 enrolment of participants. If other IVF facilities join this trial before recruitment, this will be
29
30 reported in the study.
31
32
33
34

35 **Intervention**

36
37 In each arm of the trial, which will only include intracytoplasmic sperm injection (ICSI)
38
39 cycles, oocytes and embryos will undergo continuous culture from day 0 through day 5 or 6
40
41 without medium renewal. The oocytes and resulting embryos after ICSI will be cultured in
42
43 either pHe 7.2 ± 0.02 ("Arm I"), pHe 7.3 ± 0.02 ("Arm II"), or pHe 7.4 ± 0.02 ("Arm III").
44
45
46

47 **Patient and Public Involvement**

48
49 Patients have not been directly involved in the design, planning, and conception of this trial.
50
51

52 **Randomisation and Masking**

53
54 Using an online tool, participants will be randomised to the experimental arms with a 1:1:1
55
56 allocation ratio. The allocation sequence of participants will be generated using a permuted
57
58 block randomisation of 3, 6 and 9 block sizes with unique identifiers in random order,
59
60

1
2
3 stratified by trial site. Randomisation of participants and storage of the results in sequentially
4 numbered, opaque, sealed envelopes will be performed by a secretary with no involvement in
5 patient care, and the sealed envelopes will be provided to trial sites before enrolment of the
6 first participant. Eligible participants will be allocated to the relevant arms on the day of
7 maturation trigger and the allocation result will be communicated to the laboratory team.
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 $\geq 1\%$ 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,

1
2
3 starting on day 1 after retrieval (“day 1”) to 12 weeks of gestation, if a pregnancy is
4
5 established.
6

7 **Sperm Preparation, Oocyte Denudation and ICSI**

8
9
10 Semen samples will be processed using density gradient centrifugation¹⁶ (Puresperm,
11
12 Nidacon, Sweden). The pellet will undergo once washing and incubation at room temperature
13
14 in HEPES buffered medium (AllGrade Wash, LifeGlobal). Denudation of oocytes will occur
15
16 immediately after collection in 40 IU hyaluronidase (LifeGlobal, Canada) diluted in Global
17
18 HEPES using a 170-micrometre stripper (Cook, US). Metaphase II (MII) oocytes will
19
20 undergo ICSI in Global HEPES medium under an inverted microscope as previously
21
22 described.¹⁷
23
24
25

26 **Incubator Management and pH Adjustment**

27
28 We will use only humidified benchtop incubators for this study, involving Labo C-Top
29
30 (Labotect, Germany), Minc-1000 (Cook, US), and AD-3100 (Astec, Japan). Each centre will
31
32 use only one brand to account for incubators as a variable. It is possible that dry incubators
33
34 such as ESCO (Esco Micro Pte. Ltd, Singapore) are used in some centres, but we note that
35
36 our analysis will account for differences between centres by adjusting for trial sites.
37
38

39
40 Incubators will undergo stringent control of temperature ($36.9\pm 0.1^{\circ}\text{C}$) by daily validation
41
42 using certified thermometers. Incubator CO_2 and O_2 levels will be measured daily using a
43
44 certified gas analyser to ensure that O_2 measures 5%, and CO_2 concentration is at the
45
46 prespecified level to achieve the required pH. Well-trained persons across the sites will verify
47
48 all measurements (temperature, CO_2 , O_2 , and pH levels). Incubators will be sterilised by 6%
49
50 H_2O_2 every four weeks, along with the installation of a new set of inline filters (Green,
51
52 Lifeglobal, CooperSurgical).¹⁸
53
54

55
56 A minimum of 3 incubators of a single brand within each facility is obligatory to represent
57
58 the three pH arms: 7.2 ± 0.02 pH level (Incubator A), 7.3 ± 0.02 pH level (Incubator B), and
59
60

1
2
3 7.4±0.02 level (Incubator C). The three incubators will undergo strict adjustments to maintain
4
5 the required pH using a handheld blood gas analyser (Epoc® Reader and Host; BGEM card
6
7 US). Constant pH levels will be ensured by a twice-weekly measurement of pH using the
8
9 blood gas analyser along with daily measurement of CO₂ levels of incubators. Measurement
10
11 of pH will occur following overnight incubation of 1 mL culture media in a central well dish
12
13 covered with 0.4 mL oil. Before the opening of incubators, the handheld blood gas analyser
14
15 (Epoc® Reader and Host; BGEM card US) will be prepared for pH measurement as per the
16
17 manufacturer's protocol. Briefly, after switching on the device, the temperature will be set at
18
19 37°C, sample type set as arterial, and automatic calibration will be initiated. When the device
20
21 is ready, 0.5 mL calibrated culture media will be aspirated using a 1 mL syringe attached to a
22
23 wide calibre needle. The first droplet will be discarded and the remainder of the sample
24
25 injected into the device, for the assessment of pH, and partial pressures of CO₂ and O₂. In
26
27 each laboratory, the partial pressures of CO₂ and O₂ will be compared with those on the
28
29 incubator display. The pH levels will be measured with every new batch of culture media.
30
31 Measurements of pH and CO₂ across centres will be performed using the same brand of
32
33 equipment with periodic calibration. To account for errors in measurement, one well-trained
34
35 staff member will be assigned to measure the pH and double-check the CO₂ level across trial
36
37 sites.
38
39
40
41
42
43

44 **Culture Protocol and Embryo Scoring**

45
46 Each culture dish (micro-droplet, Vitrolife) will hold 12 × 20 µl droplets of Global Total
47
48 culture medium (Lifeglobal, CooperSurgical, US) overlaid with 5 ml oil (NidOil, Nidacon).
49
50 If there is a decision to change culture media at any time during the study, this will be
51
52 performed at the same time across study sites. Dishes will be incubated overnight in the
53
54 relevant incubator adjusted to the proper pH as per randomisation. After ICSI, the injected
55
56 oocytes will undergo washing in culture medium followed by incubation from day 0 through
57
58
59
60

1
2
3 day 5 or 6 in the relevant pH arm, except for a small portion of embryos transferred on day 3.
4
5 The inseminated oocytes will undergo culture in groups of 3 each from days 0 to 5 or 6, with
6
7 the removal of the unfertilised, abnormally fertilised or degenerated oocytes at fertilisation
8
9 check. Two embryologists will perform the fertilisation check and embryo grading on day 1,
10
11 2, 3, and 5 of culture as per the Istanbul Consensus.¹⁹ All laboratories will vitrify embryos no
12
13 earlier than day 5. Embryos will be suitable for transfer or vitrification on day 5 provided
14
15 they are graded 3 1 1 as per the Istanbul Consensus.¹⁹ Images of embryos utilised for transfer
16
17 or cryopreservation will be recorded in the patient file. All the images from all centres will
18
19 undergo blind grading by two independent, experienced embryologists.
20
21
22

23 **Embryo Transfer**

24
25 One to two embryos will be transferred per cycle. Women with a reduced uterine cavity or
26
27 previous preterm birth will receive only one embryo. Trial sites will transfer blastocysts on
28
29 day 5 except for one participating site that will transfer embryos on day 3. This issue will be
30
31 accounted for by adjusting the analysis by trial site. Embryos will be transferred under
32
33 sonographic guidance using the Sydney IVF Transfer Set (Cook, US) as per the standard
34
35 transfer protocol in each site. Any remaining utilisable embryos will be vitrified for transfer
36
37 in subsequent cycles as we plan to monitor the cumulative live birth rate from fresh and
38
39 vitrified-warmed transfers within one year from randomisation. Women will test the level of
40
41 serum hCG for biochemical pregnancy 14 days after oocyte retrieval and will confirm clinical
42
43 pregnancy at \geq week 7 of gestation by detection of intrauterine sacs with a heartbeat on the
44
45 ultrasound.
46
47
48
49

50 **Outcome Measures**

51
52 Each outcome will be calculated including all randomised participants in the arms to which
53
54 they were allocated, with the exception of implantation rate, which will be interpreted
55
56 cautiously due to concerns over its validity as a measure of treatment effect, and perinatal
57
58
59
60

1
2
3 outcomes, which by definition are only available in the subset of participants achieving live
4
5 birth. This study will adopt the definitions of outcomes included in a forthcoming core
6
7 outcome set for infertility trials²⁰ where appropriate. This outcome set was developed by
8
9 means of an international consensus process involving clinicians, clinical scientists, patients
10
11 and researchers.
12
13

14 ***Primary outcome***

15
16 Live birth (delivery of one or more viable infants beyond the 20th week of gestation).
17
18

19 ***Secondary outcomes***

20
21 All secondary outcomes will be cautiously reported, since statistical significance has limited
22
23 meaning in the context of a plurality of tests.
24
25

- 26 1) Biochemical pregnancy (positive β hCG \geq 10 IU/L at 14 days after egg retrieval).
- 27 2) Clinical pregnancy (registered sacs with a heartbeat on ultrasound $>$ 7th week of gestation).
- 28 3) Ongoing pregnancy (continued viable pregnancy $>$ 20th week of gestation).
- 29 4) Miscarriage (loss of a clinical pregnancy \leq 20th week of gestation).
- 30 5) Term live-birth (i.e. delivery of one or more viable infants \geq 37 weeks of gestation).
- 31 6) Preterm birth (delivery of one or more viable infants $<$ 37th week of gestation).
- 32 7) Very preterm birth (delivery of one or more viable infants $<$ 32nd week of gestation).
- 33 8) Low birth weight babies (babies weighing $<$ 2500 gm within 24 hours of delivery)
- 34 9) Congenital malformation (delivery of congenitally malformed babies).
- 35 10) Stillbirth (delivery of nonviable babies $>$ 20 weeks of gestation).
- 36 11) Cumulative live birth (registered viable neonates after one fresh plus one vitrified-
37 warmed embryo transfer within one year of randomisation).
- 38 12) Fertilisation (presence of 2 pronuclei 17 ± 1 hr after ICSI).
- 39 13) Embryo cleavage (cleaved embryos per fertilised oocyte).
- 40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 14) Top-quality embryos on day 3 (7-8 cells with appropriate sizes blastomeres and less than
4
5 10% fragmentation by volume).
6
7
8 15) Blastocyst formation on day 5 or 6 (formed blastocysts per fertilised oocyte).
9
10 16) Top-quality blastocyst on day 5 (rounded and dense inner cell mass with many
11
12 trophoctodermal cells creating a connected zone and a blastocoel more than 100% by
13
14 volume; ≥ 3 1 1 grade per fertilised oocyte).
15
16
17 17) Cryopreservation (cryopreserved embryos per fertilised oocyte).
18
19 18) Live-birth-implantation rate (live birth per embryo transferred).
20
21 19) Utilised embryos (number of cryopreserved plus transferred embryos per fertilised
22
23 oocyte).
24
25
26 20) Top-quality utilised embryos (number of high-quality embryos transferred plus blastocyst
27
28 cryopreserved of 3 1 1 grade per fertilised oocyte).
29

30 **Statistical Analysis**

31 ***Sample size estimation***

32
33 This is a three-arm study looking at pH over a range of 7.2 to 7.4. The hypothesis to be tested
34
35 is that adjusting the pH value to the edges of this range might result in improvements to the
36
37 live birth rate, although we remain in equipoise as to whether higher or lower values will be
38
39 optimal. On the basis of internal data, live birth rates in the region of 25-35% are typical, and
40
41 our goal is to investigate whether this is associated with varying pH levels.
42
43
44
45

46
47 The study has been powered for a global test of the effect of pH, calculated using plausible
48
49 birth rates in the three groups of 25%, 30%, and 35%. 646 participants per arm yield 98%
50
51 power in this scenario, using a 5% significance level. This test makes no assumption about
52
53 the ordering of the live birth rates in relation to the ordering of the pH values. The high-
54
55 power level has been adopted to allow for some leeway in relation to the minimum effect
56
57 size. For illustration, if the birth rates were slightly less distinct, with 26%, 30%, 33% (a
58
59
60

1
2
3 spread of just seven percentage points) this sample size yields 85% power against a 5%
4
5 significance level, and 65% at a 1% significance level. We have also been conservative in our
6
7 inflation of numbers for dropout. We have allowed for 5% loss to follow up, inflating our
8
9 group size to 680. In reality, we will conduct the analysis on an intention to treat basis,
10
11 including all randomised women. Women who do not complete treatment (for example, they
12
13 do not undergo embryo transfer) will be counted as not having a live birth, and so do not
14
15 represent a reduction in sample size. The only exceptions to this will be participants who
16
17 withdraw consent for their data to be used in the study. Our inflation for loss to follow up
18
19 reflects this possibility. We also note that adjustment for site and age in the analysis will
20
21 increase power further.
22
23
24
25

26 *Analytical methods*

27
28 The study will be conducted according to the intention-to-treat approach, where each
29
30 participant randomised will be included in the analysis, regardless of protocol deviation. The
31
32 primary analysis of live birth will be conducted using logistic regression, with live birth event
33
34 regressed on pH groups, adjusted for study site and participant age, which will be
35
36 standardised before being entered as a covariate. pH will be entered as a categorical
37
38 covariate, allowing a Likelihood Ratio test of the association between pH and live birth rate
39
40 across the three groups to be performed. Secondary supportive analyses will be conducted to
41
42 try to characterise the nature of any association. This will include a test of a linear trend in
43
44 live birth rates across pH groups, which would imply an optimal pH level for the lowest or
45
46 highest value, as well as pairwise comparisons between each group (again, these analyses
47
48 will be adjusted for site and age). The pairwise comparisons will focus on size and precision
49
50 of the odds ratios. Although it would be desirable to power the study for all pairwise
51
52 comparisons as the primary outcome, this yields impracticable sample sizes (> 4000
53
54
55
56
57
58
59
60

1
2
3 participants) against realistic effects. The study has therefore been designed to represent the
4
5 most informative test of the hypothesis that pH level affects live birth, that is practicable.
6
7 For secondary outcomes, binary variables will be analysed in an analogous fashion to the
8
9 primary analysis. Count variables will be analysed using Poisson regression, with zero-
10
11 inflated models wherever the outcome is structurally undefined for some participants. Again,
12
13 these will be adjusted for site and age. In the analysis of the number of usable embryos,
14
15 implanted embryos arising from the day 3 transfer will be included as formed and good
16
17 quality blastocysts, while those that do not implant in this portion will be considered blocked
18
19 at day 3. Utilisable embryos will be calculated as the total number of transferred embryos and
20
21 formed blastocysts. A 1% significance level will be employed. Due to the short treatment
22
23 duration, it is anticipated that loss to follow up will be minimal (it is unusual for patients not
24
25 to return to clinic to have their embryos transferred, for example). However, if any loss does
26
27 occur, these participants will be analysed as having a negative status for the primary
28
29 outcome, unless consent to use data is withdrawn. The follow-up period is identified as one
30
31 year from randomisation of the last participant provided that all pregnant women have given
32
33 birth.
34
35
36
37
38
39

40 **Ethics and dissemination**

41
42 The Ethics Review Board of the Upper Egypt IVF Network relating to the participating sites
43
44 approved this trial (Approval No. 009/2016). An independent safety and monitoring
45
46 committee formed of five experts in reproductive endocrinology, reproductive biology,
47
48 embryo culture, biostatistics and trial methodology will oversee the conduct of trial. All
49
50 participants will receive independent counselling from research instructors who are not
51
52 involved in patient care or laboratory work. Participants who agree to participate will sign a
53
54 written informed consent before enrolment. This study will be conducted in accordance with
55
56 the Declaration of Helsinki.²¹ The trial reporting will be according to the CONSORT
57
58
59
60

1
2
3 statement.²² No plan exists to amend this protocol and any amendments will require approval
4
5 of the safety committee, and will undergo detailed reporting on the trial registry and in the
6
7 final manuscript.
8
9

10 **Discussion**

11
12 Given the lack of evidence for a superior pH level for human embryo culture and whether
13
14 the pH level could make a difference in live birth after IVF, a trial is warranted. This trial is
15
16 expected to fill the gap in this area, since at present embryologists must rely upon the
17
18 recommendations of culture media manufacturers rather than on a robust evidence base. This
19
20 trial is powered to a high level (>90%, with a 1% significance level) against clinically
21
22 important differences to minimize the risk for uninformative results. In the case of cleavage-
23
24 stage transfer, the calculation of formed blastocysts will be based on the assumption that
25
26 implanted embryos represent formed blastocysts, while any transferred embryos which fail to
27
28 implant will be counted as blocked at the cleavage stage. Although this definition will be
29
30 subject to some error, we believe it is a reasonable way to assess blastocyst formation without
31
32 excluding a portion of the data. This point will be further discussed when this trial is
33
34 reported. A large number of secondary outcomes will be measured and reported. This is
35
36 partially driven by adherence to a recently developed core outcome set for infertility trials,²⁰
37
38 as well as by the inclusion of some embryological variables which might shed light on the
39
40 mechanism of any effects of pH levels. As is usual in clinical trials, Type 1 error is controlled
41
42 by the fact that the study inference will be based on the primary outcome variable, live birth.
43
44 Accordingly, we will interpret the results of secondary endpoints cautiously in the final
45
46 report, since these endpoints are not subject to Type 1 error control.
47
48
49
50
51
52

53 **Funding and conflict of interest**

54
55 The study receives no funding and the authors have no conflict of interest to declare.
56
57
58
59
60

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 Ibsina 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 Ibsina 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]

- 1
2
3 5. Tarahomi M, de Melker AA, van Wely M, et al. pH stability of human preimplantation
4 embryo culture media: effects of culture and batches. *Reprod Biomed Online*
5 2018;37(4):409-14. doi: 10.1016/j.rbmo.2018.08.011 [published Online First:
6 2018/09/20]
- 7
8 6. Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J*
9 *Reprod Fertil* 1972;28(3):335-40. [published Online First: 1972/03/01]
- 10
11 7. Phillips KP, Leveille MC, Claman P, et al. Intracellular pH regulation in human
12 preimplantation embryos. *Hum Reprod* 2000;15(4):896-904. doi:
13 10.1093/humrep/15.4.896 [published Online First: 2000/03/31]
- 14
15 8. FitzHarris G, Siyanov V, Baltz JM. Granulosa cells regulate oocyte intracellular pH
16 against acidosis in preantral follicles by multiple mechanisms. *Development*
17 2007;134(23):4283-95. doi: 10.1242/dev.005272 [published Online First: 2007/11/06]
- 18
19 9. Dale B, Menezo Y, Cohen J, et al. Intracellular pH regulation in the human oocyte. *Hum*
20 *Reprod* 1998;13(4):964-70. doi: 10.1093/humrep/13.4.964 [published Online First:
21 1998/06/10]
- 22
23 10. Lane M, Baltz JM, Bavister BD. Na⁺/H⁺ antiporter activity in hamster embryos is
24 activated during fertilization. *Dev Biol* 1999;208(1):244-52. doi:
25 10.1006/dbio.1999.9198 [published Online First: 1999/03/17]
- 26
27 11. Swain JE, Pool TB. New pH-buffering system for media utilized during gamete and
28 embryo manipulations for assisted reproduction. *Reprod Biomed Online*
29 2009;18(6):799-810. [published Online First: 2009/06/06]
- 30
31 12. Hentemann M, Mousavi K, Bertheussen K. Differential pH in embryo culture. *Fertil*
32 *Steril* 2011;95(4):1291-4. doi: 10.1016/j.fertnstert.2010.10.018 [published Online
33 First: 2010/11/12]
- 34
35 13. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon
36 dioxide. *Biol Reprod* 1987;36(5):1155-63. doi: 10.1095/biolreprod36.5.1155
37 [published Online First: 1987/06/01]
- 38
39 14. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation
40 embryo: amino acids act as buffers of intracellular pH. *Hum Reprod*
41 1998;13(12):3441-8. doi: 10.1093/humrep/13.12.3441 [published Online First:
42 1999/01/14]
- 43
44 15. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse
45 embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 1998;50(4):434-
46 42. doi: 10.1002/(SICI)1098-2795(199808)50:4<434::AID-MRD7>3.0.CO;2-J
47 [published Online First: 1998/07/21]
- 48
49 16. World Health Organization. WHO laboratory manual for the examination and processing
50 of human semen. 5th ed. Geneva: World Health Organization 2010.
- 51
52 17. Palermo G, Joris H, Devroey P, et al. Pregnancies after intracytoplasmic injection of
53 single spermatozoon into an oocyte. *Lancet* 1992;340(8810):17-8. doi: 10.1016/0140-
54 6736(92)92425-f [published Online First: 1992/07/04]
- 55
56 18. Mortimer D, Cohen J, Mortimer ST, et al. Cairo consensus on the IVF laboratory
57 environment and air quality: report of an expert meeting. *Reprod Biomed Online*
58 2018;36(6):658-74. doi: 10.1016/j.rbmo.2018.02.005 [published Online First:
59 2018/04/17]
- 60
61 19. Medicine ASIR, Embryology ESIG. Istanbul consensus workshop on embryo assessment:
62 proceedings of an expert meeting. *Reprod Biomed Online* 2011;22(6):632-46. doi:
63 10.1016/j.rbmo.2011.02.001 [published Online First: 2011/04/13]
- 64
65 20. Duffy JMN, Bhattacharya S, Curtis C, et al. A protocol developing, disseminating and
66 implementing a core outcome set for infertility. *Hum Reprod Open*

- 1
2
3 2018;2018(3):hoy007. doi: 10.1093/hropen/hoy007 [published Online First:
4 2019/03/22]
5
6 21. Williams JR. The Declaration of Helsinki and public health. *Bull World Health Organ*
7 2008;86(8):650-2. doi: 10.2471/blt.08.050955 [published Online First: 2008/09/18]
8 22. Schulz KF, Altman DG, Moher D, et al. CONSORT 2010 statement: updated guidelines
9 for reporting parallel group randomised trials. *BMJ* 2010;340:c332. doi:
10 10.1136/bmj.c332 [published Online First: 2010/03/25]
11
12

13 Figure Legend: Trial plan for enrolment
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For peer review only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Couples will receive detailed information on the possible risks and potential benefits.

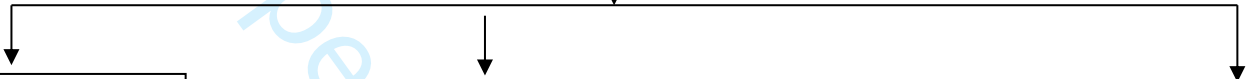


Couples with primary acceptance will be randomized to the three pH arms of the study



Randomization will occur to assign ~ 2100 participants to undergo day-5 fresh embryo-transfer utilizing the blastocysts developed in the either 7.2, 7.3 or 7.4 pH levels.

Women randomization



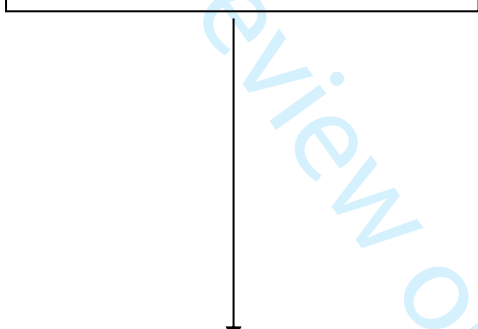
~ 700 participants will be randomized to undergo embryo culture in 7.2 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

~ 700 participants will be randomized to undergo embryo culture in 7.3 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.

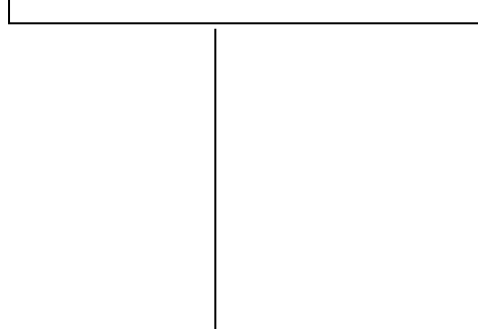
~ 700 participants will be randomized to undergo embryo culture in 7.4 pH level for 5 to 6 days and will receive fresh embryo transfer from this arm.



The intention-to-treat will include all randomized participants to 7.2 pH level group with the live birth rate as primary endpoint.



The intention-to-treat will include all randomized participants to 7.3 pH level group with the live birth rate as primary endpoint.



The intention-to-treat will include all randomized participants to 7.4 pH level group with the live birth rate as primary endpoint.

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 SPIRIT reporting 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

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
14				
15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	4
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	2 & 3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2 & 3
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	3 & 4
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	4
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
51				
52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	4
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
57				
58				
59				
60				

1	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
2				
3				
4				
5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	4 & 5
7	description			
8				
9				
10	Interventions:	#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
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	4 & 5
16	adherence			
17				
18				
19				
20				
21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
22	concomitant care			
23				
24				
25	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
26				
27				
28				
29				
30				
31				
32				
33				
34	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
35				
36				
37				
38				
39				
40	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
41				
42				
43				
44				
45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	4
46				
47				
48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#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
55	generation			
56				
57				
58				
59				
60				

provided in a separate document that is unavailable to those who enrol participants or assign interventions

1			
2			
3			
4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
6			describing any steps to conceal the sequence until interventions
7	mechanism		are assigned
8			
9			
10			
11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol
12	implementation		participants, and who will assign participants to interventions
13			
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial
15			participants, care providers, outcome assessors, data analysts),
16			and how
17			
18			
19			
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,
21	emergency unblinding		and procedure for revealing a participant's allocated intervention
22			during the trial
23			
24			
25	Methods: Data		
26	collection,		
27	management, and		
28	analysis		
29			
30			
31			
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and
33			other trial data, including any related processes to promote data
34			quality (eg, duplicate measurements, training of assessors) and a
35			description of study instruments (eg, questionnaires, laboratory
36			tests) along with their reliability and validity, if known.
37			Reference to where data collection forms can be found, if not in
38			the protocol
39			
40			
41			
42			
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,
44	retention		including list of any outcome data to be collected for participants
45			who discontinue or deviate from intervention protocols
46			
47			
48	Data management	#19	Plans for data entry, coding, security, and storage, including any
49			related processes to promote data quality (eg, double data entry;
50			range checks for data values). Reference to where details of data
51			management procedures can be found, if not in the protocol
52			
53			
54			
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary
56			outcomes. Reference to where other details of the statistical
57			analysis plan can be found, if not in the protocol
58			
59			
60			

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	11 & 12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	11 & 12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
8				
9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	4
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
19				
20				
21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	4, 11 &
23	interim analysis		including who will have access to these interim results and make	12
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	4
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
31				
32				
33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	4
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	4
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	4
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	4
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
57				
58				
59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	4
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	4
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	4
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	4
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	4
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	1
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	4
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	n/a
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

The SPIRIT checklist is distributed under the terms of the Creative Commons Attribution License CC-BY-ND 3.0. This checklist was completed on 09. September 2019 using <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)