Title:

The Effect of Constant 5% Versus Gradient (8%, 5%, and 2%) Oxygen Concentration on Development of Human Embryos to the Blastocyst Stage: A Sibling Oocyte Study

Study Type:

Prospective, Quasi-Randomized Sibling-Split Oocyte Study

Study Site:

Department of Reproductive Medicine and Gynecological Endocrinology

University Medical Centre Maribor

Slovenia

Clinical Trial Registration Number:

NCT05898178

Document Date:

April 24, 2025

Principal Investigator:

Dr. Borut kovačič

Borut.KOVACIC@ukc-mb.si

2. Study Design and Participants

Prospective sibling-split oocyte study , conducted at the University Medical Centre Maribor and included a total of 658 cumulus–oocyte complexes (COCs) collected from 44 ICSI cycles between January 2022 and January 2023. A quasi-randomisation into control and intervention groups was performed immediately after oocyte retrieval. Embryonic outcomes between different oxygen culture conditions were compared.

Eligibility Criteria

- **Inclusion**: Women <35 years old, BMI 18–30 kg/m^2, normal uterine cavity, ≥6 MII oocytes retrieved, first or second ICSI cycle, male factor infertility (non-azoospermic), blastocyst culture, GnRH antagonist protocol.
- **Exclusion**: Endometriosis, previous ovarian surgery, endocrine/metabolic disorders, PCOS.

Ethical Approval

- Institutional Ethics Committee of the University Medical Centre Maribor (No: UKC-MB-KME-1/21)
- National Medical Ethics Committee (No: 0120-405/2021/4)

All participants provided informed consent.

3. Objectives

Primary Objective

To compare the proportion of injected MII oocytes that developed into morphologically optimal blastocysts on day 5 between the control and intervention oxygen conditions.

Secondary Objectives

- Assessment of morphokinetic parameters (timing to key developmental stages)
- Incidence of abnormal cleavage patterns
- Morphometric characteristics of blastocysts (e.g., area, TE cell count)

4. Sample Size Determination

Sample size was calculated using the Sealed Envelope online tool (accessed January 2022). Based on a baseline blastocyst formation rate of 15% and a hypothesized improvement to 25% in the intervention group, 496 embryos (248/group) were required for 80% power at $\alpha = 0.05$. With a buffer for potential fertilization failures, 47 patients were targeted. Ultimately, 44 patients were enrolled, contributing 521 injected sibling MII oocytes.

5. Randomization and Blinding

A quasi-random allocation method was used post-oocyte retrieval. Group A (control) was cultured under 5% O_2; Group B (intervention) under a decreasing gradient from 8% to 2% O_2. Embryologists remained blinded through a coded system.

6. Laboratory Procedures

Sibling oocytes from each patient were divided into two groups:

- Intervention Group (8–5–2% O_2): Cultured under a dynamic oxygen gradient to mimic *in vivo* conditions. Gas levels were adjusted by stage:
    - Day 0–3: 6% CO_2, 8% O_2, and 86% N_2
    - Day 3: Oxygen concentration was reduced to 5% (6% CO_2, 5% O_2, 89% N_2).
    - Day 3–5: Oxygen was further reduced to 2% (6% CO_2, 2% O_2, 92% N_2).
- Control Group (5% O_2): Cultured under fixed conditions (6% CO_2, 5% O_2) throughout.

  Common lab procedures for both groups:

Sperm prep: Density gradient centrifugation

ICSI: Standard protocol using a Nikon Eclipse TE 2000 microscope (200x magnification).

Embryo culture: G-1 PLUS (Day 1–3) and G-2 PLUS (Day 3–5) (Vitrolife, Gothenburg, Sweden).

Monitoring: Continuous time-lapse imaging with Primo Vision system (Vitrolife, Gothenburg, Sweden).

8. Statistical Analysis Plan (SAP)

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, Illinois, USA). Continuous variables were tested for normality using the Shapiro-Wilk test. Data are presented as mean ± standard deviation (SD) for normally distributed variables or as median with interquartile range (IQR) for non-normally distributed variables. Categorical data are expressed as frequencies and percentages. Group comparisons for embryological outcomes, irregular cleavage events, and clinical outcomes were carried out using the chi-square test or Fisher's exact test, depending on the expected frequencies. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using the Baptista-Pike method for categorical variables. Morphokinetic variables were reported as median hours post-insemination (HPI) with IQR. Differences in morphokinetic timings between oxygen groups were analyzed using a linear mixed-effects model (REML), and Šídák's correction was applied for multiple comparisons at individual time points. Morphometric measurements of blastocysts graded Gardner ≥3 were compared using unpaired t-tests, with significance determined at $p < 0.05$. All statistical tests were two-sided; a p-value < 0.05 was considered statistically significant throughout the study.

9. Informed Consent Form (ICF)

9.1 Original (Slovenian language):

Obrazec izjave o zavestni in svobodni privolitvi

OBRAZEC PROSTOVOLJNE IN ZAVESTNE PRIVOLITVE PO POUČITVI

Podpisana _____, rojena _____, in

Podpisani _____, rojen _____,

sva bila pisno in ustno seznanjen/a s potekom, namenom in cilji raziskave z naslovom:

“Vpliv različnih koncentracij kisika na presnovo in razvoj človeških zarodkov in vitro in na uspešnost programa oploditve z biomedicinsko pomočjo”

Veva, kako bo poskrbljeno za najino varnost v raziskavi in da lahko kadar koli zaprosiva za dodatne informacije in jih tudi dobiva. Prav tako nama je bilo pojasnjeno, da lahko privolitev prekličeva, ne da bi morala preklic utemeljiti in ne da bi prenehanje sodelovanja v raziskavi okrnilo najino morebitno siceršnjo zdravstveno obravnavo.
S podpisom prostovoljno potrjujeva svojo pripravljenost za sodelovanje v raziskavi. Dovoljujeva tudi,
da se najini demografski in zdravstveni podatki uporabijo v anonimizirani obliki v znanstvene namene. Obrazec podpisujeva v navzočnosti raziskovalca/raziskovalke.

Podpis: _____ Podpis: _____

Ime in priimek raziskovalca/raziskovalke: _____

Podpis raziskovalca/raziskovalke: _____

Datum: _____

9.2 Translation (English language):

We, the undersigned:

Name: _____, Date of Birth: _____

Name: _____, Date of Birth: _____

have been informed in writing and verbally about the course, purpose, and objectives of the study titled:

"The Effect of Different Oxygen Concentrations on the Metabolism and Development of Human Embryos In Vitro and on the sucess of assisted reproductive technology (ART) programs.«

We understand how our safety will be ensured during the study and that we can request additional information at any time, which will be provided. We have also been informed that we can withdraw our consent at any time, without needing to provide a reason, and that discontinuing participation in the study will not affect our possible further medical care.

By signing this form, we voluntarily confirm our willingness to participate in the study. We also allow our demographic and medical data to be used in anonymized form for scientific purposes. We are signing this form in the presence of the researcher.

Signature: _____ Signature: _____

Name of Researcher: _____

Researcher's Signature: _____

Date: _____

10. Ethical Evaluation of the Submitted Research

10.1 Original (Slovenian language):

Štefanova ulica 5, 1000 Ljubljana
T: 01 478 69 06, 01 478 69 20
F: 01 251 77 55
E: gp.mz@gov.si, kme.mz@gov.si
www.mz.gov.si

Številka: 0120-405/2021/4
Datum: 28. 9. 2021

Zadeva: Ocena etičnosti predložene raziskave
Zveza: Vaša vloga z dne 31. 8. 2021

Spoštovani,

Komisija Republike Slovenije za medicinsko etiko (v nadaljevanju KME RS) je dne 31. 8. 2021 prejela vlogo za oceno etičnosti raziskave z naslovom

"Vpliv različnih koncentracij kisika na presnovo in razvoj človeških zarodkov in vitro in nauspešnost programa oploditve z biomedicinsko pomočjo"

Gre za: fiziološko – klinično raziskavo, ki bo potekala na Oddelku za reproduktivno medicino in ginekološko endokrinologijo, Klinike za ginekologijo in perinatologijo, Univerzitetnega kliničnega centra Maribor.

Namen raziskave je določiti optimalno koncentracijo kisika in čim bolj posnemati fiziološke pogoje v kulturah človeških zarodkov ter posledično izboljšati uspeh programa oploditve z biomedicinsko pomočjo (OBMP).

Spremljali bi fiziološki vpliv kisika na nivo optimalne blastulacije zarodkov, ki je trenutno 10% pri uporabljenem 5 % kisiku v atmosferi inkubatorjev. Ob hipotezi, da gojenje zarodkov pri atmosferi z 2 % izboljša optimalno blastulacijo zarodkov na 20 %, bi potrebovali skupno 398 zarodkov. Pri tem upoštevate statistično moč 80 % in a = 0,05. V raziskavo bi torej vključili največ 100 preiskovanih parov (ciklusov OBMP z vsaj 4 jajčnimi celicami). Povprečno minimalno število jajčnih celic na pacienta v Mariborski IVF enoti, je okoli 8 jajčnih celic (6 zrelih M II jajčnih celic). Stopnja oploditve po ICSI je okoli 70 %. To pomeni da boste v raziskavi razpolagali s približno 400 zarodki. Vsaka raziskovalna skupini bo vsebovala 200 zarodkov.

KME RS je na seji 21. septembra 2021 obravnavala prejeto vlogo in ugotovila, da je vloga popolna ter ocenila, da je raziskava etično sprejemljiva. S tem vam za njeno izvedbo izdaja svoje soglasje.

Peter Slatinšek, mag. biol. in ekol.
Oddelek za reproduktivno medicino in ginekološko endokrinologijo
Klinika za ginekologijo in perinatologijo
UKC Maribor
Ljubljanska 5
2000 Maribor

e-naslov: peter.slatinsek.ps@gmail.com

Pri nadaljnjih dopisih v zvezi z raziskavo se obvezno sklicujte na številko tega dopisa.

S spoštovanjem,

dr. Božidar Voljč, dr. med.
predsednik KME RS

Vročiti:
– naslovniku – po e-pošti

10.2 Translation (English language):

Republic of Slovenia Ministry of Health
Štefanova ulica 5, 1000 Ljubljana
T: +386 1 478 69 06, +386 1 478 69 20
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Reference No.: 0120-405/2021/4
Date: September 28, 2021

Subject: Ethical Evaluation of the Submitted Research
Reference: Your application dated August 31, 2021

Dear Sir/Madam,

The National Medical Ethics Committee of the Republic of Slovenia (hereinafter: NMEC RS) received your application on August 31, 2021, for the ethical assessment of the research titled

"The Effect of Different Oxygen Concentrations on the Metabolism and Development of Human Embryos In Vitro and on the sucess of assisted reproductive technology (ART) programs."

This is a physiological-clinical study, which will be conducted at the Department of Reproductive Medicine and Gynecological Endocrinology, Clinic for Gynecology and Perinatology, University Medical Centre Maribor.

The purpose of the research is to determine the optimal oxygen concentration and to best simulate physiological conditions in human embryo cultures, thereby improving the success of medically assisted reproduction (MAR) programs.

The study will observe the physiological impact of oxygen on the level of optimal blastulation of embryos, currently at 10% under a 5% oxygen atmosphere in incubators. Based on the hypothesis that culturing embryos at 2% oxygen may improve optimal blastulation to 20%, a total of 398 embryos would be required. This calculation assumes a statistical power of 80% and $\alpha = 0.05$. Accordingly, a maximum of 100 participating couples (MAR cycles with at least four oocytes each) would be included in the study. The average minimum number of oocytes per patient at the Maribor IVF unit is about 8 oocytes (6 mature MII oocytes). The fertilization rate following ICSI is around 70%. Therefore, the study is expected to involve approximately 400 embryos, with 200 embryos per research group.

At its meeting on September 21, 2021, the NMEC RS reviewed the application, found it to be complete, and assessed the study as ethically acceptable. Therefore, the Committee grants its approval for the study to be conducted.

Sincerely,

Dr. Božidar Voljč, M.D.
Chair, National Medical Ethics Committee of the Republic of Slovenia

Delivered to:
– Recipient via e-mail

Contact:
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