On developing a thesis for Reproductive Endocrinology and Infertility fellowship: a case study of ultra-low (2%) oxygen tension for extended culture of human embryos

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Abstract Fellows in Reproductive Endocrinology and Infertility training are expected to complete 18 months of clinical, basic, or epidemiological research. The goal of this research is not only to provide the basis for the thesis section of the oral board exam but also to spark interest in reproductive medicine research and to provide the next generation of physician-scientists with a foundational experience in research design and implementation. Incoming fellows often have varying degrees of training in research methodology and, likewise, different career goals. Ideally, selection of a thesis topic and mentor should be geared toward defining an “answerable” question and building a practical skill set for future investigation. This contribution to the JARG Young Investigator’s Forum revisits the steps of the scientific method through the lens of one recently graduated fellow and his project aimed to test the hypothesis that “sequential oxygen exposure (5% from days 1 to 3, then 2% from days 3 to 5) improves blastocyst yield and quality compared to continuous exposure to 5% oxygen among human preimplantation embryos.”

Keywords Blastocyst · Embryo development · In vitro fertilization · Oxygen tension · Fellowship training · Medical education

The scientific method revisited

While Sir Francis Bacon is commonly credited with the enumeration of the scientific method circa 1620 AD, its true origin dates back much further than the Enlightenment [1]. By most scholarly accounts, an Arabic physicist by the name of Ibn al-Haytham was the first to explicitly delineate what is now taught in grade-school science classrooms worldwide. In his 1021 AD “Book of Optics,” in addition to providing the basis for development of the pinhole camera centuries later, al-Haytham outlined three steps to scientific inquiry:

- (1) State a problem based on observation
- (2) Criticize a hypothesis through experimentation
- (3) Interpret the resulting data to form conclusions.

Indeed, these central research tenets have withstood the test of time and still serve as a guide for the most seasoned of investigators.

The following article describes a practical approach to research, with tips and tricks aimed to simplify the process for those, like myself, who are just embarking upon a career that includes research. I recently completed my fellowship in Reproductive Endocrinology and Infertility, which includes an 18-month research requirement. Fellowship thesis projects can be clinical, basic, or now epidemiological in nature. My primary research compared the blastulation rates in human preimplantation embryos in two different conditions for extended culture: sequential oxygen (O₂) exposure (5% from days 1 to 3, then 2% from days 3 to 5) vs. continuous 5% O₂ exposure [2]. While my work was basic, the steps described below are generally applicable to all research. The specifics may vary based on your study, mentor, or institution, but the outline should still be relevant.

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Finding a mentor

Identifying a mentor who is able to sponsor you as a fellow is paramount. Sometimes what seems to be an ideal mentoring situation turns out to be just the opposite. Early in my first year of fellowship, I rotated through a laboratory to learn more about the research opportunities there—the project I pursued was interesting and had the potential to lead somewhere, but the doctoral student I was working with was about to graduate and was not particularly interested in training me. On the scale from basic to translational to clinical, the lab was heavily anchored on the basic side with possibility to lead to more translational work years later. This is the reality of much research, and while clearly critical to advance knowledge, it felt too distant from the clinic for me personally. And perhaps most importantly, I knew the skills that I would learn there would not be relevant beyond fellowship, as I did not plan on continuing to do basic research.

I changed labs partway through my first year. My fellowship director was initially reluctant about this decision, because from the outside it looked like a perfect lab for a fellow—extremely well-funded, highly productive with visible publications in impactful journals, a star mentor. Ultimately, though, it was not a good fit for what I needed and where I wanted to go. Thankfully, I recognized this early on and still had time to pivot.

If your program allows you to do an abbreviated rotation in a laboratory or with a particular mentor, without having to commit for the duration if things do not pan out, I would encourage you to consider this option. It allows you a brief, but substantive experience to determine if the project is worth pursuing.

Following this false start, I began a rotation in the clinical IVF lab. This immediately was more in line with the type of work I wanted to do in fellowship and, indeed, for the rest of my career. Fortunately, the director of the IVF Laboratory, Dr. Catherine Racowsky, agreed to take me on for my thesis work. We had completed several other projects together during my residency and had grown to have a close working relationship.

Like any effective mentor-mentee arrangement [3], we deeply respected one another's work, shared common values, communicated regularly and without reservation, were accountable to one another, and had a personal connection. Other attributes to consider when choosing a mentor include their availability, track record in mentoring other trainees to success, their willingness to challenge you to expand your goals, to take appropriate risks, and to think both critically and creatively. Just as mentees have different strengths and weaknesses, so do mentors. It is important to be honest with yourself about your own working style and to select a mentor that enhances your strengths and likewise complements your weaknesses.

Reading up

Once I had identified a mentor, I immersed myself in the clinical IVF literature. My initial work in the lab involved quality control and dish preparation—checking incubator temperatures and pH at the start of each day, pouring media. I started there with my reading: media composition, the Henderson-Hasselbalch equation, buffering capacity, and incubator settings. I became interested in learning more about normal physiology of the peri-implantation period and how that was, or was not, reflected by laboratory conditions.

One of my most memorable professors from medical school used to say “Not reading equals death.” Perhaps his point was overstated, but the sentiment was spot on—without knowing the background for a topic backwards and forwards, it is hard to know a relevant question to ask, not to mention being sure that your idea has not previously been investigated.

When you start learning about a particular topic, do not limit yourself to the newest publications in the field. Read the old literature, too—the foundational works. During fellowship, I became on a first name basis with the medical librarian at my university. I would email her a reference that PubMed did not have linked, and she would either help track down the .pdf or scan the original version for me. I requested so many articles like this that I thought the library may stop honoring these requests. They never did. One time the librarian told me that I was one of the only trainees who regularly used this service. How could this be? It was like a virtual walk through the stacks of an old library, and my appetite for reading these early works was insatiable.

Rarely, you come across a finding in the literature that gives you pause, a finding that somehow seems to have been buried under other publications, in years of other work. You have to read it twice, three times for it register. This is the feeling that I had when I stumbled into Yedwab's description of “The temperature, pH and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle” in a 1976 volume of *Fertility & Sterility* [4]. It was right there in plain sight:

The mean peri- and postovulatory uterine PO₂ in the human was approximately 15 mmHg [4].

I calculated the conversion to atmospheres (atm); 15 mmHg divided by 760 mmHg/atm = 0.0197, or roughly 2%! Why were we culturing embryos, then, in 5% O₂? I double-checked my arithmetic. I asked my co-fellow down the hall to do the same thing and then an old Chemistry teacher. Two percent was unanimous. Maybe the research had not been done at sea-level? Tel Aviv, Israel, elevation 16.7 ft. Maybe it was a one-off finding that was never corroborated? I emailed for more articles. Thirty years after Yedwab's initial description, another report of the uterine O₂ tension in the human was published, and the findings were remarkably similar: 18.9 mmHg or 2.5% [5]. Other mammals, including the

rat, hamster, rabbit, and Rhesus monkey likewise have been reported to have uterine O₂ tensions of approximately 2%, as well [4, 6].

I finally believed there might be something to this when I came across a 1992 study in *Obstetrics and Gynecology* [7], which elegantly described the O₂ tension in the uterus through the first and early second trimester of pregnancy among patients undergoing elective termination. Surprisingly, a polarographic microelectrode inserted into the placental bed confirmed that the fetus is exposed to a hypoxic environment (17.9 mmHG or 2.4%) through 10 weeks of gestation, and by the 12th week, the same measurement had significantly increased to 60.7 mmHg or 8.0%. This was consistent with histologic data from early pregnancy hysterectomy specimens, as well, which indicated that the fetal-placental circulation is not actually patent until 11–12-week gestation [8]. It seems that for the first trimester, Mother Nature had preserved the reducing, O₂-poor atmosphere characteristic of early life on this planet. Now, we just had to turn down the setting on the incubators and follow her lead.

I next set out to determine when in normal development the human embryo traverses the tubal-uterine junction. While the traditional teaching is that the preimplantation embryo does not reach the uterus until the blastocyst stage, review of the sparse literature available suggests that the late cleavage-stage or early morula may in fact be the developmental stage that enters the endometrial cavity. This timing was first described by Croxatto et al., who serially ligated, transected, and flushed the oviducts in 54 women undergoing voluntary sterilization at various intervals following the LH surge [9]. The most advanced embryo recovered from the oviduct was a 7-cell; no morulae or blastocysts were found [10]. Furthermore, no embryos remained in the oviduct beyond 96 h post-LH peak (i.e., 80 h post-ovulation). From corresponding uterine flushing experiments, the earliest reported stages recovered from the uterus were a 12-cell and a 16-cell embryo [11, 12].

Thus, the cleavage stage embryo is likely exposed to the O₂ tension characteristic of the oviduct (5 to 8% in mammals and non-human primates) [6, 13], while the early morula and beyond is exposed to that of the uterus (2%).

Defining a question

The next step to any research project is to define a question that is feasible to investigate. Typically, the more specific the question is, the better. A specific question allows you to hone in on one aspect of a problem and to select appropriate methodology and outcomes.

In the context of developing my own project, I wondered whether it was possible that as our field moved from routine cleavage stage transfer to blastocyst transfer, our culture conditions, specifically the O₂ tension, had not been updated to

reflect normal physiology. I was familiar with the vast literature comparing 20 vs. 5% O₂ and recent meta-analyses that demonstrated a modest improvement in both clinical pregnancy (OR 1.11, 95% CI 1.04–1.18; 9 RCTs, *n* = 5,501) and live birth (OR 1.14, 95% CI 1.04–1.25; 8 RCTs, *n* = 5,401) following culture in low oxygen tension [14]. However, to my knowledge, when I began my experiments, no one had examined what effect, if any, a further reduction in oxygen tension might have on human embryo development.

In defining my question, one could pose “how does lowering the incubator O₂ tension from 5% to 2% on day 3 affect in vitro blastulation rates in sibling human embryos?” Alternatively, one might ask “is 2% O₂ superior to 5% O₂ for clinical IVF?” Both are certainly reasonable questions to ask; however, the former is more specific and provides a clearer framework than the latter as to how the experiment will be executed. This targeted thinking is important early on in study design. By committing yourself to a specific question, you will have already considered the feasibility of performing the investigation, you will have narrowed down the required methods, and identified study endpoints from the outset.

Forming a hypothesis

In an era of big data, do not overlook the power of a simple hypothesis. The hypothesis is very similar to the research question—it is just typically phrased as an affirmative or negative statement. That is, the null hypothesis for this research question is “There is no difference in blastulation rates of sibling human embryos when the incubator O₂ tension is reduced on day 3 of development.” Stated this way, your reader might be expecting a non-inferiority design with corresponding power analyses. In contrast, the alternative hypothesis might be “Reducing the incubator O₂ tension on day 3 is associated with increased (or decreased) blastulation rates in sibling human embryos.” Here, the reader might expect a study that was powered to show superiority. Ideally, a research project should be selected that is publishable whether or not the null hypothesis is rejected. Accordingly, if you choose a biologically plausible question to answer, even if you get negative results, the data are still valuable in guiding future research efforts.

Whenever you find yourself lost about how to proceed (either with designing your next experiment, choosing an appropriate statistical test, interpreting results, etc.), go back to your original hypothesis. Say it out loud. What is it, *specifically*, that you are testing? It is easy to get caught up in methodology and to lose sight of the original research goal. In those uncertain times, remind yourself of your hypothesis. It is your compass home.

Designing the study

Just as a hypothesis will serve as your compass, a well-crafted research proposal will serve as your map. Such a proposal is invaluable and well worth the time and effort required for development. Therefore, even if your program does not require it, I would encourage you to write one for your project. The basic format provides the hypothesis, background and significance, and study design (including inclusion and exclusion criteria, variables, primary and secondary outcomes, power calculations, proposed statistics, interim analyses, and stopping criteria). Empty or “shell” tables and figures are also helpful to organize how you anticipate presenting the data and writing the manuscript. Depending on your research background, it may be worthwhile to meet with a biostatistician early and often to discuss the proposal and incorporate feedback in real-time prior to even starting the study. Ask other more senior investigators, including your mentor, to review and revise the proposal as well. By investing time in this process upfront, not only will it clearly set up the planned analyses but also when you are ready to synthesize the paper, it will practically write itself.

Ultimately, for my O₂ tension study, I chose to perform a randomized study of sibling embryos that had been donated to research. The sibling pairs, in which one embryo from each pair would remain in 5% O₂ for the duration of culture and the other would be placed into a pre-equilibrated media drop at 2% on day 3, was critical to eliminate patient variability in embryo development, as each patient served as a control for herself. Variables *between* the pairs that we could control for, such as method of insemination, number of pronuclei, and whether or not the embryo had previously been cryopreserved, were included in a model using generalizing estimating equations to account for multiple embryos from the same patient. Power calculations determined how many embryos I might expect to need in order to determine if there was a significant difference in the number of usable blastocysts between the treatment groups based on the effect size observed when comparing 20 to 5% oxygen.

While it likely would have been more straightforward to use an animal model, and certainly easier to accrue pairs of sibling embryos, any finding (positive or negative) would have to be extrapolated to the human. That is, I had what seemed to be a biologically plausible hypothesis, and anything short of using human embryos would not directly answer the research question. This created numerous challenges relating not only to ensuring that we had a sufficient number of embryos for the project but also to the funding opportunities available. Due to the paucity of donated two pronuclei zygotes, in the end, the vast majority of embryos were trippronuclear, 3PN. And the choice to study human embryos informed how the research could be funded and where it legally could be conducted. Indeed, the Dickey-Wicker Amendment, a rider attached to a

1995 congressional bill, specifies that federal funds cannot be used for human embryo research [15]. This applies to all personnel, reagents, equipment, and even physical laboratory space supported by federal dollars. As a result, the research was performed in a non-federally funded laboratory and secondary outcomes for this study (mass spectrometry of spent culture media and multiplexed real-time PCR of O₂-regulated genes in the inner cell mass and trophoctoderm) were contracted out—the former to a private company out-of-state and the latter to a university core lab that was entirely funded by the Howard Hughes Medical Institute.

All of the above is to say, be creative about study design (in the case study described, using human 3PN embryos instead of an animal model) and be cognizant of how these choices will affect eligibility of study funding and how it might affect your selection of outcomes.

Getting funding

Research is typically expensive. Depending on the type and scale of the study, funding requirements may be minimal or grow to be quite considerable. This will largely be driven by study design, so again, take time in drafting a solid research proposal.

Various sources for funding are available for fellows, including extramural grants from the National Institutes of Health (e.g., T32, K grant, Loan Repayment Program for Contraception and Infertility Research, or the Reproductive Scientist Development Program); professional organizations or non-profits (e.g., American Society for Reproductive Medicine KY Cha Award in Stem Cell Technology, the Young Investigator’s Achievement Award from the Jones Institute for Reproductive Medicine, or the March of Dimes); private grants (e.g., Foundation for Embryonic Competence or pharmaceutical companies); and finally, internal funding from your local institution.

Most universities have a grants administration department that is responsible for managing grant applications and awards. When preparing a new application, be sure to notify your grant officer of any deadlines—otherwise, you may find yourself in a situation in which your portion of the application is complete, but the corresponding administrative work required to submit the grant on behalf of the institution cannot be completed by the submission deadline.

Typically, a project proposal, timeline, and itemized budget are required for application. Many funding sources likewise request an interim milestones report to ensure that the recipient is on-track and often a final presentation of findings. The funding source may also dictate how the awarded money may or may not be spent. For example, as described above, federal monies cannot be used for human embryo research. Other grants, particularly those from private sources, stipulate that

the award may only be used for direct, and not indirect, costs. Direct costs are those that are spent to perform the specific project, while indirect costs (otherwise known as facilities and administration, F&A) are those that are not and are used for overhead costs, such as general office equipment, laboratory maintenance and infrastructure, utilities, and so on. If a grant is subject to indirect costs, a percentage of the direct cost amount (as determined by the institution or funding agency) is added to the final budget line and earmarked for overhead, so is not available for research. These indirect cost rates are independently negotiated by institutions with the Department of Health and Human Services, and the rates can vary considerably from institution to institution [16]. Some private grants specifically forbid that the award can be applied to indirect costs, effectively ensuring that all money is used for the research project itself and not moved into general funds “to keep the lights on.”

Sometimes it is necessary to apply for multiple grants before being awarded funding. It is easy to become discouraged, but use any feedback from a rejection as a source for improving your project and next proposal. Continue to put your name in the hat; with time, thoughtful research design, and perhaps most importantly, identifying the most appropriate funding source, your efforts will pay off.

Executing the project

After so much preparation, performing the actual study may be the easiest, and hopefully, the most rewarding, part of your research. Clearly, it depends on the work you are doing. Most will be relieved, however, to see all of their efforts finally in motion once the first experiment is done or the first patient is recruited. The specifics of how you execute the research will be guided by your hypothesis and your study design, but will always be shaped by external pressures such as time and funding. Recognizing these external constraints allows you to address them proactively so you can focus on the research at hand.

Depending on the type of research, data may become available for analysis on a continuous basis, episodically at predetermined interim analyses (and only accessible to a Data Safety Monitoring Board) or at the conclusion of the study. Throughout this process, be sure to keep thorough records of your work and back up your data often. Once the final dataset is available, it will likely require data cleaning to ensure accuracy and to confirm outliers. In addition, if you are working with a biostatistician, compile a data dictionary that defines each variable and how it is coded in the dataset; this will be used for coding purposes and will ensure accuracy of the statistical analyses. Finally, review your study proposal one last time and confirm that all of the planned data are included in the dataset for analysis.

Publishing your work

Research is meant to be shared. This may occur at local research-in-progress meetings, regional professional organizations, or national or international conferences. All of these forums are aimed to critically evaluate the presented work and to offer suggestions for improvement or future study. I always consider feedback from the audience after a presentation as the first round of reviewer comments for a paper, and I make sure that all of these points are addressed in the final submission.

The success of a project may not only depend on the quality of the work but also how it is packaged into a story. Certainly, if the research is low quality or low impact, no amount of spin will change that; however, a good project may be made better with effective communication of a clear, take-home message.

One example of how to do this is to devise a memorable name for the project. When I first proposed “Ultra-low” O₂ to my mentor, she cringed, but with some explanation, ultimately came round. It was an accurate portrayal of the study, distinguished it from the conventional “low” O₂ moniker for 5%, sounded contemporary, and allowed people to immediately understand where this work fit within the literature. It further delineated a progression from atmospheric to low to ultra-low O₂, and anyone familiar with the antecedent work would immediately understand the motivation for the current study.

Following presentation at a conference, the next and final step for a project is to submit the work for publication. Again, incorporate feedback from meetings into the final revision of the paper in an attempt to anticipate reviewer comments. The choice of where to submit your work depends on the novelty and/or clinical significance of the findings, the intended audience (subspecialty vs. general medical interest), and its place within the existing literature. Your mentor often has the best perspective on the most appropriate journal for submission. Include key references in your citations, as reviewers are often familiar with the landmark studies in a particular field and expect to see them cited or they are the primary researchers who have conducted the previous important studies themselves.

Repeating and/or translating the findings

The hallmark of science is that it is reproducible. Your manuscript should provide sufficient detail such that an outside researcher in the same field should be able to replicate your methodologies. Data using human embryos in experimental conditions are limited, and so ultimately, larger multicenter studies or meta-analyses are often required to arrive at a definitive answer for a particular research question. As a follow-up of my preliminary findings, an ongoing laboratory study is currently being performed at Reproductive Medicine

Associates of New Jersey (ClinicalTrials.gov Identifier: NCT 02919384) in which we are assessing the effect of ultra-low O_2 on the development of sibling embryos used in clinical IVF. To be involved in this project from its inception, and to see its potential for clinical application, has been a rewarding experience. Ultimately, whether or not this will translate into a paradigm shift in how human embryos are cultured in vitro remains to be determined.

Concluding remarks

While the scientific method can be summarized by al-Haytham's three postulates: (1) state a problem based on observation, (2) criticize a hypothesis through experimentation, and (3) interpret the resulting data to form conclusions, scientific inquiry often does not occur so smoothly or in such a linear fashion. There will be starts and stops, detours, and U-turns along the way. Young investigators should rest assured, though, that meaningful work is possible with the help of an experienced mentor, a research question that is informed by both biology and the historical literature, and a thoughtful study proposal and design.

Everyone's research experience is unique. This is just one version of the story, and the specifics provided here may need to be tailored to your own work. What is important to recognize is that the purpose of all research is to cast light where there previously was none. Accordingly, make sure you are testing a novel, unexplored hypothesis. Do not be afraid to question something that is currently accepted dogma. You might be surprised by what you find.

References

1. Toomer GJ. Review: Ibn al-Haytham's Weg zur Physik. *Isis*. 1964;55(4):463–5.
2. Kaser DJ, Bogale B, Sarda V, Farland LV, Racowsky C. Randomized controlled trial of low (5%) vs. ultra-low (2%) oxygen

tension for in vitro development of human embryos. *Fertil Steril*. 2016;106(3):e4.

3. Davis OC, Nakamura J. A proposed model for an optimal mentoring environment for medical residents. *Acad Med*. 2010;85(6):1060–6.
4. Yedwab GA. The temperature, pH and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle. *Fertil Steril*. 1976;27(3):304–9.
5. Ottosen LDM, Hindkjaer J, Husth M, Petersen DE, Kirk J, Ingerslev HJ. Observations on intrauterine oxygen tension measured by fibre-optic microsensors. *Reprod Biomed Online*. 2006;13(3):380–5.
6. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil*. 1993;99(2):673–9.
7. Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol*. 1992;80(2):283–5.
8. Burton GJ, Jauniaux E, Watson A. Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd Collection revisited. *Am J Obstet Gynecol*. 1999;181(3):718–24.
9. Croxatto HB, Ortiz ME, Diaz S, Hess R, Balmaceda J, Croxatto HD. Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. *Am J Obstet Gynecol*. 1978;132:629–34.
10. Avendano S, Croxatto HD, Pereda J, Croxatto HB. A seven-cell human egg recovered from the oviduct. *Fertil Steril*. 1975;26(12):1167–72.
11. Hertig AT, Rock J, Adams EC, Mulligan WJ. On the preimplantation stages of the human ovum: a description of four normal and four abnormal specimens ranging from the second to the fifth day of development. *Contrib Embryol*. 1954;35:199–221.
12. Diaz S, Ortiz ME, Croxatto HB. Studies on the duration of ovum transport by the human oviduct. III. Time interval between the luteinizing hormone peak and recovery of ova by transcervical flushing of the uterus in normal women. *Am J Obstet Gynecol*. 1980;137(1):116–21.
13. Mastroianni Jr L, Jones R. oxygen tension within the rabbit fallopian tube. *J Reprod Fertil*. 1965;9:99–102.
14. Natri CO, Nobrega BN, Teixeira DM, Amorim J, Diniz LM, Barbosa MW, et al. Low versus atmospheric oxygen tension for embryo culture in assisted reproduction: a systematic review and meta-analysis. *Fertil Steril*. 2016;106(1):95–104.
15. Annas GJ. Resurrection of a stem-cell funding barrier: Dickey-Wicker in court. *N Engl J Med*. 2010;363(18):1687–9.
16. Ledford H. Keeping the lights on. *Nature*. 2014;515:326–9.