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Identifying the factors that influence the stability of the embryo culture system and the sensitivity of the Mouse Embryo Assay

The critical role of oil in the IVF lab

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Director de la tesi

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What will this day be like? I wonder
What will my future be? I wonder
It could be so exciting
To be out in the world, to be free
My heart should be wildly rejoicing
Oh, what's the matter with me?

I've always longed for adventure
To do the things I've never dared
Now here I'm facing adventure
Then why am I so scared?

Oh, I must stop these doubts
All these worries
If I don't, I just know I'll turn back
I must dream of the things I am seeking
I am seeking the courage I lack

The courage to serve them with reliance
Face my mistakes without defiance
Show them I'm worthy
And while I show them
I'll show me

So let them bring on all their problems
I'll do better than my best
I have confidence
They'll put me to the test
But I'll make them see
I have confidence in me

With each step I am more certain
Everything will turn out fine
I have confidence
The world can all be mine
They'll have to agree
I have confidence in me

<div align="right">

Julie Andrews, *The Sound of Music (1965)*

Lyrics by Richard Rogers

</div>

Agraïments

Ja fa sis anys que vaig decidir embarcar-me en aquesta aventura de fer UN DOCTORAT. Sis anys plens de dubtes, esforç, sacrifici i treball. Sis anys plens d'aprenentatge, satisfacció, orgull i metes superades. Sis anys compartits amb tantes persones, que es fa difícil donar-vos les gràcies.

Començaré donant-me les gràcies a mi mateix, o a l'Enric del passat. Per ser valent i atrevir-te a donar el pas, aquest i els altres. Sempre sense estar segur de res, acceptant-ho i avançant. Seguim així, tot es veu més fàcil des del davant.

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Enric

Abstract

The embryo culture system is composed by all those elements with the potential to impact embryo development. Its optimization is required to stabilize *in vitro* embryo culture and produce reliable and reproducible results in the IVF and QC laboratories. In this thesis we have shown how several components of the culture system, including but not limited to the mineral oil viscosity and volume, the relative humidity in the incubators, the culture dish design, and the type of medium and protein supplementation, are key elements to stabilize the culture conditions. In addition, we have developed a specialized Mouse Embryo Assay protocol to detect peroxide-related toxicity in mineral oil samples, and shown its effectiveness to screen multiple commercial oils used in human IVF. As a whole, the results obtained in this thesis could help to establish regulated and standardized methodologies for the future of QC testing in the IVF field.

Resum

El sistema de cultiu embrionari està compost per tots aquells elements amb el potencial d'afectar al desenvolupament embrionari. La seva optimització és necessària per estabilitzar el cultiu embrionari *in vitro* i generar resultats fiables i reproduïbles als laboratoris de FIV i Control de Qualitat. En aquesta tesi hem demostrat com diversos components del sistema de cultiu, inclosos però no limitats a la viscositat i el volum d'oli mineral, la humitat relativa a dins dels incubadors, el disseny de les plaques de cultiu, i el tipus de medi i suplement proteic, són elements essencials en l'estabilització de les condicions de cultiu. Així mateix, hem desenvolupat un protocol especialitzat de l'Assaig amb Embrions de Ratolí per detectar toxicitat causada per peròxids en mostres d'oli mineral, i hem confirmat la seva efectivitat pel cribratge de varis olis comercials utilitzats en FIV humana. En conjunt, els resultats presentats en aquesta tesi podrien esdevenir un primer pas cap a la regulació i estandardització de les metodologies utilitzades en el tests de Control de Qualitat aplicades al camp de la FIV.

Preface

Assisted reproduction is a highly relevant field in the current biomedical context, due to the increasing prevalence of infertility as well as recent social changes such as the delay of maternity or the reproductive regulation for homosexual couples and mono-parental families. While the technology, techniques and equipment applied to assisted reproduction and *in vitro* fertilization have experienced an astonishing modernization in the last decades, there is still much room for improvement, aiming to better the conditions during embryo culture in the IVF lab and, ultimately, clinical outcomes.

Despite extensive research showing the impact that culture conditions have on embryos and gametes during *in vitro* culture, scarce publications exist about which elements of the culture system are able to modulate them. In addition, even though Quality Control testing programs are essential to avoid the introduction of any embryo-toxic components into the culture system, there is a striking lack of regulation and standardization regarding testing methodologies. The introduction in this thesis describes the culture conditions that have a direct impact on gametes/embryos during *in vitro* culture, as well as the components of the culture system that have the power to modulate those conditions. Moreover, an overview on QC testing is given, specially focusing on the

Mouse Embryo Assay and the factors that may affect its sensibility.

The works collected in this thesis aimed to improve the stability of culture conditions in the IVF lab and, given the essential role that oil plays as a stabilizer of these conditions, we focused on improving its biosecurity through the development of a sensitive QC testing protocol for the screening of toxicity, and the characterization of the differences between various commercial oil brands.

Preliminary results have been presented in the following international meetings: European Society of Human Reproduction and Embryology (ESHRE 2015, 2018, 2020, 2021), American Society for Reproductive Medicine (ASRM 2021), Asociación para el Estudio de la Biología de la Reproducción (ASEBIR 2015, 2019), Kazakhstan Association of Reproductive Medicine (KARM 2018), Academy of Clinical Embryologists (ACE e-summit 2021), Arbeitsgemeinschaft Reproduktionsbiologie des Menschen (AGRBM 2021). Additionally, these findings have been collected in three original articles, accepted in peer-reviewed specialized journals (Human Reproduction and Reproductive BioMedicine Online).

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List of abbreviations

ART = Assisted Reproduction Technology

BFR = Blastocyst formation rate

BSA = Bovine Serum Albumin

CZB = Chatot Ziomek Bavister (culture medium)

DIPSO = 3-[N-bis(hydroxyethyl)arnino]-2-hydroxypropanesulfonic acid

GV = Germinal Vesicle

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HTF = Human Tubal Fluid

HSA = Human Serum Albumin

ICM = Inner cell mass

IR = Infrared

IUI = Intra Uterine Insemination

IVF = *In vitro* fertilization

KRB = Krebs-Ringer Bicarbonate (culture medium)

KSOM = K^+-supplemented Simplex Optimized Medium

L:P = Lactate/pyruvate ratio

LAL = Limulus amebocyte lysate

MEA = Mouse Embryo Assay

MI = Metaphase I

MII = Metaphase II

MOPS = 3-(N-morpholino)-propanesulphonic acid

PAO = Polyalphaolefins

pH$_e$ = Extracellular pH

pH$_i$ = Intracellular pH

POV = Peroxide value

QA = Quality Assurance

QC = Quality Control

SA:vol = Surface:volume ratio

SMA = Sperm Motility Assay

SOM = Simplex Optimized Medium

TC = Thermal Conductivity
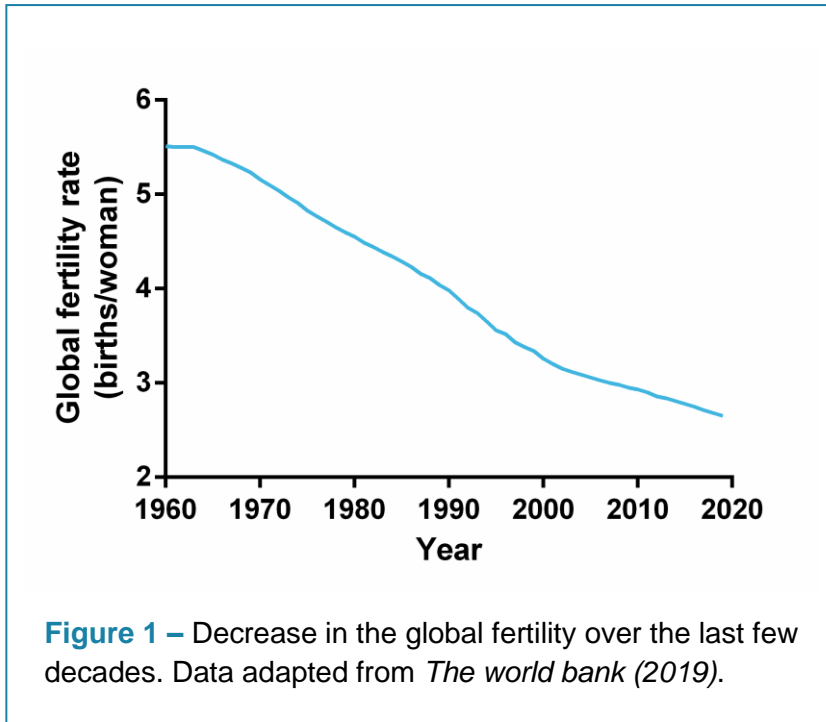
VOC = Volatile Organic Compound

INTRODUCTION

1. The need for assisted reproduction

1.1. Reproduction and infertility

Human reproduction, or the generation of offspring, is a natural and necessary desire for human beings. Surveys have shown that the majority of women and men, especially those residing in developed countries, long for parenthood at some point during their reproductive lives [1,2]. Since the discovery of the cellular requirements for human reproduction, a physical contact between an oocyte and a spermatozoon, in the XVIII-XIX centuries, scientists began to study the particulars of this biological phenomenon. Aiming to assist those couples with difficulties to achieve a successful pregnancy, and after many years of use in the agriculture industry, the first report of an artificial intra uterine insemination (IUI) on a woman was published in 1943 [3,4]: thus, Assisted Reproduction Technology (ART) in humans was born.

In the years elapsed since then, reproductive technologies have evolved as a consequence of the knowledge generated during decades of scientific research. Simultaneously, the modernization of the society has transformed the human reproductive needs and, consequently, so have changed the social requirements from the ARTs. Some prominent examples of such social changes include the demurral of pregnancy derived from the access of women to employment and the progressive law regulation of reproductive rights for same-sex couples. In addition, during the last decades an alarming

Figure 1 – Decrease in the global fertility over the last few decades. Data adapted from *The world bank (2019)*.

decrease in the human fertility has been observed worldwide [**Fig. 1**] [5–7].

Infertility's medical definition refers to the lack of conception after one year of unprotected heterosexual intercourse, and it is considered as a disease by the WHO [8]. It currently affects up to 15% of heterosexual couples, arising either from male or female-related factors, a combination of both, or an undefined idiopathic origin. Besides the underlying medical and/or environmental causes for infertility, the difficulty or impossibility of producing offspring has been identified as a prominent source of emotional and psychological distress [9–11].

1.2. *In vitro* fertilization

While in constant improvement and modernization, it would still take some more years, research and infrastructure, before our understanding allowed for the ARTs to evolve from the relatively simple IUI procedure to the much more complex *in vitro* fertilization (IVF) of human oocytes. Even after the birth of the first baby in the world produced by IVF in 1978 [12], many modifications have been applied in IVF laboratories aiming to improve insemination, assisted fertilization and handling techniques, the *in vitro* culture of human embryos and, in conclusion, achieving better clinical outcomes. Still an ever-evolving field, IVF in the modern ART clinic must be considered as a complex ontological system involving many precise actions (from the hormone treatment to the various procedures performed in the IVF lab) and professionals (embryologists, gynecologists, nurses, geneticists, counselors, etc.) [13]. Such complexity must be accounted for while keeping in mind the main goal of a successful IVF cycle: the live birth of a healthy baby.

Focusing on the IVF lab, a complex ecosystem within itself, many new techniques, consumables and pieces of equipment have been developed in the last decades specifically to be used in human IVF. As a whole, all these pieces form a puzzle known as the embryo culture system.

2. The embryo culture system

The embryo culture system is composed by all those elements that may have a direct or indirect impact on the embryos and gametes in culture. It comprises innumerable individual components that can interact between them, including but not limited to the soluble and insoluble environment; the temperature and gaseous inputs; the used consumables and pieces of equipment; the applied methodologies, standard

Figure 2 – Schematic representation of some of the different elements included in the embryo culture system.
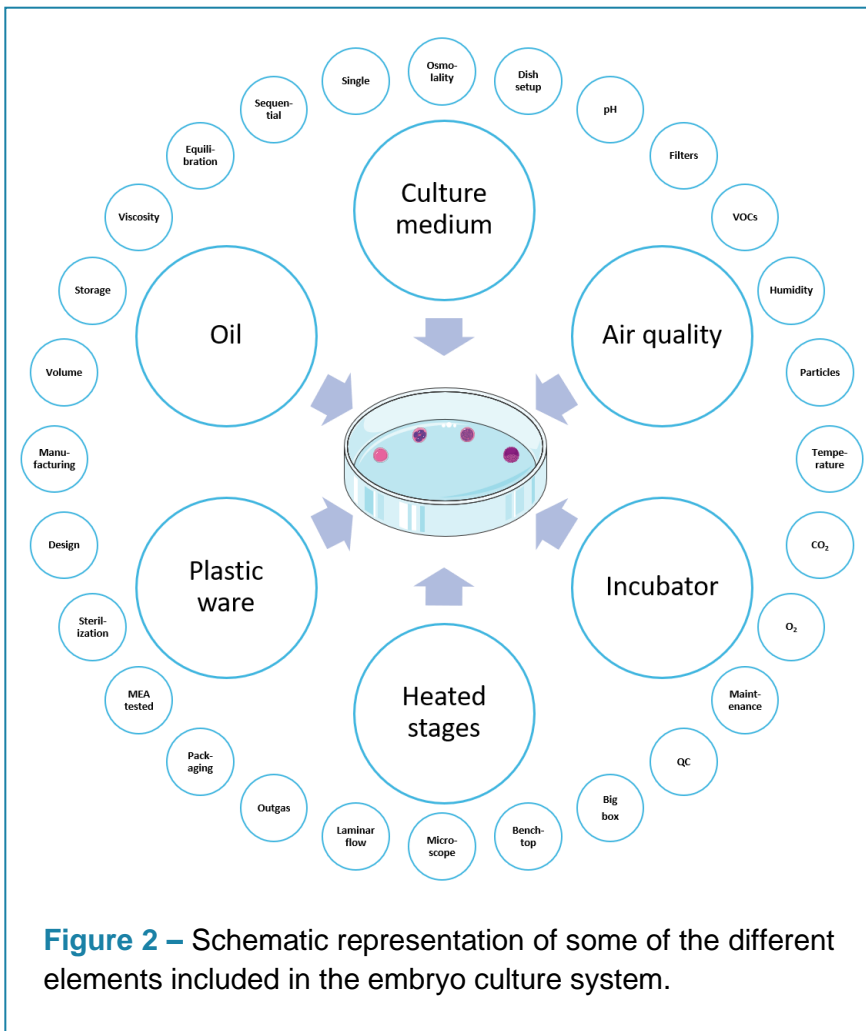
operating procedures and general workflow; etc. [**Fig. 2**]. An optimal culture system must be based on good Quality Control (QC) & Quality Assurance (QA) programs, which keep track of all the aforementioned elements and help to guarantee the stability of the critical culture conditions. Additionally, all elements comprising the culture system should be screened individually to ensure that they will not become a source of toxicity that could compromise embryo development. Only by minimizing the stress imposed on the embryos in the *in vitro* environment can the quality of the embryo culture be improved. Simple routine precautions taken before the implementation of any changes or the introduction of any new elements in the laboratory will allow the lab to optimize the variables in their culture system [14,15].

2.1. Culture conditions

Besides any contaminants that may be inadvertently introduced in the culture system, the quality of *in vitro* embryo culture mainly depends on four critical parameters: temperature, pH, osmolality and air quality. The stability of all of them and the understanding of their behavior within the culture system can minimize environmentally-induced embryonic stress and will be determinant to the success of an IVF cycle [15].

Temperature

Temperature is a highly variable parameter of the culture system as it can be affected by many elements in the IVF lab (incubators, warm surfaces, air conditioning and air handling unit, objective warmers, etc.), all of which need to be carefully calibrated to achieve and ensure an appropriate working temperature. As a prominent example, reports have described up to a 4°C difference between the expected and the measured temperature on some microscope warm stages [16,17]. Other elements, such as the dish type, drop size, oil overlay or incubator type, also play an important role in temperature stability [16,18].

The optimal temperature for the culture of human cells has been shown to be 37°C, with a noteworthy relevance during assisted fertilization techniques involving human oocytes. The meiotic spindles in the oocytes of the majority of mammals, including humans, are extremely sensitive to fluctuations in temperature, which inevitably occur during *in vitro* manipulation [19,20]. Reducing the magnitude of these fluctuations, however, is of the utmost importance considering the impact they can have on human oocytes.

The meiotic spindle of the human oocyte is composed by microtubules, cytoskeletal filamentous components that align, hold and segregate chromosomes during meiosis. Extensive published literature has shown that even temporal reductions in temperature can trigger the irreversible

depolymerization of the microtubules and result in an abnormal meiosis, and, similarly, overheating may also have a detrimental impact on the spindles. Thus, it is not surprising that temperature fluctuations are associated with the occurrence of aneuploidies or polyploidies in human oocytes and early cleavage embryos, even triggering apoptotic signals that can lead to blastomere fragmentation, irregular division and a heavily reduced implantation potential [19–23]. In addition, it has also been suggested that temperature may affect the metabolism of oocytes and preimplantation embryos in mammals [24]. By contrast, reaching and keeping a stable temperature around 37°C has been linked to increased fertilization rates, better embryo development and higher implantation and clinical pregnancy rates [17,22,25–27].

pH

pH is another of the environmental parameters which can become a potential stressor during embryo culture. As a measure of the concentration of protons, or hydrogen ions (H^+), in a solution and, thus, its acidity or alkalinity, pH is expressed in a logarithmic scale, meaning that seemingly small variations in its value can actually translate in big changes in the concentration of protons. This is an important consideration taking into account the dynamic nature of pH [14,15,28].

To understand the importance of keeping a stable pH in the culture system, it is necessary to contextualize it at the

cellular level. Several publications have tried to measure the intracellular pH (pH_i) in human oocytes, cleavage embryos and blastocysts, by using fluorescence pH markers. While initial tests reported a varying pH_i throughout development, between 7.0-7.4 [29], following a high-low-high pattern, newer studies showed that pH_i is maintained relatively stable at around 7.0-7.1 at least throughout the GV-MI-MII-zygote-cleavage stage development [14,30]. Human preimplantation embryos, similarly to other mammal species, have effective mechanisms to regulate their pH_i, a necessary cellular function to maintain their intracellular homeostasis. Intracellular alkalosis activates an HCO_3^-/Cl^- exchanger and, likewise, acidosis is regulated by a Na^+/H^+ antiporter and a second still-not-well-defined mechanism which also seems to require the presence of HCO_3^-. However, the required energy to activate these mechanisms can stress oocytes/embryos and has been shown to impact their glycolytic activity and oxidative metabolism; gene expression and epigenetics; cytoskeletal functions; mitochondria and chromosomal localization; embryo development and fragmentation; blastocyst formation, hatching and cell number; and fetus' size [14,30–38]. This supports the notion that, even though embryos have their own pH_i regulation mechanisms, efforts should be made in order to minimize the activation of such mechanisms and reduce the waste of cellular resources to optimize *in vitro* development [30]. In addition, extra care should be taken when working with denuded human oocytes, as their

pH_i-regulating mechanisms are not still functional [15]. The best way to do so seems to be through the external regulation of the medium's pH.

Extracellular pH (pH_e) has a direct impact on pH_i and so, theoretically, its value should be close to it (around 7.1). However, in order to account for the slight acidification of the medium caused by the cellular metabolic processes of the embryos in culture, 7.2 is a more commonly used threshold for pH_e. Media manufacturers typically describe an accepted range of 7.2-7.4; however, given the logarithmic scale of pH, this difference translates in more than a 60% difference in the concentration of H^+. Even though embryos have the capacity to develop within this 0.2 range, this does not mean that the quality of their development will be the same at any point of this range. Thus, it is encouraged to establish a target pH_e

Figure 3 – pH regulation in a bicarbonate-buffered medium in presence of CO_2. Adapted from *Swain (2010)*.
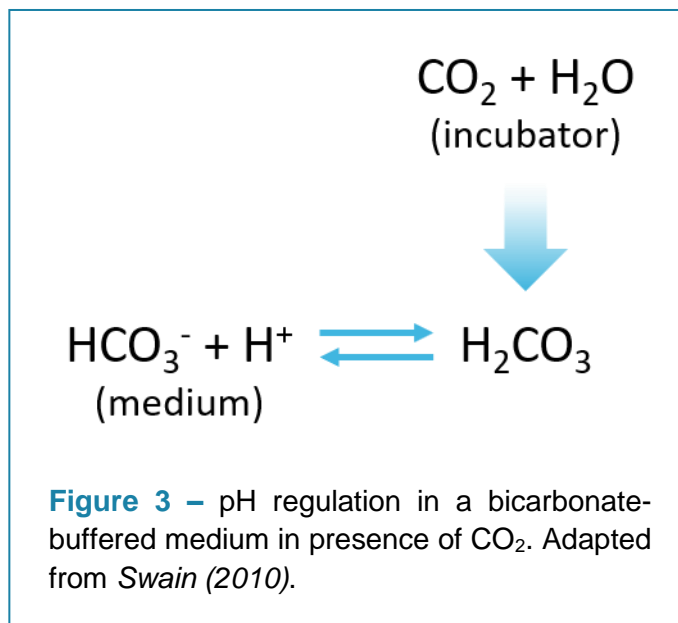
and keep its drifts within as narrow a range as possible, both during culture and handling in the IVF laboratory [14,29,39,40]. Evidently, the bigger the variations in pH_e, especially outside of the 7.2-7.4 range, the bigger the difference with the pH_i will be and the more deleterious effects the oocytes/embryos will suffer [14,41–45]. The strategy used to stabilize pH_e in the medium depends on its intended use, culture or manipulation, as the buffering capacity differs between both.

Buffers are components added to the culture medium that behave as weak acids/bases, in order to provide the medium with some resistance to pH changes via the acceptance/donation of H^+ ions [46]. The most typical buffer used in embryo culture medium is bicarbonate because of the balance it creates with carbonic acid (H_2CO_3) and the CO_2 present in the incubator atmosphere, which diffuses and dissolves in the medium [**Fig. 3**]. Since the concentration of bicarbonate in commercial media is set by the manufacturer, the easiest way to modulate pH_e in the IVF laboratory is through the regulation of the CO_2 concentration in the incubators, which follows an inverse correlation with pH. It should be kept in mind that other factors, such as the altitude of the laboratory, temperature, media evaporation, volume of media and oil and their surface area of contact, etc. can also influence this equilibrium and, consequently, the pH_e [14,15,28,46,47].

A different strategy must be applied to dampen pH_e shifts when handling oocytes/embryos outside of the incubator, where the concentration of atmospheric CO_2 is much reduced (less than 0.1%). In this environment, the use of media with bicarbonate as their only buffer can result in a rapid increase of their pH [31,39]. The most extended practice to avoid this is the use of media buffered instead with an organic zwitterionic component (containing an equal number of positive and negative-charged functional groups and able to act both as an acid or as a base), that can stabilize pH independently from the CO_2 gas input. This strategy has been shown to yield better embryo development [48–50]. While there are around 20 different "Good's" buffers with these characteristics [51,52], only those with an acid dissociation constant (typically expressed as its log, pK_a) similar to the desired pH can be a good fit to be used for IVF purposes. The two most commonly used buffers in commercial manipulation media are 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (known as HEPES) and 3-(N-morpholino)-propanesulphonic acid (MOPS), with a pK_a of 7.31 and 6.93 at 37ºC, respectively. Others, such as 3-[N-bis(hydroxyethyl)arnino]-2 hydroxyp-ropanesulfonic acid (DIPSO, pK_a of 7.35 at 37ºC), have also been proposed [39,46].

There are three main concerns derived from the use of these types of buffered-media:

1. Temperature has an important impact on the pK_a of the buffers and, consequently, on the pH_e of the medium, which increases as temperature decreases. This requires an especial consideration, since these media are commonly used for manipulation procedures outside of the incubator, where temperature is less stable.

2. The formulation of manipulation media commonly includes a much-reduced concentration of bicarbonate. This fact, added to the low concentration of CO_2 at room atmosphere, can impair the ability of the oocytes/embryos to regulate their pH_i since, as described previously, this regulation uses HCO_3^--dependent exchangers. Without the action of the membrane regulation mechanisms, the stabilization of the pH_i will depend solely on the avoidance of fluctuations of the pH_e.

3. Concerns have been raised about the potential toxicity or undesired effects caused by the organic zwitterionic buffers [53,54]. However, some publications in the last decade have demonstrated the security of the organic zwitterionic buffers at low concentrations, noting that the detrimental effects described in the past could have been caused by secondary alterations which were not considered in the original articles [39,46].

As described earlier, a rigorous thermal control should be applied to all equipment and procedures in the IVF lab, which should resolve the concerns from the first point. To bypass the other two observations, it has been proposed that a sufficient concentration of HCO_3^- could be added to manipulation media to allow the activation of pH_i regulation mechanisms, whilst the combination of two or three different buffers (e.g. HEPES, MOPS and/or DIPSO) would translate into a lower and safer concentration of each of them, with the additional advantage of a wider pK_a and increased pH modulation capacity [30,39,46].

Osmolality

Osmolality is a measure of the osmotic pressure of a solution, and another essential parameter of the culture system, due to its role in mammal cell volume regulation and embryo development [55–58]. Discussions and concerns regarding osmolality have incremented in recent years as a result of modern IVF practices, including the uninterrupted culture of embryos for up to seven days in benchtop and time-lapse incubators with a dry atmosphere. While these strategies present undeniable advantages regarding the reduced handling of the embryos and the stability of the other culture conditions, they increment the chances of osmolality being inadvertently altered by diverse external elements, such as the design of the culture dishes [59,60], the volume of the

medium microdroplets [61,62], and the use, volume and type of mineral oil [63,64].

Contrary to other parameters, osmolality always tends to increase accumulatively during culture, due to the evaporation of the water in the culture medium. As a consequence, embryos can become stressed by the osmotic shock, the modification of the original medium formulation (including the concentration of bicarbonate and the subsequent rise in pH_e), and the accumulation of toxins generated through embryonic metabolism, such as ammonium [65,66].

Using the murine model, several studies have determined the effects of osmotic stress induced by media with an osmolality higher than 300 mmol/kg. Even after just a temporal exposure to hyperosmotic media, they described an activation of stress kinase responses [67]; changes in the gene expression and epigenetics of the embryos [62,68]; an increased cell apoptotic index [69,70]; and reduced blastocyst formation rates and blastocyst cell numbers [71,72]. While the osmotic threshold in the human is not as well defined, various studies have hypothesized that it could be close to that of the mouse, due to the similarities in the physiological standards of the reproductive tracts between both species [73,74]. While the complete avoidance of media evaporation is not possible with the current technology and methodologies, there are several

strategies that allow its minimization and, thus, the reduction of hyperosmotic embryonic stress.

Firstly, some manufacturers have taken a double approach through the formulation of modern commercial media. By lowering their initial osmolality to hypoosmotic levels around 250-260 mmol/kg, hypertonicity of the media can be delayed and reduced, even assuming that some evaporation will occur anyway [68,75–77]. In addition, newer formulations have included some amino acids into the media, such as glycine and glutamine, which act as organic osmolytes and allow oocytes/embryos to regulate their intracellular osmotic pressure against changes in extracellular osmolality [78–83].

Secondly, keeping a humidity-saturated environment during culture, even in benchtop or time-lapse incubators (those that allow to work with a humidified atmosphere), is a key strategy to reduce evaporation and changes in osmolality [63,84,85].

Finally, covering media with oil, both during gamete/embryo culture and handling, has shown to be perhaps the most effective approach to reduce, but not to avoid, its evaporation [86–89]. As will be described later, other related factors, such as the thickness of the oil overlay and its viscosity, seem to affect the capacity of the oil to prevent evaporation [59,60,62,90,91].

Air quality

The quality of the air is one more critical feature that should be carefully considered due to its potential detrimental impact

on the quality of the culture system [92,93]. Particles and, especially, Volatile Organic Compounds (VOCs) are the most common air pollutants [94].

VOCs are hydrocarbon-based compounds present in the air that may originate from many different sources outside the lab (e.g. vehicles, industrial emissions, paint, glue, dissolvents, etc.), inside the lab (e.g. equipment, furniture, cleaning products, etc.), labware (e.g. polystyrene consumables, gas cylinders, etc.) and may even be brought in by staff members (e.g. perfumes, make-up, alcohol-based deodorants, etc.) [95–98]. While not all VOCs result toxic to gametes and embryos [93], multiple reports have thoroughly demonstrated that air quality, and its total VOC concentration, have a direct impact on embryo development, implantation rates and post-implantation fetal development [97,99,100].

Unfortunately, there are several reasons that have prevented the determination of a clear cut-off value for the accepted levels of individual or total VOCs in the IVF laboratory. Published literature on this subject is based mainly on retrospective analyses with limited power and varying characteristics [101–103]. However, at this point, with undeniable proof of the detriment caused by poor air quality, the design of a prospective randomized controlled trial to assess the precise effects of the VOCs on human embryos is no longer acceptable for ethical reasons. In addition, more than 300 different VOCs have been detected in a single IVF laboratory,

and the individual quantification and testing of each of them could prove insurmountable [93,96,104]. As a result, the closest the scientific community has come to a consensus is to consider a concentration of 0.2 ppm (or 200 ppb) of total VOCs as acceptable, since the lowest concentration reported as deleterious is 0.5 ppm, associated with an increased miscarriage rate [97,98,105].

Surprisingly, it has been described that the accumulated concentration of VOCs inside the IVF lab may be higher than that of unfiltered outdoors air, and may even vary through climate seasons and depending on humidity and temperature levels [96,97,99]. It seems clear that, in addition to the reduction of air particles through HEPA filters, active VOC monitoring and removal should be an integral element of every IVF laboratory. The best strategy to do so, apart from reducing the number of VOC-emitting elements within the lab, is to install efficient air-filtration systems with activated carbon and potassium permanganate ($KMnO_4$), ideally as a part of the centralized air filtration unit, or as portable freestanding units otherwise. These form a cloud of delocalized electrons that bind to the VOCs and trap them in the carbon [97,106,107]. Its use has been reported to benefit fertilization rates, embryo development and quality, pregnancy rates and miscarriage rates [98,108–112]. The use of UV light to promote the photocatalytic breakdown of the VOCs has also been proposed as a valid strategy to reduce their concentration,

although its efficiency compared to the filtration systems has not been well defined [113–115].

Apart from the VOCs in the air of the IVF laboratory, special consideration must be taken about the VOC concentration that might accumulate within the incubators. These VOCs will be in direct contact with the culture dishes during the majority of the *in vitro* cycle, and could accumulate specially in their oil overlay, affecting the medium and embryos underneath [116]. Several VOCs have been detected in the gas supply bottles, a fact that could result fatal specially in tri-gas incubators (those with a regulable concentration of both CO_2 and O_2) or incubators using a pre-mixed gas bottle, which require the majority of their internal atmosphere to be replaced with the gases provided by the bottles [96,97]. In this case, installing in-line filters in the gas input or, alternatively, placing internal recirculating filters inside of the incubators, is a good strategy to improve the quality of the air [65,85,111,112]. In addition, providing a large "sink" that can be replaced frequently, such as a humidification pan or dishes filled with oil, can also act as a limited measure against the accumulation of VOCs in the culture dishes [96].

A secondary source of multiple VOCs found inside of the incubators are the materials placed in them, mainly plastic consumables. Disposable dishes, tubes, flasks, etc., have been shown to emit high quantities of VOCs, particularly styrene. An appropriate outgassing period on a laminar flow

hood after opening the packages of all plasticware, and preferably doing so in a separate room, should help to reduce the introduction of VOCs both into the IVF laboratory and the incubators [96,117].

As Esteves summarized, the optimal definition for an IVF laboratory could be "a room in which the concentration of airborne particles and VOCs is controlled, and which is constructed and used in a manner to minimize the introduction, generation, and retention of particles and VOCs, and in which, temperature, humidity, and pressure are controlled." [111]

2.2. Incubators

All the aforementioned culture conditions may be potentially impacted by one of the most important pieces of equipment in the IVF laboratory: incubators. The principal task of an incubator is to provide a stable environment (temperature, gas, humidity and air quality) to achieve the optimal culture of gametes and embryos [85,118]. While, historically, regular cell culture incubators (or "big-box" incubators) have been used in IVF, even though they were not designed for such purposes, in the last decades new, much-smaller "benchtop" incubators have been developed to adapt to the specific requirements of the embryo culture *in vitro*. Even more recently, "time-lapse" incubators have incorporated microscopy objectives and cameras within their system, offering the chance to monitor embryo development continuously and without the need of

taking the culture dishes out of the incubator for their assessment in a traditional inverted microscope.

The heating methods used to regulate their inner temperature may vary between different incubators. "Big-box" and "small-box" incubators primarily use a water jacket or an air jacket heating system. Modern benchtop and time-lapse incubators rely on the direct contact of the culture dishes with a warm surface instead. Whilst temperature in the former two may take a long time to recover after repeated door openings, they are able to provide an increased retention of heat. By contrast, direct heat sources typically offer very fast temperature recovery rates, but they are prompt to cool quickly in case of a temporary power failure [85].

An accurate regulation and fast recovery of the CO_2 concentration within the incubator is also critical to maintain a stable pH in the medium throughout the culture. Thermal conductivity (TC) or infrared (IR) sensors are used to monitor CO_2; while TC sensors are dependent on temperature and humidity and, consequently, take a long time to stabilize after any disruption of the incubator inner environment, IR sensors are independent from other variables and offer a faster response and atmospheric recovery [85,119,120]. Similarly, galvanic or, more recently, faster zirconium sensors are used for O_2 regulation, as numerous publications have described that a reduced presence of oxygen during human gamete/embryo culture can yield better clinical outcomes [85,121–

[126]. Alternatively, some incubators insufflate gas from a pre-mixed cylinder which is already prepared with the desired concentration of CO_2 and O_2, a strategy that can help to reduce the recovery time of their inner atmospheric environment after a door opening.

As for humidity, which will have a direct impact on the potential evaporation of the medium during culture, there are also several approaches used by different incubators. Typically, incubators that work with high humidity do so by placing a water pan on their bottom surface and, as water evaporates, the incubator's internal atmosphere is saturated with vapor. However, since this strategy is prone to result in contamination, other options have been proposed by some manufacturers, such as the use of water bottles in the gas inlet or the installation of water-saturated porous stones in the incubator [14,85,127]. The differences in the levels of humidity resulting from each of these humidification systems should be noted [63].

As mentioned above, traditional incubators were not specifically designed for human IVF and, therefore, suffer important disruptions in their internal gaseous and thermal environment after each time their door is opened during routine work. Benchtop incubators offer much faster recovery times, while time-lapse incubators offer quasi-undisturbed conditions during culture. Nevertheless, while this improvement in the stability of the incubators' conditions has been thoroughly reported, not all groups have observed a significant change in

their clinical outcomes, probably because of the multiplex group of factors involved in the embryo culture system [16,118,128–133].

With all these options on the market, the selection of an incubator may be a complex task and, probably, no incubator will be perfect, as each laboratory's requirements can differ. Instead, a good quality control of the incubators might be a more important piece in the puzzle than the specific incubator model. External temperature, CO_2 and O_2 sensors can provide valuable information about the environmental dynamics within the incubator much more accurately than the incubator's own display. The periodic measurement of pH in all incubators is also an informative control of the culture stability [85,133]. In addition, proper incubator maintenance and management, including the use of separate culture and working incubators, and an appropriate distribution of patient samples between incubators to reduce their individual workload, have been shown to result in significantly improved blastocyst formation and morphological quality [134,135].

2.3. Culture medium

The immediate environment and main source of energy for oocytes/embryos under *in vitro* culture is the culture medium that surrounds them. Interestingly, commercial media used for human embryos today are only slight modifications of media originally designed to culture mouse embryos [58].

Krebs and Henseleit first developed a HCO_3^--buffered saline solution (KRB) in 1932 [136], which was later modified by Whitten in 1956, adding glucose and Bovine Serum Albumin (BSA) to it and creating the first culture medium able to support the development of cleavage-stage mouse embryos until the blastocyst stage [137,138]. In the following years a new medium, M16, would be developed, but it still would not support the complete *in vitro* development of mouse embryos from the zygote stage, which blocked after early cleavage in what was commonly named "the 2-cell block" [139]. Trying to overcome this issue, two new media were created: one developed by Chatot, Ziomek and Bavister (CZB) in 1989 [140], and a second one product of a composition-optimization process called Simplex Optimized Medium (SOM) [141]. The subsequent increment of the concentration of potassium resulted in the still commonly used K^+-supplemented SOM medium (KSOM) [79,142,143]. Whilst the main saline components in KSOM are quite similar to the original KRB or Whitten's media (mainly Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+} and SO_4^{2-}), the modification of their individual concentrations plus the introduction of EDTA, glutamine, and other amino acids, where the key factors in the development of a functional *in vitro* embryo culture medium [75,135,144–147].

The development of these media derived in the creation of the first medium specifically designed for human embryo culture, Human Tubal Fluid (HTF) [148], and, later, to the different commercial media currently on the market. The extension of the culture period to produce blastocysts *in vitro* resulted in two

contrasting approaches regarding culture medium. Single-step media follow the "let the embryo choose" principle and use a sole composition throughout all stages of embryo development [149]. By contrast, sequential media, which were developed in the nineties and especially promoted by Gardner and Lane [146,150], support the "back to nature" approach and propose the use of two or three media with slightly different compositions that mimic the changing embryonic environment found *in vivo* [151–153]. Many analyses and meta-analyses have been published comparing both types of medium; however, the lack of good study designs, conflicting results, and distinct methodologies have made it impossible to draw reliable conclusions on the appropriateness of one over the other [154–157].

Regardless of their approach, the composition of the human commercial media typically includes salts, protein, energy sources (pyruvate and lactate), amino acids and antibiotics. However, their exact composition varies between brands, a non-trivial fact considering that culture medium has been directly linked, in humans and other mammals, to modifications of embryo quality and metabolism; fertilization, implantation, pregnancy and live birth rates; and perinatal outcomes [158–167]. Unfortunately, while media manufacturers must disclose the components of their media, they typically do not share the precise concentration of each of them, complicating the proper study of each of their individual ingredients.

In recent years, Morbeck and colleagues analyzed the exact composition of several commercial media [58], with surprising differences found between brands. They detected significant variations in the number of mouse embryos able to develop to the blastocyst stage, which had been reported previously [168], and found that these differences were protein and oxygen-dependent. As a prominent example, the presence of lactate and pyruvate, the main source of energy for early embryos, as well as the ratio between their concentrations (L:P), play a critical role on embryonic metabolism, reductive-oxidative balance and, ultimately, may have an impact on embryo viability and even on the offspring's health [159,164,166,169–171]. Morbeck reported a surprisingly wide range in the L:P ratio of the analyzed media, between 8 and 126, showcasing the undisclosed disparity in the composition of some of the commercial media available for IVF. While it is clear that human embryos have the capacity to adapt to a variety of environments and, therefore, the ultimate medium composition may not exist, the long-term effects of the composition and concentrations of the currently-used media are still unknown [58].

2.4. Protein supplementation

A secondary required component of the embryos' *in vitro* environment is the protein utilized to supplement the culture medium with. Human serum was commonly used until human serum albumin (HSA) was described as an appropriate

replacement for media supplementation, and has since become the standard protein used in clinical IVF [172]. Although typically utilized at a working concentration of 5 mg/ml, some pre-supplemented "ready-to-use" media may contain a higher or lower concentration of protein and, likewise, some laboratories might choose to work with a particular concentration of their choice. In addition, some "complex" protein supplements have been developed which include other components besides HSA, such as α and β-globulins [173,174].

Unfortunately, protein supplements are poorly defined and have a noteworthy documented lot-to-lot variability [175–180], that can even affect the development, gene expression and viability of the embryos [178–184]. Whilst little is known about the source of this variation, it has been hypothesized that the traces of the stabilizers used as protein-protectors during the heated viral-inactivation of the serum, such as octanoic acid, may be a potential cause of fluctuations in the quality of the commercial protein supplements [180,184,185].

Similarly to culture media, Morbeck published a comparative analysis of several protein supplements [184], in which they considered protein as an "important source of inter and intra-clinic variation". In addition, they also detected an unexpected high concentration of amino acids in some of the analyzed complex supplements, which were not present in the simple albumin solutions [186]. These amino acids could tamper with the amino acid concentration present in the culture media as part of their original formula [187].

The use of protein and an increased presence of amino acids is not trivial. Some amino acids, especially free L-glutamine, may spontaneously break-down into ammonium [188]; similarly, the degradation of the protein used as a supplement can also lead to the formation of ammonium [177,184,189–193]. Several reports in the human have showed that a build-up of ammonium during culture negatively affects blastocyst formation, metabolism and gene expression [194–196]. As a consequence, the majority of manufacturers have substituted L-glutamine with a more stable dipeptide form (glycyl-glutamine or alanyl-glutamine), which has successfully reduced, but not completely avoided, the accumulation of ammonium [66,191,196–200]. Kleijkers and colleagues described that the build-up of ammonium as a result of the degradation of amino acids and proteins was increased during culture at 37ºC. Interestingly, they also found a low-progressive increase of ammonium in ready-to-use media (which already includes protein in their original formulation) while in storage at 2-8ºC, especially if it is stored for several weeks prior to its use. They were able to establish an inverse association between the time elapsed since the medium's production and the birthweight of the babies born after a successful IVF cycle [66,201,202].

2.5. Plasticware

The majority of the plasticware used in the IVF lab, including culture dishes, is made from polystyrene, a material obtained from crude oil through radical polymerization. Whilst all dishes

and other plasticware should be embryo-tested prior to their release onto the market, they are still known to emit VOCs which, as explained previously, may result harmful to gametes and embryos [93,96,110]. A useful strategy to reduce potential embryo-toxicity derived from plasticware is off-gassing it in a laminar flow hood for at least 48-72 hours before using it [203,204]. In addition, some sterilization processes (e.g. gamma irradiation) and packaging (e.g. ventilation paper strips) can also help to avoid plasticware-related embryo-toxicity.

The design of the plasticware, especially culture dishes, can also be of importance within the embryo culture system. Traditional Petri dishes typically present a plastic rim on their perimeter which slightly elevate their base to avoid suction. However, during the temperature-dependent techniques used in the IVF lab, the thin layer of air created between the dish's base and the heated surfaces has been shown to result in a steep loss of temperature. The recent development of culture dishes specifically designed for IVF has taken on this matter by removing the plastic rim and allowing the direct contact of their base with the heated surface [18]. On a similar note, time-lapse dishes present highly specialized designs which allow the perfect fit of each dish in their intended time-lapse incubator. The characteristics of these dishes, such as the number and volume of its culture wells or the volume of oil that it can hold, could also prove important and may require for other elements of the culture system to be adapted in order to ensure the stability of temperature, pH and osmolality.

Unfortunately, there are not many studies that have looked into the stability of the culture conditions on these specific dishes.

2.6. Heated stages

Heated surfaces are used in laminar flow hoods and inverted microscopes to keep the in-drop temperature as stable as possible during the handling of the dishes outside of the incubator in the IVF lab. Nonetheless, each lab should analyze and adjust the temperatures of their heated surfaces due to the generally observed differences between the set-point of the equipment and the actual surface or in-drop temperature. For example, if using the aforementioned classic Petri dishes with a plastic rim on their base, a higher temperature in the heated surfaces might be required than if using modern dishes with direct contact [18].

Special consideration must be taken when choosing the heated stage for the inverted microscope. Traditional glass stages allow the comfortable observation of the gametes and embryos without having to reposition the dish after moving the microscope's stage. By contrast, metal stages with a small hole on their central area require the dish to be repositioned when moving to the next drop or well in the culture/manipulation dish. However, since glass is a much worse temperature conductor than metal, it is not surprising to find bigger differences in temperature between areas of the heated stage in the former than in the latter. In addition, using peripheral stage heaters

that surround the central insert may help to reduce temperature fluctuations and improve its stability [**Fig. 4**].
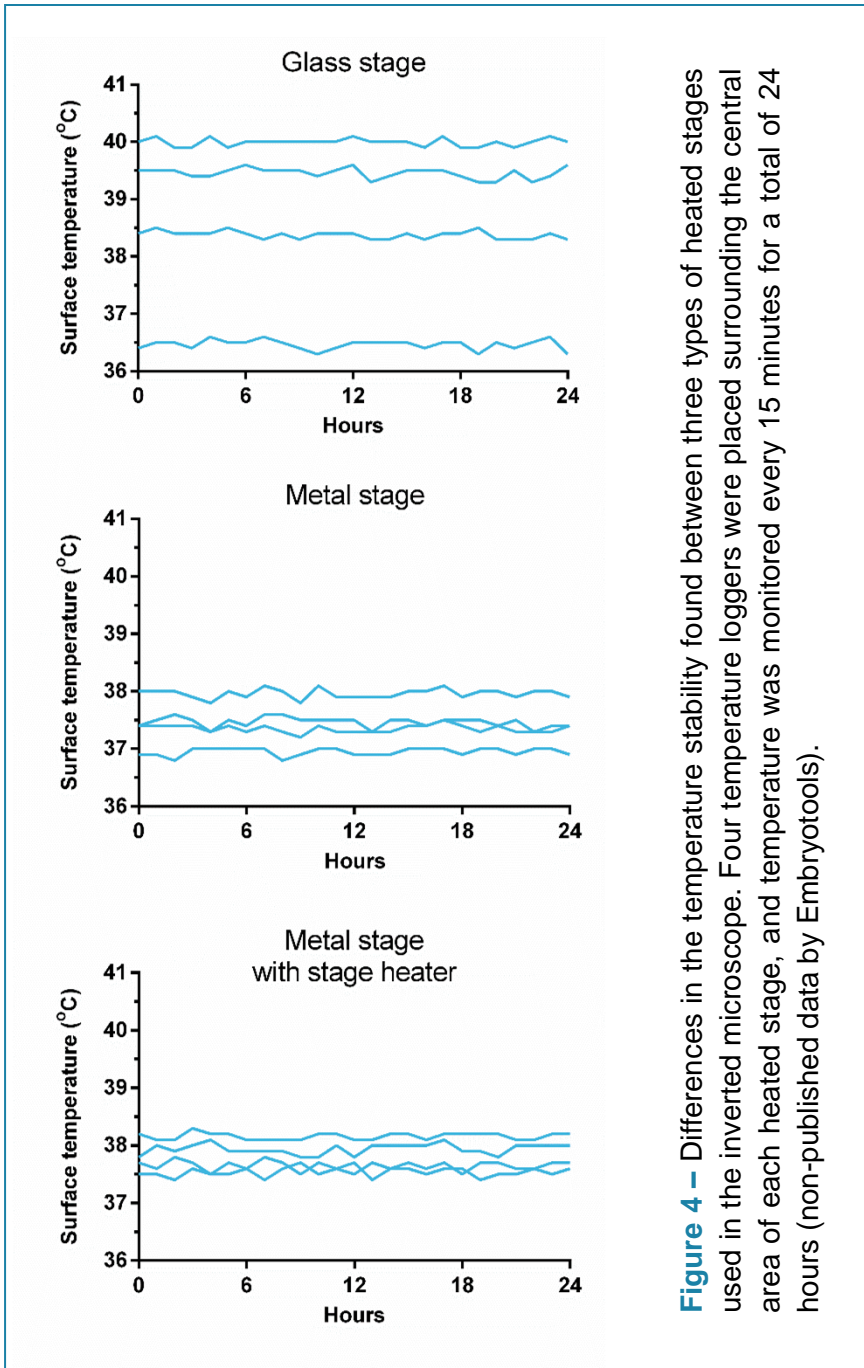
Figure 4 – Differences in the temperature stability found between three types of heated stages used in the inverted microscope. Four temperature loggers were placed surrounding the central area of each heated stage, and temperature was monitored every 15 minutes for a total of 24 hours (non-published data by Embryotools).

2.7. Oil in the IVF lab

The use of oil in the IVF lab was first described by Brinster in 1963 [55]. Since then, oil has become a standard consumable in IVF labs used as a cover to protect the water-based media underneath, allowing embryos to be cultured in medium micro-droplets. As previously mentioned, its properties as a protector against fluctuations of the culture conditions (temperature, pH and osmolality) have been widely reported [61,86,87,205–207].

Types of oil

By definition, oil is a non-polar chemical substance, which is found in a viscous liquid form at room temperature or when slightly warmed, and presents both lipophilic and hydrophobic characteristics. There are many different types of oil, mainly depending on their source of origin.

Organic oils have an animal or plant origin. These are often not completely refined and prone to oxidation, and are therefore not used for biomedical applications, which commonly rely instead on mineral oil [208].

Even though its name seems to indicate otherwise, mineral oil is also organic in its origin. The source of mineral oil is petroleum or crude oil which, in its own, originates from fossilized organic materials [209,210]. The production of mineral oil is relatively easy and cost-effective. However, being a derivate from petroleum, it requires extensive refinement and

purification steps before being considered apt for biomedical applications.

Recently, synthetic oils have been proposed as an alternative to mineral oils. These are commonly produced through the oligomerization of polyalphaolefins (PAO), but still require several additional purification steps. Since the molecular composition of synthetic oils is much more defined than in mineral oils, a theoretical higher resistance to oxidation was expected from the former compared to the latter. Surprisingly, some tests have shown an earlier onset of oxidation in PAO-derived synthetic oils compared to mineral oils [211]. This, added to the increased cost of production of synthetic oils, suggests that additional research is still required to evaluate any effective advantages of synthetic oils over mineral oils, which can tilt the balance regarding their popularity in IVF and other biomedical applications [208].

Mineral oil

Refined mineral oil is mainly composed by saturated hydrocarbon molecules, which may be straight or branched (known as alkanes or paraffins), or cyclic (alicyclics or naphthenes). The presence of unsaturated chains (alkenes or olefins) or aromatic cyclic hydrocarbons must be minimized through distillation, refinement and hydrogenation steps included in the oil's production, as they are highly reactive molecules that may result in the posterior oxidation of the oil [**Fig. 5**]. The oils used for biomedical applications are known

Figure 5 – Main types of hydrocarbons found in mineral oil. Adapted from *Prince (2010)*.
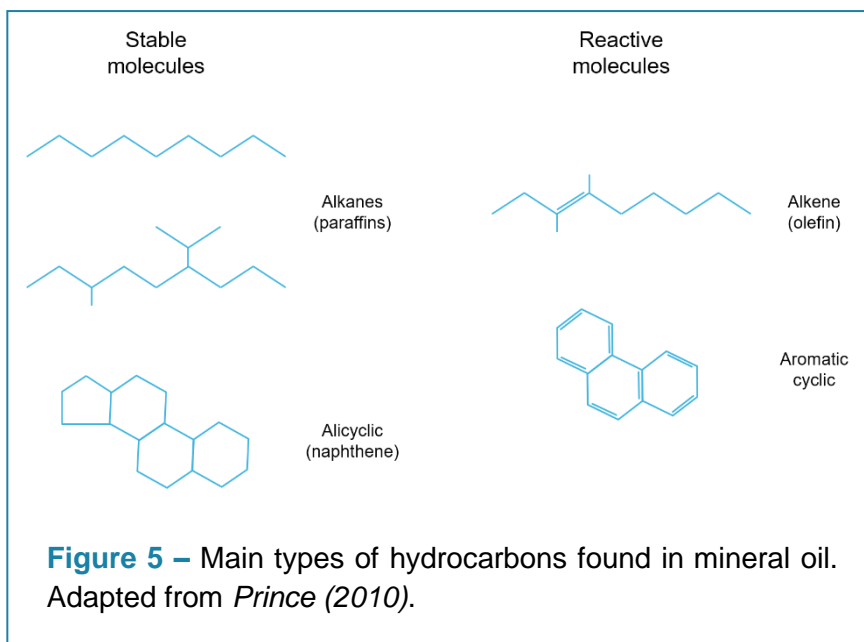
as "white oils" and are theoretically composed exclusively by saturated compounds, having removed any alkenes or aromatics with thorough hydrogenation. Of note, even when sharing the same molecular weight, the hydrocarbons' molecular structure and length result in markedly different physical and chemical characteristics, such as their viscosity and chemical stability [205,206,212,213].

Nowadays, several IVF manufacturers offer at least one type of oil in their product portfolio. The oil's characteristics differ greatly between some brands, both in their physico-chemical properties as in their quality. Manufacturers typically refer to their oils as "heavy" or "light" to indicate their high or low viscosity, respectively; however, there exists no clear threshold in viscosity to determine which oils should be

considered heavy or light. In addition, there is a lack of standardization in the nomenclature of the oils. While some manufacturers prefer to label their product as "Paraffin oil", suggesting an increased refinement and purity, there seems to be no difference in their chemical composition compared with other mineral oils on the IVF market. It has been suggested that this difference in nomenclature may instead originate from the different regulations from the US and European Pharmacopoeias, or even just from marketing strategies [62,65,214,215].

Within the context of the embryo culture system, mineral oil is deemed as one of its most heterogenous products, with marked differences between brands, lots and even bottles within the same production batch. In addition, while undoubtedly providing numerous advantages, oil can also become a source of toxicity caused by contaminants, such as Triton X-100 or zinc, or its own oxidation. It has, in fact, been reported as the source of toxicity detected in clinical IVF laboratories, after the conventional QC bioassays failed to identify its embryo-toxicity prior to the oil's release into the market [116,216–221]. Hence, it is essential to optimize the detection of toxicity in the oil, so that the many advantages derived from its use are not overshadowed by its potential detrimental effects.

Peroxides are the most common source of toxicity in mineral oil, and are generated by the oxidation of the reactive double-

bond carbons found in alkenes and aromatic hydrocarbons. While fully saturated molecules should remain inert, it has been shown that peroxides may build-up over time, and their generation is exacerbated when the oil is exposed to sunlight and/or high temperatures [217]. This means that even after undergoing successful peroxide value (POV) determination with sensitive specialized methodologies [222], and QC testing through sensitive bioassays, oil samples may become embryo-toxic at a later time, especially if their transportation and/or storage conditions are inadequate. Taking this information into account, there is once again a surprisingly wide variation in the packaging (plastic *vs.* glass bottles; transparent *vs.* opaque bottles; protective cardboard box), shelf life (from a few months up to two years) and storage recommendations (room temperature *vs.* refrigeration) between the oil produced by different manufacturers, especially considering that all of these factors may have a direct impact on the quality and toxicity of the oil [205,223–228].

Why and how to use oil

The introduction of oil into the culture system allowed for embryo culture to be carried out in micro-droplets of medium [55], which resulted in better clinical outcomes compared to previous methodologies of culture in big volumes of medium placed in tubes or dishes [229,230]. However, while an oil overlay will reduce the variations in temperature, pH and osmolality [**Fig. 6**], information about the effects that different working

methodologies (e.g. volume of oil used) or different oils' characteristics (e.g. diverse viscosity or density) may have on the oil's functionality is scarce.

The few publications that have tried to describe functional differences between oils have mainly focused on the analysis of media evaporation. These have described that increasing the thickness of the oil overlay (and, thus, the volume of oil used) that separates the media from the air is an important factor in reducing its evaporation [59,60,208], and, similarly, oils with a high viscosity seem to be more effective in preventing the media's evaporation [62,64,90]. The differential behavior of oils in the modulation of temperature and pH remains understudied and has not been described in detail yet; nonetheless, some data suggests that the stability of these variables would be influenced not only by the oil's own characteristics, but also by its interactions with other elements of the culture system, such as the design of the culture dishes, the humidity in the incubators or the temperature of the heated surfaces [16,18,60].

Figure 6 – Ten-minute analysis of the temperature, pH and osmolality in culture medium with and without an oil overlay, after overnight equilibration without a lid in a big-box incubator with humidified atmosphere (non-published data by Embryotools).
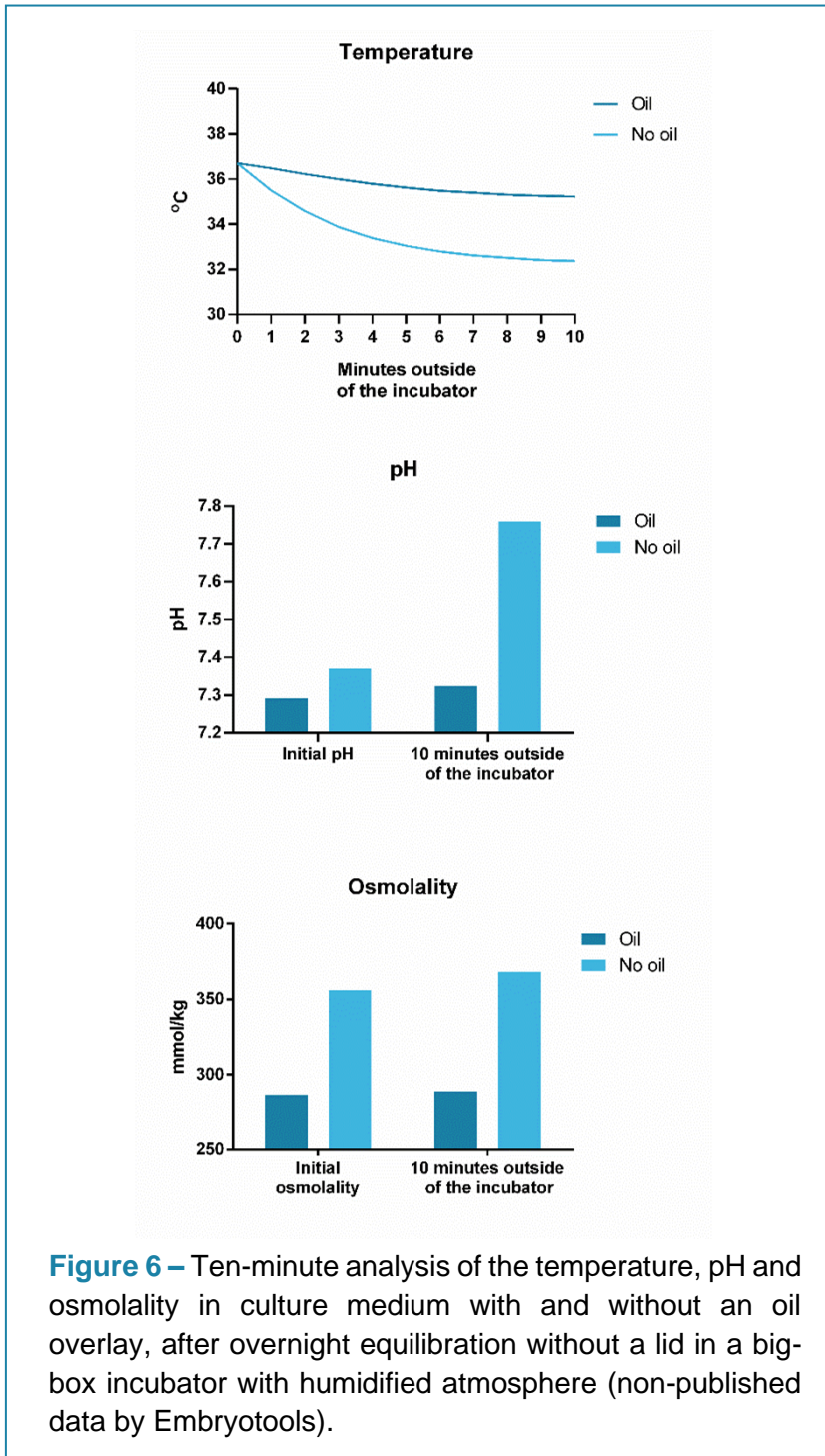
3. Quality Control testing

The biological samples in the human IVF lab, gametes and embryos, have a high biological, ethical and economical value, mainly due to their unique genetic characteristics, limited number and the difficulties associated to their collection. The conditions in the IVF laboratory must therefore be carefully optimized and, similarly, the introduction of any potentially detrimental equipment, reagent or consumable into the lab must be avoided. Thus, a robust QC testing system is of paramount importance in order to identify any embryo-toxic element prior to their introduction in the culture system.

The main three tests included in an IVF QC program are the Sperm Motility Assay (SMA), used to detect sperm-specific toxicity, the Limulus Amebocyte Lysate assay (LAL), to quantify the presence of endotoxins, and the Mouse Embryo Assay (MEA), which has been the current gold standard test to identify embryo-toxic samples during the last decades. Unfortunately, the lack of regulation and standardization on testing protocols arises concerns about the appropriate methodology to test each type of sample, the sensitivity of the bioassays and the reliance of their results [219,228,231,232]. In fact, insufficient sensitivity, associated with inappropriate testing protocols, has led to the recall of several consumables after their release to the market [233–236]. Increasing the sensitivity of the QC bioassays, in particular the MEA as the most common one, remains a top objective in the QC and IVF fields.

3.1. The Mouse Embryo Assay

The MEA briefly consists in exposing a group of mouse embryos to a test product (plasticware, media, oil, incubators, etc.), analyzing their development and comparing it to a control group, which is not exposed to the sample [**Fig.7**]. However, while most notified bodies require the products used in human clinical IVF to be "MEA tested", there exists no regulation on the methodologies nor the end-points concerning the MEA, and a surprising variability in the applied methodologies between testing centers has been reported [237].

In January of 2021, the United States Food and Drug Administration issued a document with their recommended guidelines to perform Mouse Embryo Assays [238]. While this is definitely a first step in the right direction regarding the regulation and standardization of the MEA, it offers only a set of non-mandatory recommendations, and it still lacks concreteness regarding the testing methodologies that should be used. As discussed below, these may have a huge impact on the sensitivity of the assay and, thus, its capacity to detect low and moderate levels of embryo-toxicity.

Mouse strain

Three types of mouse strains have been proposed to be used for toxicity screening through the Mouse Embryo Assay [239]:

1. Inbred strains, obtained through the consecutive breeding of brother x sister or parent x offspring mice,

for at least 20 generations. The individuals will present at that point less than 0.1% heterozygosity, and so they can be considered as genetically identical at all loci. These may help to reduce experimental variability and increase reproducibility.

2. Outbred strains, in which no two individuals share the same genotype. Breeding with close relatives is avoided to maintain the maximum heterozygosity in a random genetic population. These are often used for toxicology studies, to represent a wider range of genotypes and phenotypes.

3. Hybrid strains, the first generation from the breeding between two inbred strains.

While inbred strains are not typically used to perform MEAs, due to their limited genotypic range, there have been discussions since the beginning of human IVF and QC testing about the advantages or disadvantages comparing outbred and hybrid strains. While some groups have reported an increased sensitivity of the assay when using embryos from an outbred strain, such as CD-1 [224,228], these are prone to suffer from arrested development at the 2-cell stage, and generally present poor and unstable developmental rates.
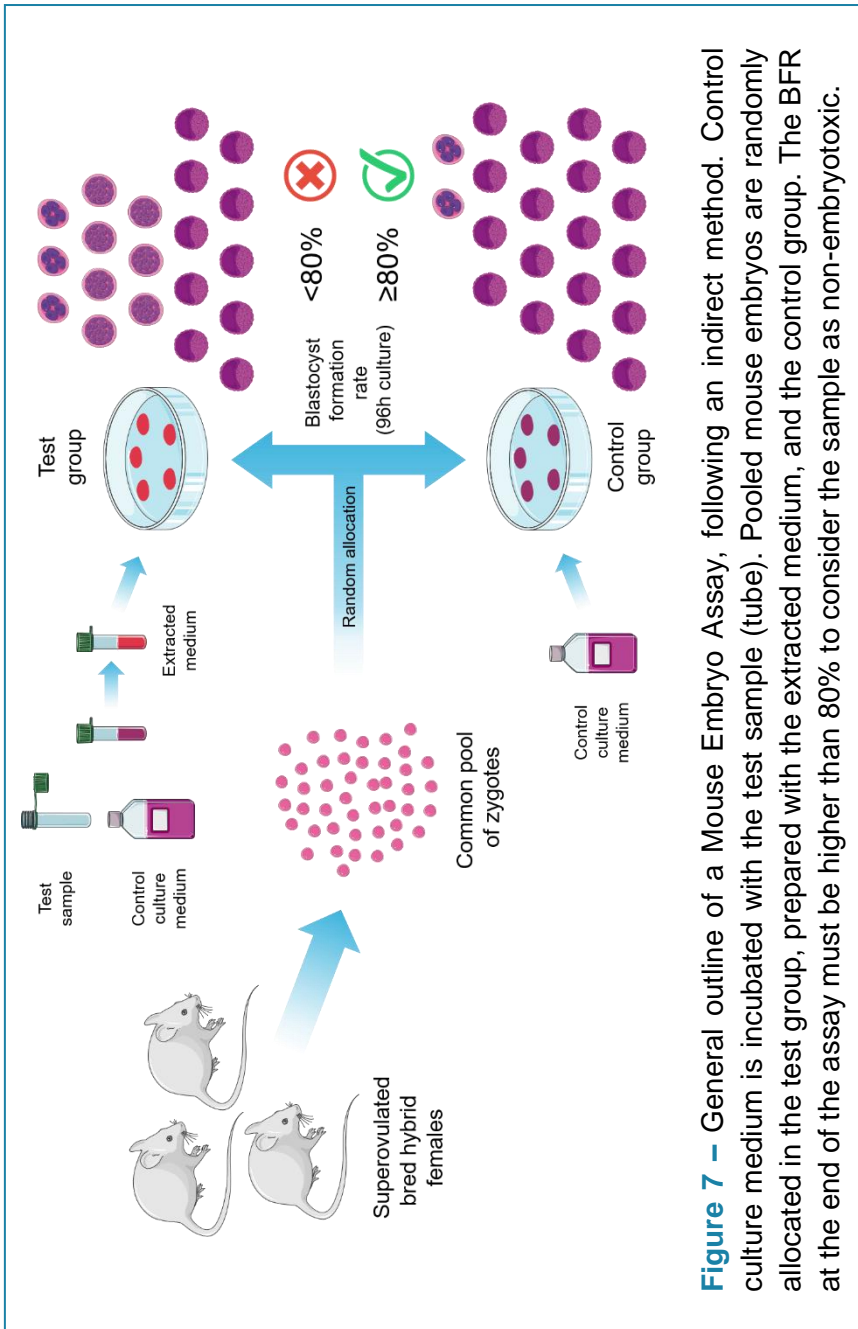
Figure 7 – General outline of a Mouse Embryo Assay, following an indirect method. Control culture medium is incubated with the test sample (tube). Pooled mouse embryos are randomly allocated in the test group, prepared with the extracted medium, and the control group. The BFR at the end of the assay must be higher than 80% to consider the sample as non-embryotoxic.

Instead, hybrid strains show a much more stable and reproducible superovulation response and embryo development, while still maintaining a good sensitivity to toxicity, and are currently the most common strains used for QC, as recommended by the FDA [227,240–242].

Starting embryo stage

Because of the historical 2-cell block phenomenon when trying to culture mouse zygotes *in vitro* in early-generation culture media [139], it was common practice to collect 2-cell embryos after *in vivo* fertilization and early cleavage. This strategy is still used by some groups to perform the MEAs starting at the 2-cell stage. While there is extensive bibliography describing a notorious depletion in the assay's sensitivity by following this method, instead of starting the assay with embryos at the 1-cell stage (zygotes) [224,227,232,235,236,241–244], the FDA's recommended guidelines still considered both approaches as valid [238].

Embryo density

Embryo density, defined as the number of embryos divided by the volume of culture medium, is one more variable that can affect the outcomes of the Mouse Embryo Assay. However, being a variable that depends on two different parameters, there are two scenarios worth considering:

- Culturing embryos in large groups is a strategy that may be thought of to reduce both the required time and

economical costs associated with the MEA. However, in the mouse, as in other mammals, it has been proven that group culture promotes embryo development, possibly due to an accumulation of autocrine and paracrine factors secreted by the embryos. This may reduce the sensitivity of the assay and potentially result in a false negative result [232,244,253,245–252]. While the FDA acknowledges in their guidelines the loss of sensitivity associated to embryo culture in groups, no advice is given regarding an appropriate embryo density.

- The volume of culture medium used in each micro-droplet takes special relevance in micro-well dishes or those dishes using a well-of-the-well system, as is usually the case for time-lapse dishes. It has been hypothesized that even the culture of a single embryo could also result in the build-up of autocrine factors, creating a micro-environment in the reduced droplet volume. Similarly to the culture in groups, this could prove counterproductive for the detection of toxicity [230,248,250].

Oxygen concentration

The concentration of oxygen in the Earth's atmosphere is roughly around 21%. Early studies showed that *in vitro* embryo culture was improved if the oxygen's concentration was reduced to 5%, both in terms of blastocyst formation and

the number of cells forming the blastocysts [254,255]. Recently, Kelley and Gardner showed that oxygen concentration and embryo density are interacting factors [247], and hypothesized that the impact of oxygen on the embryos' metabolism and the secretion of cytokines and embryotrophic factors into the media could result in the alteration of the microenvironment derived from a particular embryo density [256–259]. However, while the reduced concentration of oxygen is associated with an improved embryo development, further studies are needed to determine whether it would lead to a reduction of the MEA's sensitivity or, by contrast, it could improve the stabilization of the culture system's conditions which, in turn, would allow the modification of other parameters of the MEA that helped to increase its sensitivity even further.

Protein supplementation

Historically, protein-free culture medium has commonly been preferred for QC applications, as protein is thought to chelate some of the potential toxins present in the media and thus mask their detrimental effects [260,261]. However, in the last decades it has been suggested that HSA-based supplementation could, in particular cases, enhance the impact of some toxic components, such as peroxides in oil samples, by binding to them and easing their way through the embryos' zona pellucida [220].

End-point

Due to the lack of regulation by any notified body, the end-point of the Mouse Embryo Assay has traditionally been set, as a scientific consensus, at an 80% blastocyst formation rate (BFR) or, in other words, a minimum of 80% of the cultured mouse embryos must reach the expanded blastocyst stage or further by the end of the assay, in order to consider the test sample as not-embryo-toxic and suitable for clinical use. The duration of the assay is typically set at 96 hours (five days) when culture starts at the 1-cell stage, or 72 hours (four days) then starting with 2-cell embryos.

The main issue with the traditional BFR end-point is that the raw ratio of embryos developing to the expanded blastocyst stage does not account for the quality of those blastocysts, and is therefore not reliably associated with their viability. Consequently, low toxicity levels could impart sublethal damage on the embryos and traditional end-points would not be able to detect it [232]. Some modifications to the assay have been proposed as potential improvements for the interpretation of the MEA's results:

- **Extended MEA:** increasing the culture period up to 120 or even 144 hours could improve the sensitivity of the assay [231]. However, the validity of such an approach could be disputed if the expanded blastocyst is still used as the cut-off stage for valid embryo

scoring, as the additional culture time could allow bad quality blastocysts to expand [146].

- **Cell counts:** the fixation and staining of the blastocysts allows the determination of their total cell number, which may be considered as a quantitative measure of the blastocysts quality that is affected in a dose-dependent manner by the presence of toxins during culture [146,227,231,236]. A differential staining of the pluripotent cells in the inner cell mass (ICM) and the number of apoptotic cells could provide additional information on the embryos' quality [163,232].

- **Cell division timings and morphokinetics:** analyzing the different stages of embryo development throughout the culture period could prove more informative than simply relying on the embryo scoring at the end of the assay [236]. Morphokinetics, the study of the timings for each cell division and embryo stage, allowed by the introduction of time-lapse incubators in the IVF laboratories, is also being considered as a powerful tool to detect sub-lethal toxicity [187].

- **Embryo transfer:** the ideal methodology to test any product related with IVF would be to perform several series of embryo transfers after *in vitro* culture, as this is the protocol with the highest resemblance to a clinical IVF cycle. While this would allow to assess the

chronic effects manifested after transfer and birth, unfortunately such a detailed approach is typically very time-consuming and has too many associated costs to be used in routine QC testing. However, it remains a good option for the validation of new types of products, prior to their accreditation by the notified bodies or their release into the market [227,243].

Other considerations

Other factors that could play a role in the results of the Mouse Embryo Assay or its sensitivity are:

1. Using freshly collected or frozen-thawed zygotes. While the use of frozen embryos allows a better adjustment of the laboratory's daily needs and number of test groups, it might lead to a slightly decreased embryo development and altered developmental timings. This could prove misleading if the effects caused by the freezing-thawing procedure were instead attributed to the test sample.

2. The testing procedure for the MEA varies depending on the nature of the test sample and its intended use. A "direct" MEA implies a direct contact of the test sample with the embryos (e.g. when testing culture media, oil or dishes) and, by contrast, an "indirect" test consists in the previous exposure of the culture medium to the test sample, after which the medium is

extracted and used to culture the embryos for the duration of the assay. Some samples can be processed by any of the two methods, and it will depend on each laboratory's expertise and preferences to choose one or the other. The FDA recommends to apply a testing protocol similar to a worst-case-scenario when using the test sample in a clinical IVF setting [238].

3. Related to the previous point, the volume of medium used to perform a toxicity extraction is of paramount importance, since an excessively large volume of medium could result in the dilution of the potential toxicity, which could then go undetected.

4. *In vitro* fertilization in the mouse typically shows worst outcomes than when embryos are directly collected at the zygote stage after *in vivo* fertilization. However, for those samples specifically used for *in vitro* fertilization in the human, such as fertilization medium, this methodology could prove useful as it would provide with the closest protocol to the one intended during clinical use.

OBJECTIVES

The principal objective of this project was to better understand the performance of the embryo culture system, studying in-depth some of its parameters or components that play a direct role in the stability and quality of the culture system and, ultimately, in clinical outcomes. Three aims were set out, which translated into the publications presented in this thesis:

1. Stabilizing the culture conditions in a QC testing lab to heighten the reliance of the assays performed in it. We selected osmolality as the most unknown factor during culture (and, thus, the one with the widest room for improvement) and analyzed which components of the culture system had the biggest impact on its stability.

2. Increasing the sensitivity of the bioassays used to test IVF consumables. The Mouse Embryo Assay, or MEA, is the gold standard bioassay used in the ART field. By adjusting some elements of the culture system, we aimed to maximize the assay's sensitivity, increasing its capacity to detect embryo-toxicity. In particular, we focused on improving the testing of mineral oil, as it is considered a highly heterogenous product and a common source of toxicity in the embryo culture system.

3. Screening various commercial oils currently used in human IVF laboratories. By comparing several oil brands, we intended to correlate the oil's characteristics with its capacity to modulate critical parameters of the

culture system (namely, temperature, osmolality and pH). In addition, to assess whether there were differences in their overall quality, we used the knowledge generated in the two previous studies to strictly test and compare all the selected oils.

RESULTS & PUBLICATIONS

Chapter I

Mestres E, García-Jiménez M, Casals A, Cohen J, Acacio M, Villamar A, Matia-Algué Q, Calderón G, Costa-Borges N
Factors of the human embryo culture system that may affect media evaporation and osmolality

Factors of the human embryo culture system that may affect media evaporation and osmolality

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Study question: Which lab-related factors impact the culture system's capacity to maintain a stable osmolality during human embryo culture?

Summary answer: Incubator humidity, the volume of mineral oil, the type of culture media and the design of time-lapse dishes have been identified as important parameters that can cause an impact on media evaporation and consequently osmolality during culture.

What is known already: Culture medium is a critical component in human embryo culture. Minimizing its evaporation during culture is an adequate strategy to stabilize osmolality and, as a result, improving culture conditions and clinical outcomes.

Study design, size, duration: The studied variables included media composition and supplementation; volume of mineral oil; incubator humidification; and the type of dish and incubator used. Additionally, six time-lapse dish models were compared in their ability to prevent evaporation.

Participants/materials, setting, methods: Dishes were incubated in parallel to analyze osmolality during culture between groups: KSOM versus HTF medium; protein versus no protein supplementation; dry versus humid atmosphere; high versus low volume of mineral oil. Additionally, media evaporation was compared between six models of time-lapse dishes with distinct designs, cultured in a joint incubator. Two of them were retested in their corresponding incubator to analyze the dish-incubator fit. Daily osmolality measurements were compared between groups. Linear regression was performed to analyze evaporation rates.

Main results and the role of chance: Protein supplementation did not significantly affect evaporation. Contrarily, humidity levels inside the incubators, the volume of mineral oil, and the type of culture media, played an important role in osmolality stabilization. The design of time-lapse dishes and their recommended preparation protocol heavily influenced their evaporation rates, which were further altered by each incubator's characteristics. Media with initially high osmolalities had a bigger risk of reaching hypertonic levels during culture.

Limitations, reasons for caution: While numerous, the studied variables are limited and therefore other factors could play a role in osmolality dynamics, as well. Incontrollable atmospheric factors could also result in some variation in the observed results between different centers and laboratories.

Wider implications of the findings: Published literature has extensively described how hypertonic media may impair embryo development and negatively affect clinical outcomes; therefore, maintaining a stable osmolality during culture should be considered essential. This work is of interest both for embryologists when analyzing their culture system and methodologies, as well as manufacturers in charge of designing IVF consumables.

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Introduction

Culture medium, a critical component of an *in vitro* culture system, is the substrate in which gametes and embryos can be cultured for prolonged periods of time. In particular, media osmolality, a measure of the osmotic pressure of a solution, has been shown to be a key factor in mammal cell volume regulation and embryo development (Brinster, 1963; Miyoshi *et al.*, 1994; Liu and Foote, 1996; Morbeck *et al.*, 2014). Unfortunately, osmolality may inadvertently be affected by many factors in the lab, such as dish preparation and microdroplet volume (Swain *et al.*, 2012; Yumoto *et al.*, 2019), use of mineral oil (Holmes and Swain, 2018; Yumoto *et al.*, 2018), design of the dish (Carpenter *et al.*, 2018; Swain *et al.*, 2018), or humidity in incubators (Swain *et al.*, 2016). Additionally, due to the development of new benchtop and time-lapse incubators, generally lacking atmosphere humidification, and the popularity of uninterrupted embryo culture systems based on using single-step media, concern has arisen regarding the osmolality changes that may derive from this practice. Uninterrupted culture systems offer the advantage of reducing handling and increasing the stability of the culture; however, as culture periods lengthen, the osmolality in the medium rises accumulatively as well (Swain, 2019). In a dry atmosphere, significant evaporation can in some cases be detected as early as after one day of incubation. On the contrary, incubation in humid atmospheres, although possibly linked to a higher chance of contamination,

may prevent this (Geraghty *et al.*, 2014; Swain, 2014; Holmes and Swain, 2018).

As a consequence of water evaporation, the initial formulation of embryo culture medium may change, resulting in higher concentrations of all its components, increased pH (due to the elevated concentration of bicarbonate) and the possible accumulation of toxic elements, such as ammonium derived from amino acids break-down (Kleijkers *et al.*, 2016). It is important to identify all the variables of the culture system that may contribute to medium evaporation to prevent possible adverse consequences on embryo development. In the mouse model, hyperosmotic stress induced by media of >300 mOsm has been associated to low blastocyst formation rates (Hay-Schmidt, 1993; Dawson and Baltz, 1997), low cell number, high apoptotic indexes (Xie *et al.*, 2007; Bell *et al.*, 2009), and altered epigenetics and gene expression (Wang *et al.*, 2011; Yumoto *et al.*, 2019). Even temporal exposure to high osmolalities may be enough to trigger stress kinase responses and induce physiological changes in the embryos (Davidson *et al.*, 1988). Thus, appropriate precautions should be taken to minimize osmolality changes in all procedures performed in the lab (Davidson *et al.*, 1988; Van Winkle *et al.*, 1994; Bell *et al.*, 2009).

The use of a mineral oil overlay throughout embryo culture and manipulation can reduce medium evaporation (Mori *et al.*, 2010; Olds *et al.*, 2015). However, certain characteristics of oil

have been shown to have a correlation with its capacity to reduce osmolality changes, for example the thickness of the oil overlay (Carpenter *et al.*, 2018; Swain *et al.*, 2018), its viscosity (Swain, 2018; Yumoto *et al.*, 2019) and its pre-humidification (Swain *et al.*, 2016; Yumoto *et al.*, 2019). Importantly, oil does not completely avoid evaporation and, even if used appropriately, some increment in osmolality may still occur (Iwata *et al.*, 2016; Fawzy *et al.*, 2017).

The composition of culture media is another factor that may play an important role in osmolality stability. Firstly, commercial media have tended to reduce their initial osmolality to hypotonic levels when compared to those in the reproductive tract of mammals. However, embryo requirements *in vivo* differ from those in an *in vitro* setting and, consequently, modern formulations do not necessarily replicate the natural environment. A lower starting osmolality will delay hypertonicity if some evaporation occurs (Lawitts and Biggers, 1991; Collins and Baltz, 1999; Wang *et al.*, 2011; Baltz, 2012). Secondly, the addition of certain amino acids, such as glutamine and glycine, helps to protect embryos against osmotic stress. Their role as organic osmolytes allows embryos to regulate their intracellular osmotic pressure in hypertonic media (Arakawa and Timasheff, 1985; Van Winkle *et al.*, 1990; Biggers *et al.*, 1993; Kwon and Handler, 1995). On a similar note, the emulsification properties of albumin and some amino acids should also be taken into consideration as

a possible factor in osmolality instability (Haque and Kinsella, 1988; Yiase, 2015).

The aim of this study was to assess how different lab-related variables, such as media composition and protein supplementation, the use of mineral oil, atmosphere humidity, culture dish design and incubator type impact the osmolality of the culture system. In addition, osmolality changes were also evaluated in several time-lapse dishes with different designs and preparation protocols.

Materials and methods

Unless otherwise specified, 35 mm Petri dishes were prepared following the wash-drop method (Swain *et al.*, 2012). Briefly, nine 5 µl microdrops of medium (EmbryoMax® KSOM, Millipore, USA) supplemented with 5 mg/ml human serum albumin (FertiCult HSA, FertiPro, Belgium) were placed onto the dish and immediately covered with 4 ml of low-viscosity light mineral oil (Sydney IVF Culture Oil, COOK Medical, USA). The drops were then removed and replaced with 20 µl of the same medium. For each experiment, dishes were placed in the same incubator and taken out one at a time for osmolality assessment. Readings were performed in triplicate on three different droplets per day and replica, using a last-generation vapor pressure osmometer (Vapro5600®, Wescor, USA; accuracy ± 1%). Outliers of more than ± 5 mmol/kg compared to the rest of readings for that group/day/replica were discarded. The osmometer was calibrated daily prior to starting

the experiments, and readings were always performed at the same time of the day.

Room temperature and relative humidity were controlled with a continuous monitoring system (Octax Log&Guard™, Vitrolife, Germany) and were stable at 23.5ºC ± 0.8 and humidity of 44.0% ± 13.4 (mean ± SD) throughout the experimental period.

Experiment 1: the role of amino acids and albumin

The effect of amino acids on osmolality was assessed by incubating in parallel, in a dry benchtop incubator, a group of culture dishes prepared with a basic saline medium without amino acids (Human Tubal Fluid [HTF], Fujifilm Irvine Scientific, USA) and a second group with control KSOM medium, containing both essential and nonessential amino acids. Only commercially available media were used. To study the impact of protein presence, both media were tested without any protein supplementation and with 20 mg/ml HSA. Measurements were taken daily from 0 to 168 hours (equivalent to day 7 of embryo culture).

Experiment 2: dry versus humid incubation

Two benchtop incubators were selected due to their optional chamber humidification (MINI MN-2, Astec, Japan; CUBE AD-3100, Astec, Japan). Dishes, prepared with KSOM medium (+5mg/ml HSA) and overlaid with 4 ml of light oil, were cultured in parallel in a dry and in a humidified chamber, and osmolality

was compared between them on a daily basis from 0 to 168 hours (day 7). The relative humidity inside the incubators was measured continuously every minute for 24 hours using Bluetooth wireless portable hygrometers (essentim GmbH, Germany; accuracy ±3%).

Experiment 3: oil volume in Petri dishes

Dishes were prepared with medium microdroplets overlaid with the minimum amount of oil required to completely cover them, which was found to be 2.7 ml, and compared with the 4 ml control group. Both groups were cultured in the same dry benchtop incubator, and osmolality assessed daily between 0 and 168 hours (day 7).

Experiment 4: use of oil in well-dishes

The setup for these experiments was designed to represent common methodologies used during lab procedures such as oocyte pick-up or embryo transfer. Three wells of a 5-well type dish (Vitrolife, Sweden) were prepared with 700 µl of culture medium and immediately overlaid with 1) no oil (control); 2) 300 µl of oil; 3) 700 µl of oil. In a second set of experiments, the same type of dish was also used to evaluate osmolality in wells prepared with 20 µl microdroplets of medium overlaid with 1) 700 µl of oil; 2) 1300 µl of oil. Osmolality was measured in all groups after 16 hours of overnight culture in an incubator with humidified atmosphere (Heracell 150i, Thermo Scientific, USA). Each dish was kept for an additional 5 minutes on a

heated surface (37.5ºC) in a laminar flow hood, after which osmolality was measured again.

Experiment 5: comparison of time-lapse dishes

A total of six different models of commercially available time-lapse dishes, each of them with a unique design, were tested, including: Primo Vision® (Vitrolife, Sweden), EmbryoSlide® (Vitrolife, Sweden), EmbryoSlide+® (Vitrolife, Sweden), 38Special GPS® (CooperSurgical, USA), Geri® (Genea Biomedx, Australia) and CultureCoin® (Esco Medical, Denmark). Each type of dish was prepared according to their own manufacturer's instructions. All dishes were cultured in parallel in the same dry benchtop incubator. Osmolality was measured immediately after preparation and after 72, 120 and 168 hours of culture.

Experiment 6: comparison of time-lapse incubators

Two of time-lapse dishes that had been previously tested in experiment 5 were re-studied again, this time using the time-lapse incubator they are designed for. In these cases, results were compared with those of the previous experiment to determine the potential role of the dish-incubator fit and atmosphere on medium evaporation.

Statistics

Statistical analyzes were performed with GraphPad Prism 6 software (GraphPad Software, USA) and, unless otherwise stated, statistical significance was set at 5% (alpha = 0.05).

Pairs of parametrical datasets were compared with unpaired t-tests. Kruskal-Wallis analysis was applied in group tests between non-parametric datasets, followed by Dunn's post-hoc method for multiple comparison and p-value adjustment. An Analysis of Variance was performed in group analyses of parametric datasets with Tukey procedure for multiple comparisons. When studying evaporation rates, a linear regression was performed and the slopes analyzed for statistical divergence between groups.

Results

Experiment 1: The role of amino acids and albumin

Four groups were included in this experiment: KSOM 0 mg/ml HSA, KSOM 20 mg/ml HSA, HTF 0 mg/ml HSA and HTF 20 mg/ml HSA. For each group, osmolality was first compared between the medium aliquot used on each replicate and the microdrops at 0 h (immediately after dish setup) with non-significant differences found between samples, hence confirming that evaporation did not occur during dish preparation. The osmolalities at 0 h for each group, expressed as mean ± SD, were 252.8 ± 4.4, 256.3 ± 3.4, 287.4 ± 4.8 and 280.7 ± 8.3 mmol/kg, respectively; after 168 h in culture, a rise of osmolality was observed in all groups: 304.4 ± 4.0, 308.3 ± 3.7, 326.6 ± 3.9 and 325.8 ± 3.7 mmol/kg, respectively. In the groups with KSOM medium, regardless of the presence of protein, a statistically significant increase in osmolality in relation to time 0 was first detected after 96 hours of culture

(p=0.0077 and p=0.0097 with 0 and 20 mg/ml HSA, respectively), whilst no statistically significant differences were found in the groups with HTF until the 120-hour time point (p=0.0031 and p=0.0023).

A linear regression was performed to represent the evaporation rate throughout the whole experiment and compare the slopes between groups (**Figure 1A**). Regardless of the medium type, no differences were found when comparing groups with and without protein. However, evaporation rates were significantly different (**Figure 1B**) between KSOM and HTF media (p<0.0001 in groups without protein, p=0.007 with protein).

Experiment 2: Dry versus humid incubation

For the rest of the experiments, only KSOM medium supplemented with 5 mg/ml HSA was used. In this test, two distinct incubators were used with and without a water reservoir, resulting in four testing groups: CUBE humid, CUBE dry, MN-2 humid and MN-2 dry. Respectively, the initial osmolality (mean ± SD) for each of them was 251.8 ± 6.6, 254.8 ± 9.4, 252.6 ± 1.3 and 252.3 ± 2.1 mmol/kg; the final values after 168h of culture were 271.1 ± 4.7, 296.4 ± 3.7, 257.9 ± 4.6 and 301.2 ± 5.7 mmol/kg (**Figure 1C**). Significant differences in osmolality between humid and dry culture were quickly detected, after only 24 h in the CUBE (p=0.03) and 48 h in the MN-2 incubator (p<0.0001). Indeed, when analyzing the linear regression for each group, evaporation rates were

significantly divergent between humid and dry incubation (p<0.0001; **Figure 1D**). Interestingly, a difference was also found when comparing both incubators as a dry system (p=0.02) and as a humid system (p<0.0001); this was consistent with the analysis of the relative humidity inside each of the incubators, which revealed statistical differences (p<0.0001) between both dry incubators (mean ± SD of 32.5% ± 1.2 and 20.5% ± 2.5 for the CUBE and MN-2, respectively), as well as between both humid incubators (79.1% ± 0.4 in the CUBE, 99.8% ± 0.7 in the MN-2; **Figure 1E**).

Figure 1 – Osmolality changes according to media and relative humidity. Daily osmolality and linear regression comparing HTF and KSOM culture media, with and without protein supplementation (A), as well as their compared evaporation rates during culture in a dry benchtop incubator (B). Differences in daily osmolality measurements (C) and evaporation rates (D) depending on the relative humidity inside each of the tested incubators (E).
Significance is represented as *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

Experiment 3: Oil volume in Petri dishes

Two groups were studied to compare the use of 2.7 versus 4 ml of mineral oil. After 168 hours of culture, osmolality had risen significantly more in the group with the 2.7 ml oil overlay (mean ± SD of 307.8 ± 3.1 mmol/kg) than in the group with 4
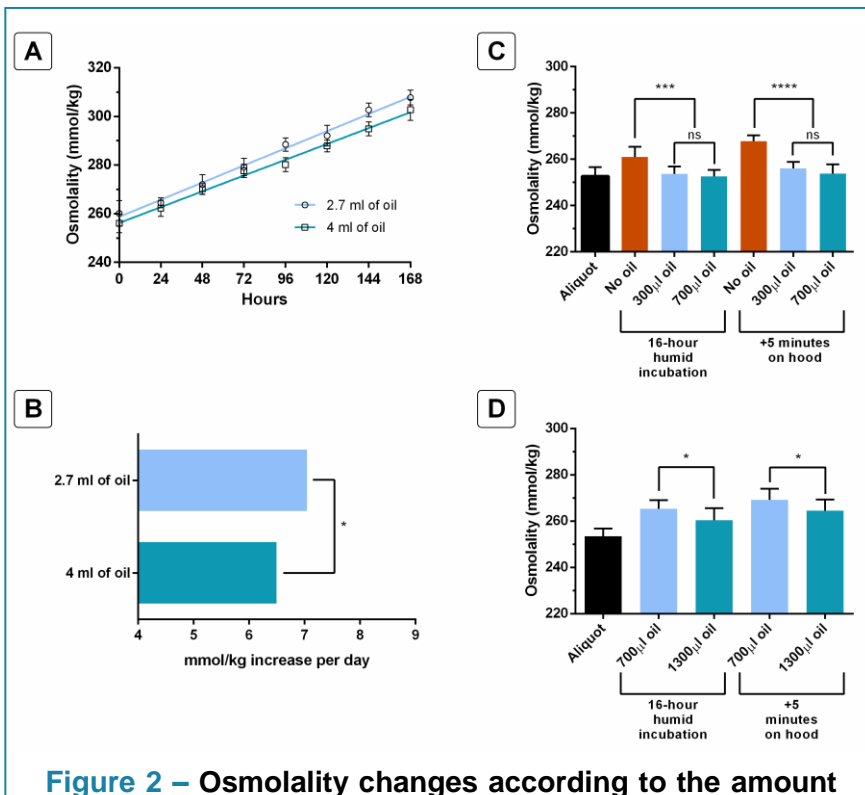
Figure 2 – Osmolality changes according to the amount of mineral oil. Comparison of daily osmolality measurements (A) and evaporation rates after linear regression (B) of culture medium using a low versus high volume of mineral oil, using Petri dishes in a dry benchtop incubator. Differences in osmolality after 16 hours in a humid incubator using 5-well dishes with either 700 μl of culture medium (C) or 20 μl microdroplets (D).
Significance is represented as *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001; ns = non-significant (p>0.05).

ml (302.8 ± 4.2 mmol/kg [p=0.0034]; **Figure 2A**). The linear regression analysis also showed that evaporation during culture was significantly decreased when a higher volume of oil was used (p=0.0145; **Figure 2B**).

Experiment 4: Use of oil in well-dishes

A first experiment was performed using a large volume of medium (700 µl). After overnight culture in a humid incubator, no differences were found in osmolality when comparing a 300 versus a 700 µl oil overlay (253.7 ± 3.2 and 252.6 ± 2.8 mmol/kg, respectively). Without any oil, evaporation occurred much quicker and osmolality rose to 261 ± 4.4 mmol/kg (p=0.0005), as shown in **Figure 2C**. After 5 minutes on a warm surface, osmolality increased further in all three groups: 267.8 ± 2.6 (no oil), 256.1 ± 2.8 (300 µl of oil) and 253.8 ± 4.0 (700 µl of oil), although statistically significant differences were only found in the no-oil group (p<0.0001).

In a second experiment, 20 µl medium microdroplets were covered with either 700 or 1300 µl of mineral oil. In this case, using a larger volume of oil significantly reduced evaporation both after overnight incubation (p=0.0233) and after the additional 5 minutes on a warm surface (p=0.0318; **Figure 2D**).

Experiment 5: Comparison of time-lapse dishes

The studied time-lapse dishes were codified for publication to avoid any commercial repercussion. The possibility of medium evaporation happening during setup was considered and

	TL dish 1	TL dish 2	TL dish 3	TL dish 4	TL dish 5
TL dish 2	<0.0001	-	-	-	-
TL dish 3	<0.0001	ns	-	-	-
TL dish 4	ns	<0.0001	<0.0001	-	-
TL dish 5	<0.0001	ns	ns	<0.0001	-
TL dish 6	0.005	<0.0001	<0.0001	0.0002	<0.0001

Table I - P-values summary after the statistical analyses of evaporation rates between different models of time-lapse dishes. In this case, statistical significance was set at p=0.0083 to account for multiple group comparison.

assessed by comparing osmolality between the medium aliquot and each of the dishes immediately after preparation (0 h). The medium used had an initial osmolality of 254.9 ± 3.9 mmol/kg (mean ± SD). After dish setup, osmolality was statistically significantly higher for dish designs 1, 2 and 3 (258.7 ± 2.0 [p=0.02], 259.8 ± 3.8 [p=0.02], 259.4 ± 2.9 mmol/kg [p=0.02], respectively), but not in dishes 4, 5 and 6 (257.3 ± 3.7 [p=0.21], 257.3 ± 3.7 [p=0.20], 257.9 ± 4.1 mmol/kg [p=0.14]; **Figure 3B**). A multiple comparison showed that the final osmolality after 168 h of culture was radically different (p<0.0001) depending on the dish type: 294.0 ± 3.3, 280.4 ± 3.1, 277.7 ± 4.6, 289.4 ± 4.1, 280.1 ± 3.4 and 299.7 ± 2.7 mmol/kg, for dishes 1 to 6, respectively (**Figure 3A**). Comparing each group's slope after linear regression revealed that the design of the dish has a huge impact in the evaporation rate (**Figure 3C**; **Table I**). Generally, those dish designs that

allowed for greater volumes of medium in each well and a deeper oil overlay experienced a lower evaporation than the rest.

Experiment 6: Comparison of time-lapse incubators

Time-lapse dishes number 2 and 4 were used to repeat the osmolality assessment during a 168h period but, instead of using a regular benchtop incubator, they were placed in their

Figure 3 – Assessment of osmolality in time-lapse dishes. Osmolality was measured in six time-lapse (TL) dish models during parallel culture in a common dry benchtop incubator (A), comparing the changes in osmolality during dish preparation in each of the models (B) and its daily increase during culture (C). Two of the previous dishes were retested in the time-lapse incubator they were designed for (D), and their evaporation rates compared to those obtained in the benchtop incubator (E).

Significance is represented as *p≤0.05; **p≤0.01. In graphs (A) and (C), different superscripts represent statistically different groups.

intended time-lapse incubator they are meant to be used in (**Figure 3D**). The analysis of the evaporation rates after linear regression for dish number 4 showed that evaporation had occurred significantly faster in its corresponding time-lapse incubator than in the benchtop incubator (p=0.006; **Figure 3E**). This difference was not observed for dish number 2 and its matching incubator (p=0.224).

Discussion

The field of assisted reproduction has undergone major changes in recent years. The development of new types of incubators, modern single-step media formulations, and the implementation of more robust quality control programs have allowed IVF centers to adopt uninterrupted embryo culture strategies. Unfortunately, longer periods of culture without media renovation may result in the evaporation of the culture medium, potentially causing osmotic stress that can impair the developmental potential of gametes and embryos in culture (Hay-Schmidt, 1993). In human clinical practice, preventing osmolality changes may contribute to reducing the stress levels for embryos, and has been associated with better embryo development and improved clinical outcomes (Fawzy *et al.*, 2017; Del Gallego *et al.*, 2018). Thus, the methodologies used in the laboratory should focus on minimizing evaporation to avoid changes in medium osmolality during manipulation and gametes/embryo culture.

The threshold above which osmotic stress is triggered *in vitro* in the mouse has been described at approximately 300 mOsm but it has not been so clearly defined in human. Nonetheless, the similarities in the physiological standard between the human and murine reproductive tracts could be indicative that the *in vitro* osmolality requirements may also be comparable in both species (Borland *et al.*, 1980; Harris *et al.*, 2005). To avoid reaching detrimental osmolalities above such levels, there are several factors in the laboratory that can be modified.

Covering culture media with an appropriate type and sufficient volume of mineral oil will create a physical barrier and has been reported to prevent evaporation (Swain *et al.*, 2018). Using oil should be encouraged even during brief procedures such as oocyte pick-up or embryo transfer, as significant evaporation may occur otherwise. Some laboratories may try to spare oil during routine setup to reduce economic costs or for other reasons such as avoiding accidental oil spillage or the possible sealing of the dishes' lids, which could interfere with their correct gas equilibration. However, our results show that increasing the thickness of the overlay between the medium and the air by using higher amounts of oil can help to reduce evaporation and, likewise, larger volumes of medium will result in minor changes in osmolality, even if some evaporation occurs. High viscosity oils are easier to manipulate and are less likely to result in spillage or dish-lid sealing, making it more feasible to increase the volume of oil used. In addition, oils with high viscosity have been shown to be better at preventing

evaporation than those with lower viscosity (Swain, 2018; Yumoto *et al.*, 2019); our study used low-viscosity oil aiming to exacerbate changes in osmolality in order to identify other parameters that could play a role in osmotic stabilization. Unfortunately, most commercial oils currently lack standardization in their nomenclature ("mineral/paraffin" oil, with no definition of their chemical differences, if any) and their physical description ("heavy/light" oil, without quantifying its viscosity or density) (Swain, 2019). By providing clearer and more detailed information, oil manufacturers could aid researchers in correlating each of the oil's characteristics with its effect on the culture system. Another concern which may arise with the use of mineral oil is its biosecurity, as it has historically been considered as one of the components with a higher potential of introducing toxicity into the culture system (Morbeck and Leonard, 2012). However, the improvement of both oil manufacturing and quality control bioassays have vastly reduced this possibility in the commercially available products nowadays (Mestres *et al.*, 2019).

Choosing the right medium to work may also be critical to avoid osmotic stress, as our results suggest that some culture media are more prone to evaporate than others. In one of the studied media, with an approximate initial osmolality of 280 mmol/kg, osmolality peaked up to over 325 mmol/kg after seven days of culture; by using media with a lower initial osmolality (around 255 mmol/kg) hypertonicity at the end of culture could be reduced. In addition, significant differences were observed in

the evaporation rate between the two studied media, regardless of their supplementation with albumin. While the exact reason for this disparity remains unknown, we postulate that the presence of some amino acids in KSOM medium but lacking in HTF, such as glutamine, could be a possible explanation due to their described emulsifying properties (Summers *et al.*, 2000; Yiase, 2015). Amino acids play another important role in culture medium by acting as organic osmolytes, which gametes and embryos can transport through specialized mechanisms to regulate intracellular pressure, thus reducing the detrimental effects of hypertonicity (Steeves *et al.*, 2003; Tartia *et al.*, 2009). In our setting, with 20 µl microdrops in 35 mm Petri dishes filled with 4 ml of mineral oil, we observed a significant increase of osmolality after 96 hours of culture in a dry atmosphere, which contrasts with the data published by other groups describing an earlier detection (Yumoto *et al.*, 2019). However, given the great pool of variables that engage in osmolality maintenance, differences between laboratories with diverse methodologies are expected.

The effect of humidification during incubation was investigated by comparing osmolality between a humid and a dry chamber in the same incubator. This had major consequences on medium evaporation, and a significant effect on osmolality was detectable after only 24 hours of culture. This concurs with previous work showing the importance of humidity in reducing evaporation (Holmes and Swain, 2018). However, the majority

of modern incubators work with a dry atmosphere and, while this may not be detrimental per se, it must be considered in order to adapt the working conditions and methodologies (Swain, 2019). Additionally, our results showed significant differences of more than 20% between the internal relative humidity of two humid incubators and more than 10% between two dry ones. Even though small variations in the internal humidity of the incubators might be attributed to atmospheric fluctuations, the gases injected into the incubators (typically CO_2 and N_2) may displace a big part or the totality of the atmospheric air within the incubator, and so the end effect of the atmospheric conditions would be diminished. Nonetheless, the described difference in humidity found between apparently similar incubators showcases that not all incubators can be considered equal in this regard, and simply distinguishing between "dry" and "humid" incubators may not be informative enough. Our results, especially when comparing both humid incubators, suggest that when aiming to minimize evaporation, humidity should be as high as possible. Commercial "humid" incubators use diverse methodologies to humidify their inner atmosphere, from water pans to porous stones or water bottles in the gases inlet, all resulting in varying levels of humidity. Since a clear correlation between exact humidity % and evaporation rates is still lacking, the use of hygrometers could prove useful in future studies to detail the most appropriate humidity for embryo culture. Furthermore, some groups may try to humidify their dry incubators by placing water or medium-

filled dishes in them. Previous publications have shown that, while a modest improvement could be achieved, this strategy remains insufficient, with humidity never surpassing 45% (Holmes and Swain, 2018).

Ideally, the dynamics of osmolality during prolonged periods of culture should be analyzed in each laboratory's setting, to account for differences in media, oil and dish preparation, and independently in every incubator model. This was further illustrated when contrasting the measurements taken in the same time-lapse dish in their own incubator and in a standard benchtop: both studied dishes showed a tendency to suffer additional evaporation in their time-lapse incubator than in the benchtop. The improved heat transmission derived from a precise dish-incubator fit could explain the variation in media osmolality.

We have shown that, through correct preparation and by considering important aspects described in the literature (Swain *et al.*, 2012), changes in osmolality during dish setup can be prevented. However, the same aspects might not be applicable to time-lapse dishes, as they have a unique design that may vary in terms of microwell volume, number of microwells and volume of mineral oil supported. In addition, each manufacturer will typically indicate a detailed protocol to prepare their dish. Even after taking the necessary precautions, preparing the dishes one at a time, on a non-heated stage and with low air flow, significant evaporation

during setup still occurred in half of the time-lapse dishes selected for this study. Therefore, in these cases osmolality was already elevated prior to starting the actual culture and hence the probability of reaching hypertonicity levels after some days of incubation was higher. This has great importance considering that time-lapse incubators seldom allow for humidity in their chambers and are commonly used in clinical practice for up to 7-day uninterrupted culture. In suboptimal conditions, the increase of osmolality could be so high as to counteract any beneficial effects derived from time-lapse technology itself (Yumoto *et al.*, 2019). We were able to distinguish evaporation rates between one dish design with high evaporation, two dishes with intermediate evaporation and three dishes with low evaporation. Elevated evaporation rates may be caused by inherent flaws in design of the dish but, in some cases, evaporation might be reduced simply by adjusting the recommended preparation protocol. Specifically, increasing the volume of mineral oil and the depth of the layer between the medium and the atmosphere could potentially help to prevent evaporation (Carpenter *et al.*, 2018; Swain *et al.*, 2018).

In conclusion, osmolality is one of many critical parameters of culture that can potentially affect embryo viability. Humidity, media composition, mineral oil, and adequate techniques (such as preparation speed and preparing only one dish at a time) allow to reduce evaporation. However, the design and preparation of the dishes, especially those used in time-lapse

incubators, have a noteworthy effect on osmolality stability. Each IVF laboratory should carefully define the conditions that allow to maintain the culture medium's osmolality as stable as possible in their own center, keeping in mind that any modification in the culture system could result in an alteration of evaporation and, potentially, clinical outcomes. Manufacturers should also understand the importance of osmolality and its dynamics, in order to optimize existing or new consumables to guarantee a stable culture system.

Author's roles

EM, NCB, JC, GC and MGJ participated in the study conception and design. EM, MGJ, AC, MA, AV and QMA performed all experiments and acquired the data. EM analyzed the data, which was discussed and interpreted by EM, MGJ and NCB. EM drafted the manuscript and all authors participated in its revision.

Conflict of interest

The authors have no conflicts of interest to declare in relation with the current work.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Chapter II

Mestres E, Garcia-Jiménez M, Faes L, Vanrell I, Bogaert V,
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*Parameters of the Mouse Embryo Assay that affect detection
of peroxides in mineral oil*

Parameters of the Mouse Embryo Assay that affect detection of peroxides in mineral oil

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Research question: Can culture conditions influence the sensitivity of a Mouse Embryo Assay and its potential to detect peroxide-related toxicity in mineral oil samples?

Design: Protein type and concentration, embryo density and culture dish design were selected as the variables in the culture system with the potential to influence the assay's sensitivity. Fresh 1-cell mouse embryos were cultured under mineral oil samples with known peroxide concentrations. Protein type (HSA + α/β-Globulins vs. HSA vs. BSA), concentration (5 mg/ml vs. 0.5 mg/ml), embryo density (25 vs. 3 µl/embryo) and culture dish (Petri vs. micro-well dish) were adjusted to define the culture conditions with the highest sensitivity.

Results: High concentrations of peroxides can be easily detected by current quality control standards. However, for oil samples with lower presence of peroxides, supplementing culture medium with 5 mg/ml of HSA + α/β-Globulins or HSA resulted in an increased detection of embryo-toxicity compared with when BSA was used as protein supplement. The sensitivity of the assay was greatly reduced when embryos were cultured in groups and when certain micro-well dishes were used.

Conclusions: Current quality control protocols may not be sensitive enough to identify low presence of peroxides which, if undetected, can increase over time and become potentially harmful during gamete and embryo culture. The different parameters established in our study allow to optimize the sensitivity of the MEAs to specifically detect peroxides in mineral oil samples, prior to their release into the market and their broad use in human IVF.

Introduction

Mineral oil is a petroleum-derived component widely used in IVF laboratories worldwide since it was first adopted for mouse embryo culture in 1963 (Brinster, 1963). Commonly used as a barrier between culture medium and air, oil stabilizes temperature and pH during culture, as well as minimizes changes in osmolality by preventing the evaporation of culture medium (Morbeck and Leonard, 2012; Prince, 2010; Swain *et al.*, 2012).

Many studies have considered culture medium as the most important component in a culture system, disregarding mineral oil as a second-line factor (Sifer *et al.*, 2009). However, mineral oil has been identified as a source of toxicity in previously published reports (Otsuki *et al.*, 2007; Turner, 2010; Wolff *et al.*, 2013). Unfortunately, toxicity detection in mineral oil samples through Quality Control (QC) assays is challenging, due to the heterogeneity in harmful components and, more importantly, the variation in toxicity levels between samples, even within the same lot. Peroxides, generated by the oxidation of reactive double-bond carbons, are the most common source of embryo-toxicity in mineral oil. Additionally, peroxidation may increase over time, and is exacerbated by inadequate storage conditions (high temperature and exposure to solar/UV light). Thus, even after undergoing QC testing, toxicity may arise afterwards during the oil's shelf life. (Ainsworth *et al.*, 2017; Chronopoulou and Harper, 2015; Khan

et al., 2013; Morbeck, 2012; Morbeck *et al.*, 2012, 2010; Morbeck and Leonard, 2012; Otsuki *et al.*, 2009; Provo and Herr, 1998).

Although specialized techniques have improved peroxide detection (Eertmans *et al.*, 2013), current QC bioassays still lack effectiveness and may not always detect low levels of toxicity. Thus, increasing sensitivity has become the main focus in protocol optimization for QC, particularly in the Mouse Embryo Assay (MEA), the current gold standard for embryo-toxicity detection in IVF reagents and consumables. However, lack of standardization has led to controversy on how MEAs' protocols should be performed or optimized (Ackerman *et al.*, 1985; Ainsworth *et al.*, 2017; Davidson *et al.*, 1988; Khan *et al.*, 2013; Morbeck *et al.*, 2010; Parinaud *et al.*, 1987; Scott *et al.*, 1993; Wolff *et al.*, 2013). Our hypothesis is that MEAs' sensitivity could be maximized by carefully defining certain culture conditions.

The inconsistent embryo development *in vitro* of outbred mouse strains deem them an inadequate model to work with; instead, hybrid strains allow for more consistent results whilst still maintaining a good sensitivity (Ackerman *et al.*, 1983; Fleetham *et al.*, 1993; Gardner DK, Reed L, Linck D, Sheehan C, 2005; Khan *et al.*, 2013; Morbeck, 2012). The use of mouse embryos at the 1-cell stage, rather than at 2-cell stage, is an additional step towards increasing sensitivity (Ackerman *et al.*, 1984; Davidson *et al.*, 1988; Fleetham *et al.*, 1993; O'Neill,

1997; Scott *et al.*, 1993; Weiss *et al.*, 1992). Group culture reduces the assay's sensitivity, as it generally yields higher blastocyst formation rates and blastocyst cell number (Dai *et al.*, 2012; Hughes *et al.*, 2010; Kato and Tsunoda, 1994; Kelley and Gardner, 2017; Lane and Gardner, 1992; O'Neill, 1998, 1997; Paria and Dey, 1990; Reed *et al.*, 2011). Similarly, micro-well dishes (typically used in time-lapse incubators) may result in microenvironments rich in embryo-secreted factors (Chung *et al.*, 2015; Dai *et al.*, 2012; Kelley and Gardner, 2017, 2016; Pribenszky *et al.*, 2010; Sifer *et al.*, 2009; Vajta *et al.*, 2008), and thus their possible effect on sensitivity should be analyzed.

Albumin is known to chelate toxins present in culture medium and mask their effect (Bavister, 1995; Gardner DK, Reed L, Linck D, Sheehan C, 2005; Meintjes *et al.*, 2009; Morbeck *et al.*, 2014; Scott *et al.*, 1993; Weiss *et al.*, 1992). However, recent studies have shown that protein supplements with Human Serum Albumin (HSA) could actually be of use to detect peroxide-related toxicity in mineral oil (Morbeck *et al.*, 2012; Otsuki *et al.*, 2009).

The end-point in the MEA has traditionally relied uniquely on blastocyst formation rates, which do not necessarily relate to their viability. New protocols, such as extending the duration of culture until 120 or 144 hours, or end-points better related to embryo viability, like total cell counts and morphokinetics, should be considered as potential improvements for sensitive toxicity detection (Ainsworth *et al.*, 2017; Gardner DK, Reed L,

Linck D, Sheehan C, 2005; Hughes *et al.*, 2010; Kelley and Gardner, 2017; Scott *et al.*, 1993; Wolff *et al.*, 2013).

The aim of this study was to explore the effect of different culture conditions (protein type and concentration, embryo density and culture dish design) on the ability of the MEA to detect peroxides in mineral oil samples. The optimization of the aforementioned culture conditions was proposed as a means to maximize sensitivity and advance towards the standardization of testing protocols.

Materials and methods

Animals

All procedures and animal care were conducted as approved by the Ethics Committee on Animal Research (reference number 10133, 20th July 2018) in Barcelona Science Park (PCB).

B6CBAF1/JRj hybrid mice were purchased from Janvier Labs and housed with a 12-hour light/dark cycle (08-20 h.), controlled temperature and ad libitum access to food and water. Five to nine-week-old females were super-ovulated by intra-peritoneal injection of 7.5 IU of pregnant mare serum gonadotrophin (PMSG, Folligon®, MSD), followed 48h later by 7.5 IU of human chorionic gonadotrophin (hCG, Veterin Corion®, Divasa-Farmavic). Immediately after hCG administration, females were placed with males from the same

genetic background (8 to 20-week-old, with previously proven fertility) in individual cages overnight. Mating was ascertained on the following morning by the presence of a vaginal plug.

Females were euthanized by cervical dislocation 22 hours post hCG administration. Zygotes were collected by locating and tearing the walls of the ampullae under the stereomicroscope (SZX10, Olympus), in hyaluronidase micro-droplets (80 IU/ml, LifeGlobal®), to remove cumulus cells. Fertilized zygotes were identified by the clear presence of two pronuclei and two polar bodies. All procedures were performed on a heated surface, to ensure adequate temperature maintenance within the micro-droplets. The totality of the experiments was performed with fresh embryos, starting at the one-cell stage, to enhance sensitivity (Davidson *et al.*, 1988; Fleetham *et al.*, 1993; Gardner DK, Reed L, Linck D, Sheehan C, 2005; Hughes *et al.*, 2010; Morbeck, 2012; O'Neill, 1997; Scott *et al.*, 1993; Weiss *et al.*, 1992).

Induced peroxide formation

Mineral oil samples were produced and provided by a specialized ART manufacturer (FertiPro N.V., Belgium), and exposed for controlled periods of time to UV light, to induce peroxide formation (Otsuki *et al.*, 2007). Peroxide value (POV) was determined by potentiometric titration, an adaptation of the iodometric titration protocol described by the European Pharmacopeia (Eertmans *et al.*, 2013), which allows for the detection and quantification of peroxides in much lower

concentrations (≥0.1 mEq/kg) than previous protocols. Five mineral oil samples were produced, with the following POVs: <0.1 mEq/kg (control sample, FertiCult Mineral Oil, FertiPro N.V.), 0.128, 0.198, 0.503 and 0.756 mEq/kg. Treatment groups were evaluated blindly throughout the study, until all experimental series were completed.

Protein supplementation

Five experimental groups were designed according to their medium protein supplementation type and concentration, including: HSA + α/β-Globulins (4.4 + 0.6 mg/ml), HSA + α/β-Globulins (0.44 + 0.06 mg/ml), HSA (5 mg/ml), BSA (5 mg/ml) and BSA (0.5 mg/ml). Commercial culture medium (Global®, LifeGlobal®) was supplemented with HSA + α/β-Globulins (LGPS, LifeGlobal®) or BSA (A3311-10G, Sigma-Aldrich®) to reach the aforementioned concentrations. Ready-to-use culture medium was used for the HSA 5 mg/ml group (Global® total® LP, LifeGlobal®). Before allocation in each treatment group, embryos were rinsed in an additional dish prepared with the same medium/oil combination.

Experiment 1: Effect of protein supplementation on peroxide detection

Each oil sample was tested with the five protein supplementation groups described above. To avoid changes in osmolality derived from evaporation, culture dishes were prepared by placing 50 µl medium micro-droplets directly

under a 5 ml oil overlay. Dishes were equilibrated overnight at 37.3ºC, 7% CO_2 and 7% O_2, prior to use. Zygotes (total n = 1070) were cultured in groups of two, resulting in a low embryo density (25 µl/zygote). Embryo development was followed daily until Day 6 (120 h. of culture), and the number of expanded blastocysts was recorded both on Day 5 and Day 6. The obtained blastocysts were fixed and stained for total cell counts. Each culture group was tested in triplicate.

Experiment 2: Effect of group culture on peroxide detection

Given the results of the first series of experiments, only the oil samples with lower peroxide concentrations were used in the second series (<0.1, 0.128 and 0.198 mEq/kg). The same protein supplementation groups were maintained. The micro-droplets' volume was reduced to 21 µl and zygotes (total n = 756) were cultured in groups of seven, in order to achieve a high embryo density of 3 µl/zygote. Embryo development was assessed daily until Day 5 (96 h. of culture). Once again, expanded blastocysts were fixed and stained for total cell counts, and every group tested in triplicate.

Experiment 3: Effect of dish design on peroxide detection

To determine the effect that micro-wells might impart on toxicity assessment, zygotes (total n = 240) were placed in time-lapse dishes (Embryoslide®, Vitrolife®), in wells filled with 25 µl of culture medium and a 1.5 ml oil overlay. All five protein

supplementation groups were maintained. Culture was carried out in a time-lapse incubator (Embryoscope®, Vitrolife®) for 96 hours, and blastocysts graded based on morphological criteria. For this series of experiments, only two oil samples were used (<0.1, 0.128 mEq/kg), and each group was tested in duplicate.

Staining and cell counts

Cell nuclei were stained by incubating them in a 10 μg/ml bisbenzimide solution (Hoechst 33342, Sigma-Aldrich®) for 10 minutes at room temperature. Stained blastocysts were washed and mounted on a slide in a 1:1 PBS-glycerol droplet and flattened with a cover slip. Upon observation under UV-2A filter fluorescence microscopy (Nikon Eclipse E1000), their total cell number was assessed manually with an image processing software (ImageJ).

Statistical analysis

GraphPad Prism® 6 (GraphPad Software, La Jolla, CA, USA) statistics software was used to perform all statistical analyzes. Fisher's exact test ([F]) was applied to compare blastocyst formation rates between groups. Mann-Whitney test ([M-W]) was used for the comparison of total cell numbers, which did not follow a normal distribution. Statistical significance was set at 5% ($\alpha = 0.05$).

Results

Experiment 1: Effect of protein supplementation on peroxide detection

High blastocyst formation rates (>80%) were obtained by day 5 with the control mineral oil sample (<0.1 mEq/kg), regardless of the type and concentration of protein used. By contrast, in samples with high peroxide concentrations (0.198, 0.503, 0.756 mEq/kg) all embryos degenerated by day 3 of culture.

Interestingly, protein supplementation had a high impact when an oil sample with a peroxide concentration close to the detection limit was used (0.128 mEq/kg). For the same amount of protein (5 mg/ml), supplementation with HSA or HSA + α/β-Globulins resulted in significantly increased sensitivity to peroxides, compared with BSA (p=0.0001^F). However, with a lower protein concentration (0.5 mg/ml), embryo development rates were comparable between HSA + α/β-Globulins and BSA. No differences were detected between high/low protein concentration in BSA groups. (**Fig. 1A**) (**Fig. 2**)

The extension of the culture period from 96h. to 120h. only resulted in a significantly higher sensitivity in a single group, that was supplemented with 0.5 mg/ml HSA + α/β-Globulins (p=0.0016^F). (**Fig. 1B**)

For all protein groups tested, total cell number of the obtained blastocysts by day 6 was compared between control (<0.1

mEq/kg) and test (0.128mEq/kg) oil samples. (**Table 1**) Cell number was significantly lower only in the 5 mg/ml BSA supplementation group ($p=0.0091^{M-W}$); however, the reduced number of blastocysts in the rest of protein treatments might have prevented a statistical difference from being detected.

Figure 1 - Effect of protein supplement on peroxide detection sensitivity. A) Blastocyst formation by 96 hours of culture. Different superscripts indicate a statistically significant difference (p < 0.05) in sensitivity.
ns = not significant B) Results comparison between standard (96 hours) and extended end-points (120 hours). $**p = 0.0016^{F}$.

Protein supplement	HSA + α/β-Glob. (5 mg/ml)		HSA + α/β-Glob. (0.5 mg/ml)		HSA (5 mg/ml)		BSA (5 mg/ml)		BSA (0.5 mg/ml)	
Mineral oil sample	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg
Initial n	63	63	63	63	63	63	63	63	63	63
Blastocysts 120h n (%)	61 (96.8%)	0 (0%)	62 (98.4%)	10 (15.9%)	62 (98.4%)	2 (3.2%)	60 (95.2%)	47 (74.6%)	57 (90.5%)	29 (46.0%)
Total cell counts (median [IQR])	183 (154 – 217.75)	N/A	175 (123.25 – 209.25)	150.5 (140 – 159.25)	193 (165 – 222)	146.5 (N/A)	182 (156 – 205)	164** (148 – 184)	164 (128 – 191.5)	162 (128 – 198.75)

Table 1 - Blastocyst formation rate and total cell number assessed in blastocysts obtained after 120 hours of culture in pairs (** p = 0.0091 $^{M-W}$).

Figure 2 - Embryos obtained with test oil (POV = 0.128 mEq/kg) and each protein supplement, after 96 hours of culture in pairs.
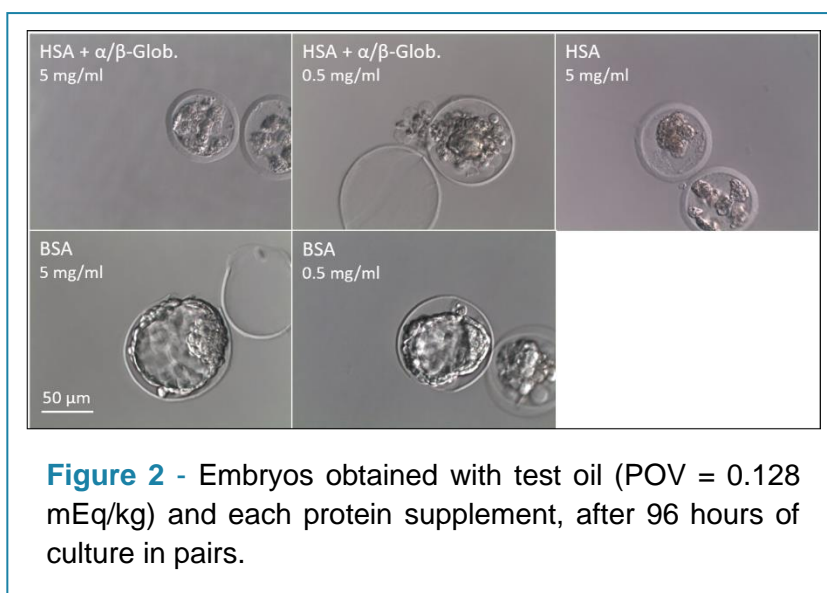
Experiment 2: Effect of group culture on peroxide detection

For the second experimental series, three mineral oil samples were tested (<0.1, 0.128 and 0.198 mEq/kg), as the ones with higher POVs were not included due to their extreme toxic effects. Similarly to the previous experiment, control oil sample yielded blastocyst formation rates higher than 90% in all protein groups. The sample with 0.198 mEq/kg POV resulted in null or negligible blastocyst rates, as the majority of the embryos degenerated by day 3 in all groups, and only 2 blastocysts (3%) formed in 0.5 mg/ml BSA group.

When using mineral oil containing 0.128 mEq/kg of peroxides, group culture (7 embryos in 21 µl) resulted in a significant loss of sensitivity to the assay, as shown by highly increased

developmental rates for all protein groups compared with the previous experiment (2 embryos in 50 µl) (p=0.0001[F]; Figure 3). The group with 5 mg/ml of HSA + α/β-Globulins maintained enough sensitivity to detect peroxides by the current end-points (<80% blastocyst formation). Supplementing with 5

Figure 3 - Effect of embryo density and culture dish on peroxide detection sensitivity by 96 hours of culture. Statistical significance is indicated as **, *** and **** for p-values <0.01, <0.001 and ≤0.0001, respectively. A) Comparison between low (2 embryos in 50 µl) and high embryo density (7 embryos in 21 µl). B) Compared results between culture in regular Petri dishes and in micro-well dishes.

mg/ml of HSA also retained some sensitivity compared with other groups, but with results very close to the standard acceptance criteria for MEAs (80% expanded blastocyst formation rates). (**Fig. 3A**) (**Fig. 4**)

Blastocysts from all protein groups were fixed and stained to compare the total cell number between control and test group (<0.1 and 0.128 mEq/kg, respectively). Results did not differ in BSA groups, regardless of their protein concentration. By contrast, in HSA-based supplements, blastocysts in the control oil presented a significantly higher number of cells than those in the test oil sample (p=0.0132^{M-W} for 5 mg/ml HSA + α/β-Globulins; p<0.0001^{M-W} for 0.5 mg/ml HSA + α/β-Globulins and 5 mg/ml HSA). (**Table 2**)
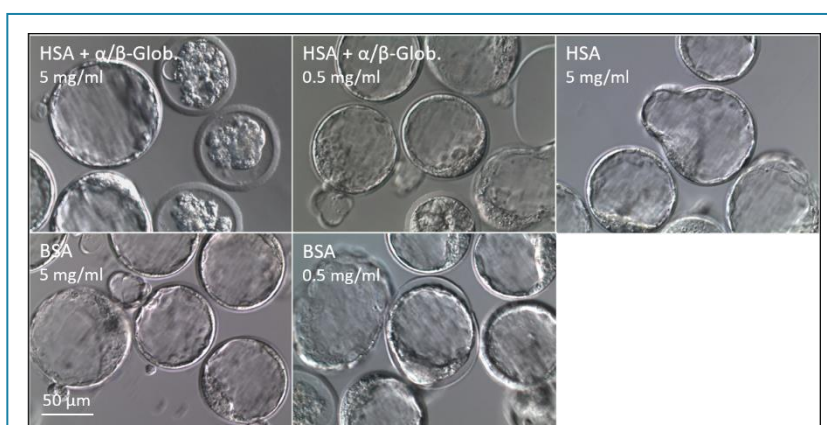
Figure 4 - Blastocysts obtained with test oil (POV = 0.128 mEq/kg) by 96 hours of group culture with all protein supplements.

Protein supplement	HSA + α/β-Glob. (5 mg/ml)		HSA + α/β-Glob. (0.5 mg/ml)		HSA (5 mg/ml)		BSA (5 mg/ml)		BSA (0.5 mg/ml)	
Mineral oil sample	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg	Control (<0.1 mEq/kg)	0.128 mEq/kg
Initial n	63	63	63	63	63	63	63	63	63	63
Blastocysts 96h. n (%)	63 (100%)	38 (60.3%)	62 (98.4%)	61 (96.8%)	61 (96.8%)	48 (76.2%)	63 (100%)	63 (100%)	59 (93.7%)	56 (88.9%)
Total cell counts (median [IQR])	139 (122.5 – 154.5)	126* (101.75 – 144.75)	143 (132 – 159)	123**** (110 – 133.5)	147 (127.25 – 165.75)	125**** (97.25 – 148)	149.5 (129 – 164.75)	140 (120.75 – 153)	130 (109 – 146.5)	124 (103.5 – 139.5)
p-value	0.0132^{M-W}		<0.0001^{M-W}		<0.0001^{M-W}		ns		ns	

Table 2 - Total cell number assessed in blastocysts obtained by 96 hours of group culture, reported for each group as the median and the interquartile range (IQR).

Experiment 3: Effect of dish design on peroxide detection

The effect of micro-well design on the assay's sensitivity was studied by culturing the same five protein groups on a time-lapse incubator. All embryos cultured with the control mineral oil sample developed to the blastocyst stage, regardless of the type or concentration of protein used (100% blastocyst formation rates).

This assay was expected to be at least as sensitive to peroxides as Experiment 1 (culture in pairs), since micro-well culture was strictly individual and implied a low embryo density (1 embryo in 25 µl). On the contrary, we found the assay's sensitivity to be decreased in micro-well culture when testing 0.128 mEq/kg POV oil. Blastocyst formation rates were significantly higher in all protein groups, compared with the results in Experiment 1 ($p \leq 0.005^F$). In three groups (0.5 mg/ml HSA + α/β-Globulins and both 5 mg/ml and 0.5 mg/ml BSA), this resulted in the assay not being able to successfully detect embryo-toxicity. (**Fig. 3B**)

Discussion

Despite its beneficial stabilizing effects, mineral oil can also act as a source of potentially embryo-toxic elements, such as peroxides (Ainsworth *et al.*, 2017; Chronopoulou and Harper, 2015; Khan *et al.*, 2013; Morbeck *et al.*, 2010; Morbeck and Leonard, 2012; Otsuki *et al.*, 2007; Provo and Herr, 1998). Low concentrations of peroxides, represented in this study by the

sample with 0.128 mEq/kg, may go undetected if tested inadequately and increase over time. Even embryo-tested oil can become embryo-toxic during storage. Thus, it is of utmost importance to detect even trace amounts of peroxides during QC testing, as well as strictly maintaining adequate storage conditions.

In this study, we aimed to design an optimized MEA protocol for peroxides detection, that could allow for standardization of mineral oil testing. We used a hybrid mouse strain, which ensured a more consistent embryo development *in vivo* than outbred strains (Ackerman *et al.*, 1984, 1983; Fleetham *et al.*, 1993; Gardner DK, Reed L, Linck D, Sheehan C, 2005), and to start all tests at the one-cell stage, extensively described as the most sensitive starting point for QC assays (Davidson *et al.*, 1988; Hughes *et al.*, 2010; Morbeck, 2012; O'Neill, 1997; Scott *et al.*, 1993; Weiss *et al.*, 1992).

When embryos were cultured in pairs (2 embryos in 50 µl), protein supplementation played a major role in the assays' results. BSA supplement resulted in heavily decreased sensitivity, with almost 70% of the embryos developing to the blastocyst stage. By contrast, using 5 mg/ml of HSA or HSA-based supplements, we were able to detect trace amounts of peroxides, with only about 3% blastocyst formation. A concentration of 0.5 mg/ml HSA equated results to those in BSA groups for the same dose. Although protein has been previously described to chelate toxins in the medium and hide

their detrimental effects (Gardner *et al.*, 2005; Weiss *et al.*, 1992), our results display the contrary effect when specifically testing mineral oil. In agreement with our study, other groups proposed that HSA could bind to free radicals present in suboptimal oil samples and ease their transfer through the zona pellucida, thus increasing the toxic effect on embryo cells and the assay's sensitivity (Otsuki *et al.*, 2009; Scott *et al.*, 1993; Weiss *et al.*, 1992). Nonetheless, the presence of octanoic acid and reactive metals in complex protein supplements has been described (Fredrickson *et al.*, 2015; Morbeck *et al.*, 2014), and their possible effect on the assays' results cannot be disregarded.

Performing total cell counts is a recommended means to increase the sensitivity of the MEA (Gardner DK, Reed L, Linck D, Sheehan C, 2005; Hughes *et al.*, 2010; Kelley and Gardner, 2017; Scott *et al.*, 1993). In our experience, cell counts only increased sensitivity significantly in the 5 mg/ml BSA group. Granted, this was the group with a lowest sensitivity in terms of blastocyst formation and, consequently, cell counts were required to reach a higher detection power. Considering the time, training, materials and infrastructures needed to perform cell counts, achieving equal levels of sensitivity purely by optimizing protein supplementation would be an extremely convenient and cost-effective alternative. As proposed by Ainsworth *et al.* 2017, we also tried extending the assay's end-point from the regular 96 hours of culture until 120 hours. Extended culture was only significantly advantageous in the

0.5 mg/ml HSA + α/β-Globulins group. Once again, an extra measure had to be taken to enhance an initially low sensitivity, which would not be necessary if 5 mg/ml were used instead. In our setting, a 144-hour culture was not informative, as blastocyst deterioration by that time-point was too advanced to be analyzed successfully.

Group culture may help to reduce the costs and time associated to toxicity screening. However, it can result in an extremely diminished toxicity detection, due to the accumulation of embryonic autocrine and paracrine factors in the culture medium (Canseco *et al.*, 1992; Chung *et al.*, 2015; Hughes *et al.*, 2010; Kelley and Gardner, 2017, 2016; Melin *et al.*, 2009; O'Neill, 1997; Reed *et al.*, 2011; Vajta *et al.*, 2008). Our results confirm that a low concentration of peroxides (0.128 mEq/kg) can remain undetected as a direct consequence of group culture (7 embryos in 21 µl). Only supplementation with 5 mg/ml HSA + α/β-Globulins was able to confidently detect such levels of peroxides despite the high embryo density.

Due to the generally reduced sensitivity in group culture, total cell counts were considered again as an additional method to increase detection power. Interestingly, cell number was significantly decreased in groups supplemented with 5 or 0.5 mg/ml HSA + α/β-Globulins and 5 mg/ml HSA. By contrast, sensitivity in BSA groups was so diminished that not even the

addition of cell counts was able to successfully detect peroxides presence.

Lastly, we studied the effect that culture dish design may play on MEAs' sensitivity. Embryos were cultured individually in micro-wells in time-lapse dishes (Embryoslide®, Vitrolife), covered with the mineral oil containing low concentration of peroxides (0.128 mEq/kg). Surprisingly, a significantly higher number of blastocysts was obtained in all protein groups by culturing in micro-well dishes, compared with regular Petri dishes. As in the previous experiment, toxicity was masked in some protein groups, with more than 80% of the embryos reaching the blastocyst stage. Again, 5 mg/ml of HSA-based supplements proved to be the optimal choice to maximize sensitivity. The micro-environment created by micro-well culture and the reduced medium-oil contact surface could be a possible explanation for these results. This agrees with the results published by Wolff *et al.*, (2013) which described blastocyst formation as an insufficient marker for toxicity in oil testing when working with a well-of-the-well culture. Instead, they achieved higher sensitivity by comparing morphokinetics timings between test and control groups.

In summary, peroxides may form in mineral oil bottles after inadequate manufacturing or handling, and their detection is fundamental to avoid adverse effects on embryo development. Testing protocols must be standardized and optimized to detect even minimal traces of peroxides. Inadequate testing

may result in embryo toxicity going undetected, and potentially harming batches being released and used in a clinical IVF setting. By culturing in low embryo densities and using high concentrations of HSA-based supplements, we were able to maximize the sensitivity of the MEA for peroxide detection, without the need of additional end-points, such as total cell counts or extended culture periods. Nonetheless, these add-in end-points may prove valuable in conditions different than those tested in this study, or to detect toxicity caused by components other than peroxides. Furthermore, our results suggest that a reduction in the assay's sensitivity may occur in micro-well culture.

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Chapter III

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Characterization and comparison of commercial oils used for human embryo culture

Characterization and comparison of commercial oils used for human embryo culture

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Study question: Are there significant differences between the available commercial oil brands used for human IVF?

Summary answer: Important differences have been detected among the tested oil brands in their potential to stabilize culture conditions and, more importantly, in their direct effect on embryo development and viability.

What is known already: Mineral oil is a critical component of the human culture system due to its protective and stabilizing roles during *in vitro* embryo development. Many different oils are available on the market, with differences in their viscosity, density and overall quality.

Study design, size, duration: Thirteen different commercial oil brands were compared.

Participants/materials, setting, methods: Each oil was firstly analyzed to assess its viscosity, density, peroxide value and potential oxidation. Secondly, the capacity of each oil to reduce pH, osmolality and temperature fluctuations during embryo culture and manipulation was compared. Lastly, a sensitive mouse embryo assay (MEA) protocol, previously optimized to detect toxicity in oils samples, was used to compare the overall quality of the different brands in terms of embryo developmental rates up to the blastocyst stage. At the end of the MEAs, a triple labelling protocol was applied to analyze Oct4+ cells, apoptotic cells and total cell counts in the blastocysts obtained by fluorescence microscopy.

Main results and the role of chance: Significant divergences were detected in the rise of osmolality and the equilibration and stability of pH between different oils, which could be correlated to their physico-chemical characteristics. In particular, oil samples with a higher viscosity tended to offer an additional protection against fluctuations in the culture conditions, however, the differences in temperature stability between oils were minor. Two out of the thirteen oil samples, which were commercially available, were identified as embryo-toxic by applying the MEA protocol with increased sensitivity for toxicity detection. Additionally, substantial differences in the total number of cells and the number of cells in the inner cell mass of the obtained blastocysts were also detected between oil groups.

Limitations, reasons for caution: A single lot of oil was used for each brand and, thus, lot-to-lot variations in oil quality could not be determined. However, several bottles from the same oil were included to account for potential intra-lot variability.

Wider implications of the findings: Commercial oils differ in both their physical characteristics and their performance in maintaining the stability of the culture conditions during *in vitro* embryo culture. Oil selection is important for embryo culture success. Additionally, the detection of embryo-toxic oils which had already been released to the human IVF market showcases the importance of applying sensitive MEA protocols for a better detection of toxicity in this type of samples.

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Trial registration number: N/A

Introduction

The success of an IVF cycle, defined as the birth of a healthy baby after *in vitro* embryo culture and transfer, is highly dependent on the quality and stability of the embryo culture system. In the last decades, many pieces of equipment, consumables and techniques have been designed specifically for human IVF purposes, aiming to increase the stability of the culture system. One of these products, culture oil, was first introduced in the ART field in 1963 as a protective layer placed on top of culture medium (Brinster, 1963), and has since become a standard methodology used for *in vitro* manipulation and culture of embryos and gametes. Among its various properties, oil has been shown to stabilize temperature, pH and osmolality during culture (Bavister, 1987), thus allowing embryo culture to be carried out in microdroplets of medium (Brinster, 1963), a strategy shown to yield better clinical outcomes (Kane, 1987; Vajta *et al.*, 2008).

Several manufacturers currently include one or multiple types of oil as part of their product portfolio, which presumably differ in their physico-chemical characteristics. However, these differences have not been properly described due to the scarce information provided by the manufacturers, as well as a notorious lack of standardization in the nomenclature of the oils on the market. As a prominent example, viscosity quantification is typically not provided by manufacturers, which commonly rely on a binary definition referring to oils with a high viscosity

as "heavy oils" and oils with a low viscosity as "light oils"; however, no clear threshold exists that defines what is considered a high or a low viscosity. Without this and other currently-missing information about commercial oils, no correlation can be established between any oils' characteristics and their behavior or performance in the culture system.

Oil is considered a highly heterogenous and not well characterized component of the culture system and, even though the advantages derived from its use are undeniable, it can also act as a source of contaminants and toxins that can migrate to the culture medium and be detrimental for embryos and gametes (Fleming *et al.*, 1987). In fact, several published reports have identified batches of mineral or synthetic oil contaminated with toxic components, such as zinc (Erbach *et al.*, 1995), Triton X-100 (Morbeck *et al.*, 2010) or peroxides, which are the most common cause of oil-related toxicity (Otsuki *et al.*, 2007). In some cases, the affected batches of commercial oil were recalled only after their toxicity was detected during clinical use, causing irreparable damage to human embryos/gametes that could have been avoided through a better production, characterization and testing of the oil (Otsuki *et al.*, 2007; Turner, 2010). In addition, numerous commercial oils have been shown to produce significant differences in clinical outcomes in the human (Sifer *et al.*, 2009). In recent years the quality of the oil used in ART has been improved, partly due to the exhaustive and specialized

quality control testing that oil must go through before being released into the market (Mestres *et al.*, 2019), which has reduced the chances of contaminated or embryo-toxic lots of oil actually being used in clinical practice. In addition, washing oil is a strategy applied by some manufacturers and end-users to remove toxicity and saturate it with water. While its capacity to reduce the presence of toxins is well known (Fleming *et al.*, 1987; Morbeck *et al.*, 2010), its permeation with water and potential effect on media evaporation is unclear and still a subject of devate due to contradicting reports (Swain *et al.*, 2016; Yumoto *et al.*, 2019), and hence not all commercial oils currently available are pre-washed.

Refined high-quality mineral oil is mostly composed of saturated straight chain hydrocarbons, with a minor presence of saturated cyclic molecules. Incomplete refinement or hydrogenation during manufacturing may result in the presence of unsaturated and aromatic cyclic molecules in the oil (Morbeck and Leonard, 2012). These are highly reactive and may oxidise over time, thus degrading the oil and deriving in the rise of toxicity, especially when the oil is transported and/or stored under suboptimal conditions, such as exposed to light or high temperatures (Otsuki *et al.*, 2007). In addition, the length and shape of the hydrocarbon molecules is related to the viscosity and the density of the oil (Sattarin *et al.*, 2007; Morbeck and Leonard, 2012), and is highly variable between brands. Some publications have looked into the differential behavior of oils during culture, especially regarding fluctuations

in media evaporation (Swain, 2018; Yumoto *et al.*, 2019; Mestres *et al.*, 2021; Mullen, 2021); however, the effects of different oils on other parameters of the culture system have not yet been described in detail.

The aim of this study was to describe the differences between several oil brands, commonly used in clinical human IVF laboratories, detailing their 1) physical characteristics (viscosity and density); 2) molecular stability (proneness to peroxidation); 3) capacity to minimize fluctuations of the culture conditions (temperature, pH, osmolality); and 4) potential toxicity and overall quality when used for *in vitro* embryo culture.

Materials and methods

Selected oil samples and culture medium

Thirteen oils were selected for this study: LifeGuard, LiteOil, Paraffin oil (LifeGlobal, CooperSurgical, USA); Liquid paraffin (Origio, CooperSurgical, USA); Oil for tissue culture (Sage, CooperSurgical, USA); Ovoil, Ovoil heavy (Vitrolife, Sweden); Hypure oil light, Hypure oil heavy (Kitazato, Japan); Mineral oil (FertiPro, Belgium); Sydney IVF oil (COOK Medical, USA); Oil for embryo culture (Irvine, USA); Mineral heavy oil (Millipore Sigma, USA). All the selected brands were commercially available for human IVF purposes at the beginning of the present study, save for one (Millipore Sigma's Mineral heavy oil), which is not approved for human clinical practice.

Information regarding the original source of the oil (mineral/synthetic) and the washing of the oil was collected from the product inserts and instructions, if available (**Table I**). All oils were tested at the same time to minimize variations in the results originated by environmental factors. The oils'

Oil (alphabetical)	Origin	Pre-washed	Initial peroxide concentration (mEq/kg)
Hypure oil heavy (Kitazato)	Mineral	Yes	<0.1
Hypure oil light (Kitazato)	Mineral	Yes	<0.1
LifeGuard (CooperSurgical)	Mineral	Yes	<0.1
Liquid paraffin (Origio)	Mineral	Yes	<0.1
Liteoil (CooperSurgical)	Mineral	Yes	<0.1
Mineral heavy oil (Sigma)	Mineral	No	<0.1
Mineral oil (FertiPro)	Mineral	Yes	<0.1
Oil for embryo culture (Irvine)	Mineral	No	<0.1
Oil for tissue culture (Sage)	Mineral	No	<0.1
Ovoil (Vitrolife)	Mineral	No	<0.1
Ovoil heavy (Vitrolife)	Synthetic	No	<0.1
Paraffin oil (CooperSurgical)	Mineral	Yes	<0.1
Sydney IVF oil (COOK medical)	Mineral	Undisclosed	<0.1

Table I - Description of the included oils and results on their initial POV determination.

names were codified (1 – 13) in the results section to avoid any commercial repercussion. In all the experiments requiring the use of culture medium, KSOM (EmbryoMax MR-107-D, Millipore Sigma, USA) with 5mg/ml protein supplementation (FertiCult HSA, FertiPro, Belgium) was used. A new aliquot of medium, prepared on the day prior to the experiment, was used for each round of pH and osmolality experiments.

Viscosity and density

The physical characteristics of each of the selected oils were described by measuring both their viscosity and their density. Viscosity was quantified with a shear viscosity meter (DV-II+Pro viscometer, Brookfield, USA; accuracy ± 2%) and was tested in duplicate. Density was measured by weighing 10ml of oil with an analytical balance (HT/HTR-CE, Vibra, Japan), and each oil was tested four times. Both measurements were performed after pre-warming the oil samples up to 30ºC.

Peroxide value determination

The initial peroxide value (POV) was measured in each of the oils with a potentiometric titration test (accuracy ± 5%, limit of quantification = 0.1 mEq/kg) (Eertmans *et al.*, 2013). Afterwards, 40ml of each oil were aliquoted in transparent polystyrene flasks and all the aliquots were placed together on an outdoor surface and exposed at the same time to direct sunlight for two weeks. The concentration of peroxides was subsequently measured again to evaluate the proneness of the oils to oxidation when exposed to potentially suboptimal

storage/transportation conditions. Oil no. 8 could not be exposed to the sunlight at the same time as the rest of oils; to rule out differences caused by the methodology, this sample was excluded from the POV-increase analyses.

pH equilibration

The time required to reach complete pH equilibration was assessed with a continuous pH measurement system (pH Online, Log&Guard, Vitrolife, Germany), which uses a fluorometric sensor placed at the bottom of one of the wells of a 4-well dish. The sensor-well was filled with 700µl of culture medium and immediately overlaid with 500µl of oil, both not pre-equilibrated and used at room temperature and room atmosphere. Following preparation, the dish was placed in a big box incubator (Heracell 150i, Thermo Scientific, USA), set at 37.3ºC, 6% CO_2 and 7% O_2 (balanced with N_2), and pH was monitored every 15 minutes for 24 hours.

pH stability

Petri dishes with a 35mm diameter were prepared in triplicate with 4ml of oil and four 50µl microdroplets of medium. After overnight equilibration (20-24 hours) in a benchtop incubator set at 37.3ºC, 6% CO_2 and 7% O_2, all three dishes were taken out of the incubator and placed on a warm laminar flow hood (37.5ºC, air flow set at low intensity). pH was measured immediately with a blood gas analyzer (epoc®, Siemens Healthineers, Germany), and the reading was repeated after

15 and 30 minutes. All measurements were repeated four times for each of the oils.

Temperature loss and recovery

A 35mm Petri dish was prepared for each group with a central 20µl microdroplet overlaid with 4ml of oil. After overnight incubation in a benchtop incubator set at 37.3ºC, the dish was moved onto the warm surface of a laminar flow hood (37.5ºC, air flow set at low intensity) and a fine-gauge K-type thermocouple was immediately put in place to monitor the in-drop temperature, using an automatic data logger (TC-08, Pico technology, UK) to perform continuous readings every 10 seconds for 15 minutes. The thermocouple probe was pre-warmed in a separate oil dish to avoid artifacts in the determination of the in-drop temperature. During the readings, an external surface thermologger (BrightSentinel, The Netherlands; accuracy ± 0.05ºC) was placed on the warm surface to evaluate its stability.

Afterwards, the dish was returned to the benchtop incubator and the recovery of temperature was monitored again every 10 seconds for 15 minutes. All measurements were performed at least in triplicate, and outliers with more than 0.5ºC of difference with the rest of replicates were discarded and repeated.

Osmolality

Petri dishes (35mm) were set up with 4ml of each test oil and nine 20µl culture medium microdroplets, which were prepared following the wash-drop method previously described in Swain *et al.*, 2012. Briefly, droplets of 5µl were placed on the empty dish, immediately covered with oil, removed and replenished with 20µl of fresh medium. The dishes were placed in a dry-atmosphere benchtop incubator (AD-3100, Astec, Japan) and cultured for seven days (168h). Osmolality was measured daily in a minimum of three droplets with a vapor pressure osmometer (VAPRO® 5600, Wescor, USA; accuracy ± 1%), and three replicas were prepared for each oil group on different days. Outliers, identified by a difference of more than 5mmol/kg compared to the rest of measurements of the same group and replica, were discarded. The osmometer was calibrated with known-osmolality standard solutions prior to each round of readings. Additionally, room temperature and relative humidity were monitored during the duration of the experimental rounds with an appropriate thermometer/hygrometer (Log&Guard, Vitrolife, Germany).

Embryo *in vitro* culture and fluorescence cell analysis

Each oil was tested following a Mouse Embryo Assay (MEA) protocol that had been previously optimized to increase the sensitivity of the bioassay on detecting toxicity specifically in oil samples (Mestres *et al.*, 2019). Each oil group was programmed to include a total of 126 embryos (1638 mouse

embryos in total) and, when possible, a new bottle of oil was used for each replicate. The number of embryos that reached the expanded blastocyst and the hatched blastocyst stages after six days of culture (120h) were used as the primary and secondary outcomes to compare the development results between oils.

In addition, a triple staining protocol (adapted from Fouladi-Nashta *et al.*, 2005) was applied to quantify and compare the mean total cell number, the number of pluripotent cells (Oct4+) forming the inner cell mass (ICM), and the number of apoptotic cells per blastocyst. The obtained blastocysts were fixed with 2% paraformaldehyde at room temperature for 20 minutes, subsequently permeabilized with 2.5% Triton X-100 for 30 minutes at 37ºC, washed with a blocking solution and stored at 4-8ºC overnight. On the next day, embryos were exposed to a primary Oct4 antibody (sc-5279, Santa Cruz Biotechnology, USA) and stored again at 4-8ºC overnight. Afterwards, the blastocysts were washed and incubated with a green secondary antibody (A-21202, Thermo Fisher Scientific, USA) for three hours at 37ºC, washed with blocking solution and stored in the fridge overnight. Finally, they were incubated in a TUNEL apoptosis-detection solution (*in situ* cell death detection kit, TMR red, Roche, Switzerland) for 60 minutes at 37ºC and in a 10µg/ml bisbenzimide solution (Hoechst 33342, Millipore Sigma, USA) for 10 minutes. They were then mounted on a microscope slide in a 1:1 PBS-glycerol droplet and

flattened with a cover slip. Photos were taken under epifluorescence microscopy (BX43F, Olympus, Japan).

Statistical analyses

All data and statistical analyses were performed with GraphPad Prism 9 (GraphPad Software, USA). To compare qualitative data, Fisher's exact two-tailed test was applied and, when required, Bonferroni correction for group comparisons was included. Quantitative data sets were analyzed with D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests to assess the normality of their distribution. Subsequently, Student's t-test and ANOVA and Tukey's post-hoc tests were applied for pair and group comparisons of parametric data sets, respectively; Mann-Whitney test and Kruskal-Wallis followed by Dunn's post-hoc tests were used to compare pairs and groups of data sets which did not follow a parametric distribution, respectively. To evaluate the differences in the osmolality rise, linear regression was used to compare the slopes between groups. Linear and non-linear regression was also applied to determine the correlation of the studied variables with the viscosity and density of the oils. The required sample size for the Mouse Embryo Assays was calculated in order to detect a 13% difference in the blastocyst formation rate with a 90% power. For all analyses, statistical significance was set at 5% (alpha = 0.05).

Ethical use of animals

All procedures involving animal care were performed as approved by the Ethics Committee on Animal Research of Barcelona Science Park (reference number 10133).

	Viscosity (cP at 30ºC) mean ± SD	Density (mg/ml at 30ºC) mean ± SD	Described by manufacturer as
Mineral heavy oil (Sigma)	110.7 ± 9.8	866.8 ± 7.0	Heavy
Oil 2	108.7 ± 8.6	866.5 ± 4.3	Heavy
Oil 3	102.1 ± 2.3	868.1 ± 3.2	Heavy
Oil 4	53.3 ± 1.7	854.6 ± 2.8	Medium viscosity
Oil 5	43.1 ± 2.2	831.1 ± 5.5	Heavy
Oil 6	22.4 ± 2.1	851.0 ± 4.4	Light
Oil 7	22.3 ± 0.4	850.7 ± 5.6	Light
Oil 8	22.1 ± 0.6	846.4 ± 7.3	Light
Oil 9	21.2 ± 0.6	838.6 ± 3.5	Light
Oil 10	21.2 ± 0.3	838.8 ± 8.3	Light
Oil 11	18.1 ± 0.1	842.7 ± 4.4	Light
Oil 12	16.6 ± 0.4	848.7 ± 3.0	Light
Oil 13	12.0 ± 0.6	835.4 ± 5.8	Light

Table II - Characterization of the viscosity and density of the oil samples, correlated with the description given by the oils' manufacturers.

Results

Viscosity and density

The viscosity of the studied oils ranged widely, between 12.0 and 110.7 cP at 30°C, and their density varied between 835.4 and 868.1 mg/ml (**Table II**). The three oils with the highest viscosities also showed the highest densities of the cohort; however, a good linear correlation could not be established between the two variables when including the totality of the groups (**Fig. 1A**). A control group was included in the density experiment using ultrapure water, with an expected density of approximately 1000 mg/ml, obtaining a result of 999.1 ± 5.5 mg/ml (mean ± SD).

Peroxide value determination

The initial presence of peroxides was lower than the limit of quantification of the peroxide test in all the studied oils (0.1 mEq/kg, **Table I**). After exposing an oil aliquot from each group to direct sunlight for two weeks, the POV determination was repeated. Peroxides heavily rose in all groups, with varying results between 1.249 and 0.394 mEq/kg, but the disparity in the increase of peroxides was not associated to the oils' viscosity/density (**Fig. 1B**), nor with whether the oils had been pre-washed by the manufacturer (**Fig. 1C**).

pH equilibration

Once the sensor dish was placed inside of the incubator, the necessary incubation time for pH to reach a value of 7.4 was
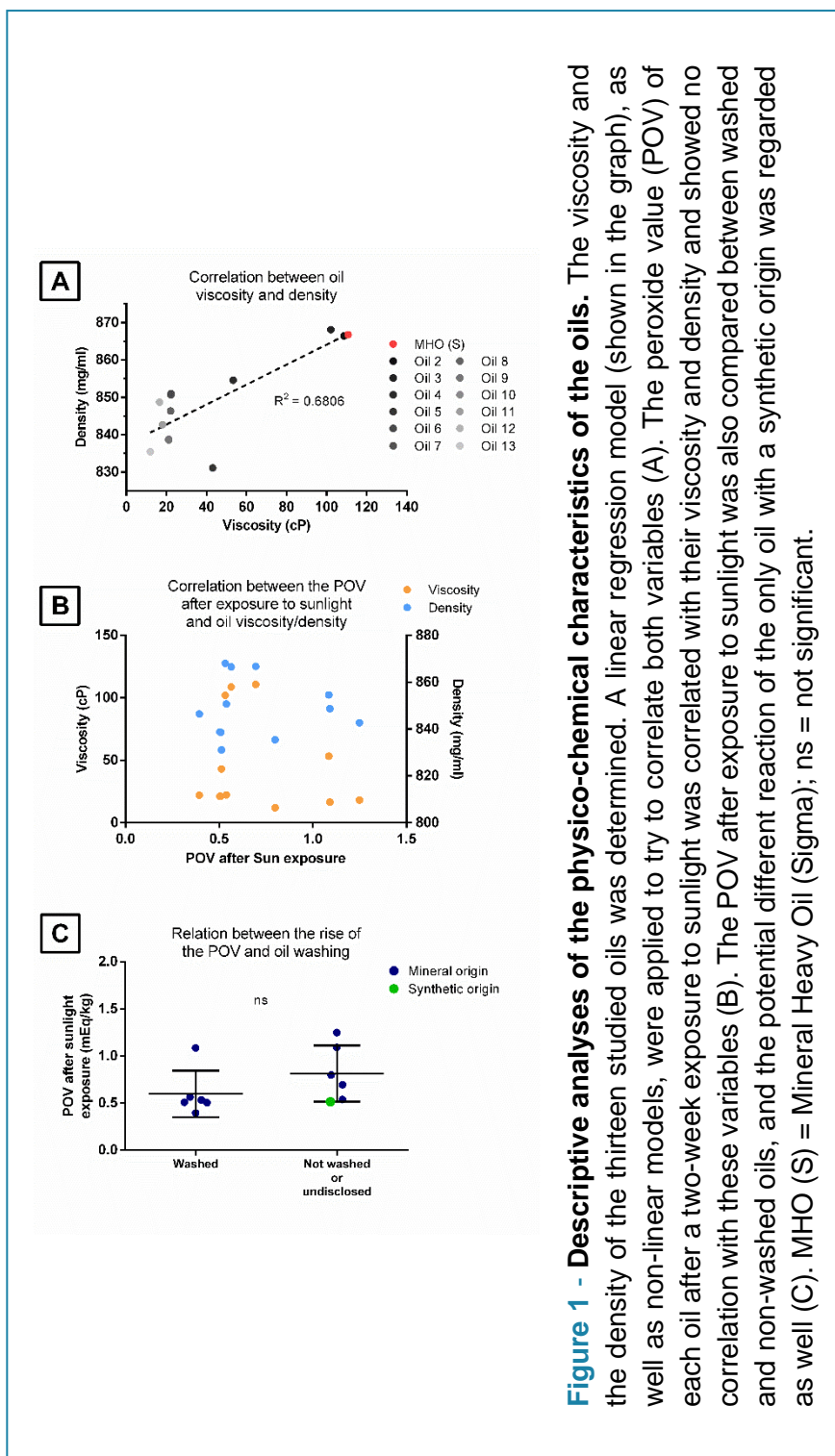
Figure 1 - **Descriptive analyses of the physico-chemical characteristics of the oils.** The viscosity and the density of the thirteen studied oils was determined. A linear regression model (shown in the graph), as well as non-linear models, were applied to try to correlate both variables (A). The peroxide value (POV) of each oil after a two-week exposure to sunlight was correlated with their viscosity and density and showed no correlation with these variables (B). The POV after exposure to sunlight was also compared between washed and non-washed oils, and the potential different reaction of the only oil with a synthetic origin was regarded as well (C). MHO (S) = Mineral Heavy Oil (Sigma); ns = not significant.

used as the value of interest to compare the equilibration of the dishes. This threshold value was selected following the recommendations of most media manufacturers, which indicate an optimal pH range between 7.4 and 7.2. The required incubation period varied between 2.5 and 6 hours, depending on the oil that was used (**Fig. 2AB**). A slight correlation could be established between the equilibration period and the viscosity or density of the oils (**Fig. 2C**), with a

Figure 2 - Media pH equilibration and its relation to the viscosity/density of the oil. The pH in a 4-well dish was monitored during 24h after preparing the dish and placing it in an incubator. Some differences were noted in the required time to achieve pH equilibration depending on the used oil, particularly during the first hours of incubation (A, B). Whilst both the oils' viscosity and density seemed to be somewhat related to the equilibration speed, viscosity proved to be a better fit after applying a linear regression model (C).
MHO (S) = Mineral Heavy Oil (Sigma).

moderate prediction power (R^2 = 0.8134 and 0.7290, respectively). Some additional time was required in all cases to achieve complete equilibration, which occurred between a narrow 0.05 range among all groups (7.24-7.29); in the slower group, it took a total of approximately 16 hours of incubation for pH to finally plateau.

pH stability

There were no differences in the initial pH of the culture medium between all the oil groups after overnight equilibration (7.277 ± 0.019, mean ± SD; p = 0.7884). After 15 minutes out of the incubator with room atmosphere, pH had increased in the majority of the groups and some significant differences appeared between groups (**Fig. 3A; Suppl. Table I**); after a total of 30 minutes out of the incubator, variations in the pH between groups had exacerbated (**Fig. 3A; Suppl. Table II**). For two of the oils, number 2 and 3, the final pH had not significantly increased compared to their initial pH (7.288 ± 0.011 and 7.284 ± 0.027 at time 0; 7.314 ± 0.005 and 7.320 ± 0.014 at time 30, respectively [mean ± SD]; p = 0.6487 for oil 2 and p = 0.1622 for oil 3).

Interestingly, there seemed to be a tendency for oils with a high viscosity to reduce the rise in pH over time; the Mineral Heavy Oil from Sigma, however, was the only exception which did not follow the same distribution as the rest of the studied oils. By treating this group as an outlier, a non-linear correlation could be established between the oils' viscosity and their capacity to

reduce pH fluctuations (R^2 = 0.8098). By contrast, the density of the oils did not seem to be directly related to the stabilization of pH (**Fig. 3B**).

Figure 3 - Stability of the pH outside of the incubator, related to the viscosity/density of the oil. The capacity for each oil to maintain a stable pH after taking the dish out of the incubator was assessed at time 15 and 30 min (A); a different superscript within the same time-point indicates a statistically significant difference between groups (the complete intergroup comparisons at both time-points can be found in the supplementary tables I and II). The increased pH after 30 minutes followed a non-linear correlation with the oils' viscosity, but did not seem to be related to their density (B).

MHO (S) = Mineral Heavy Oil (Sigma); ns = not significant.

Temperature loss & recovery

The initial in-drop temperature at the starting point was 37.35 ± 0.12°C (mean ± SD), with no differences between any groups (p > 0.05). Temperature was monitored for a total of 15 minutes, and its dynamics could be divided in three differential stages: an initial steep loss of temperature (first 5 minutes), a second stage with a slower cooling rate (between 5 and 10min) and a final stage in which temperature plateaued (from 10 minutes onwards) (**Fig. 4A**). The initial 5-minute fraction, with the most acute loss of temperature, was selected as the primary variable of interest for comparison analyses. After performing a linear regression for each of the groups, a cooling rate per minute could be established and was compared between groups. Oils 7 and 12 were the ones with a faster cooling rate (-0.22°C/min in both cases) and, in contrast, oil 13 was the one that better maintained temperature within those five minutes (-0.14°C/min) (**Fig. 4BC; Suppl. Table III**). There was no apparent correlation between the oils' viscosity/density and the cooling rate. The temperature of the heated surface was controlled with an external surface probe and remained at a stable 37.6 ± 0.2°C (mean ± SD) for the duration of the experimental rounds.

The same experiment was repeated after the dishes were returned to the benchtop incubator. Once again, the starting temperature was the same between all groups (36.00 ± 0.12°C, mean ± SD; p > 0.05). In this case, however, tempera-
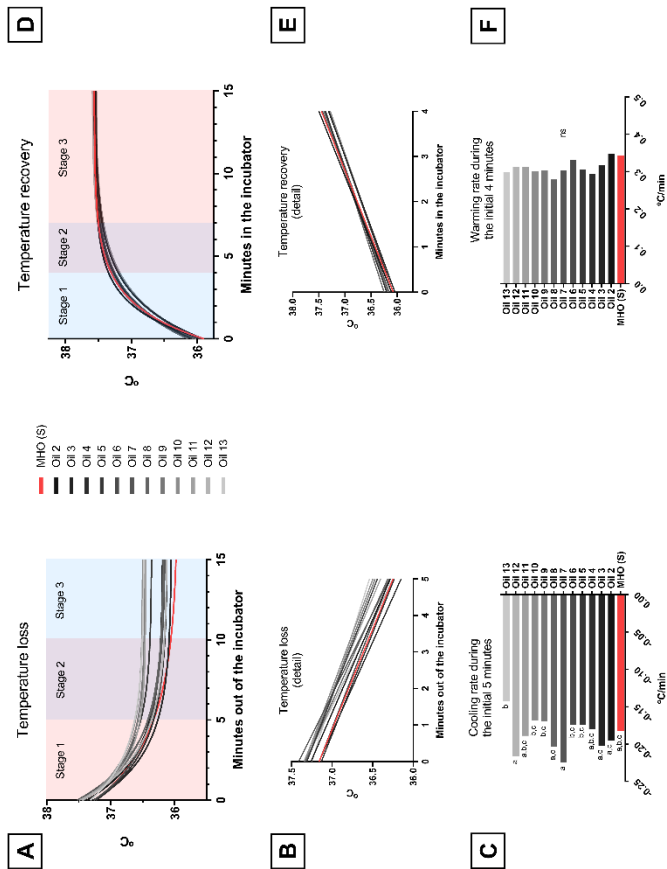
Figure 4 - Assessment of the in-drop temperature decline after taking a dish out of the incubator, and temperature recovery after placing it back. Temperature loss was measured during 15 minutes, and an initial 5-minute steep decrease was noted and analyzed in detail (A, B). The cooling rate per minute during this initial phase was calculated (C), and compared between groups. A different superscript indicates a statistically significant difference between groups (the complete p-values for all comparisons can be found in the supplementary table III). For temperature recovery, the fastest warming rate occurred during the initial 4-minute period (D, E), but no differences were detected between any groups (F). The p-values for all pair-to-pair analyses can be found in the supplementary table IV.

MHO (S) = Mineral Heavy Oil (Sigma); ns = not significant.

ture rose quickly during approximately the initial 4 minutes, followed by a slower recovery stage for an additional 3 minutes until temperature completely recovered and plateaued by 7 minutes after returning the dish to the incubator (**Fig. 4D**). Linear regression was applied to the first 4-minute period, with no detectable differences in the slope between any of the groups (**Fig. 4EF; Suppl. Table IV**).

Osmolality

The initial osmolality of the culture medium was 256.6 ± 3.1 mmol/kg (mean ± SD), with no statistical differences among the study groups (p = 0.2318). As evaporation of the medium occurred over time during culture, its osmolality increased gradually, albeit with different rates depending on the oil used (**Fig. 5AB; Suppl. Table V**). The oil that best prevented evaporation was oil number 2, with a mean osmolality rise of +2.796 mmol/kg/day; by contrast, the evaporation rate observed in group 13 doubled that of group 2, with +5.580 mmol/kg/day. Consequently, the medium's osmolality at the end of the experiment, after seven days (168h) of culture, was much higher in group 13 than in group 2 (295.2 ± 3.0 and 276.7 ± 5.5, respectively [mean ± SD]). Interestingly, a non-linear correlation with a good prediction power (R^2 = 0.8573) could be established between the oils' viscosity and the final osmolality in the culture medium for each of the groups; by using the oils' density instead, neither a linear nor a non-linear model were a good fit with a similarly comparable correlation

(**Fig. 5C**). No statistical difference was found in the daily evaporation rate when comparing washed and non-washed oils (**Fig. 5D**).

Figure 5 - **Study of the medium's osmolality during 7 days of culture in a dry benchtop incubator.** Evaporation over time during culture occurred in all groups (A), but the daily evaporation rate differed between some of the studied oils (B). A different superscript indicates a statistically significant difference between groups; the p-values for the all the comparisons can be found in the supplementary table V. The capacity of each oil to prevent evaporation could be correlated to its viscosity, following a non-linear model; by contrast, oil density was not as good a predictor for evaporation prevention (C). The daily evaporation rate was also compared between oils that had been pre-washed by the manufacturer and those that had not or had this information undisclosed (D).
MHO (S) = Mineral Heavy Oil (Sigma); ns = not significant.

Room temperature and relative humidity were monitored during the duration of the osmolality experimental rounds, and were stable at 24.4 ± 0.9°C and 36.2 ± 9.5%, respectively (mean ± SD).

Embryo *in vitro* culture and fluorescence cellular analysis

A total of 126 mouse embryos were used to perform MEAs on each oil group. The majority of the studied oils successfully reached the accepted criteria generally applied to the MEAs, defined by a minimum of 80% of the embryos in culture developing until the expanded blastocyst stage or further. Oils number 11 and 13, however, yielded significantly lower results than the majority of the other groups and, in fact, did not reach the acceptance threshold and should thus be deemed as embryo-toxic samples (**Fig. 6A; Suppl. Table VI**). When using oil 13, major degeneration occurred between day 4 and day 5 of *in vitro* culture, so the experiments were stopped sooner than scheduled and only 74 embryos were included in this group.

The number of blastocysts that had completed hatching by day 6 of development was used as a secondary outcome to compare embryo development between groups. Statistically significant differences were observed between oils, ranging between 50.0% and 28.6% of hatched blastocysts in groups 3 and 9, respectively (**Fig. 6B; Suppl. Table VII**). Only 1.4% of the embryos cultured in group 13 completed hatching, due to the generalized degeneration of the embryos. Both the

expanded blastocyst rate and the hatching blastocyst rate were equal between the oils that had been pre-washed and the ones that were not (**Fig. 6C**).

Blastocysts were triple stained applying a novel protocol, as a means of quantifying embryo quality (**Fig. 7A-G**). The analysis of the total number of cells, the number of Oct4+ pluripotent cells, and the number of apoptotic cells per blastocyst also showed significant differences between groups (**Fig. 7H-J; Suppl. Table VIII-X**). The highest number of total cells was observed in group 5 (175.0 ± 43.5, median ± IQR) and, as expected, the two lowest cell counts corresponded to oils number 11 (108.0 ± 67.6) and 13 (87.5 ± 49.3). A similar distribution was valid for Oct4+ cells, with oils number 3 and 5 showing a greater presence of pluripotent cells in the ICM, whilst oils 11 and 13 were the ones with the lowest number of Oct4+ cells (41.0 ± 15.5, 41.0 ± 21.0, 15.0 ± 20.0 and 12.0 ± 12.75, respectively [median ± IQR]). Smaller differences were observed regarding the number of apoptotic cells, as only one group, oil number 2, showed a slightly increased number of apoptotic cells compared to some of the other groups. Interestingly, oils 11 and 13, which were identified as subpar in terms of embryo development, total cell number and Oct4+ cell number, did not present a significantly higher number of apoptotic cells than the rest of groups; however, the number of stained embryos for oil 13 was very low due to the scarce quantity of blastocysts obtained in this group and,

consequently, the statistical power was very diminished in this case.
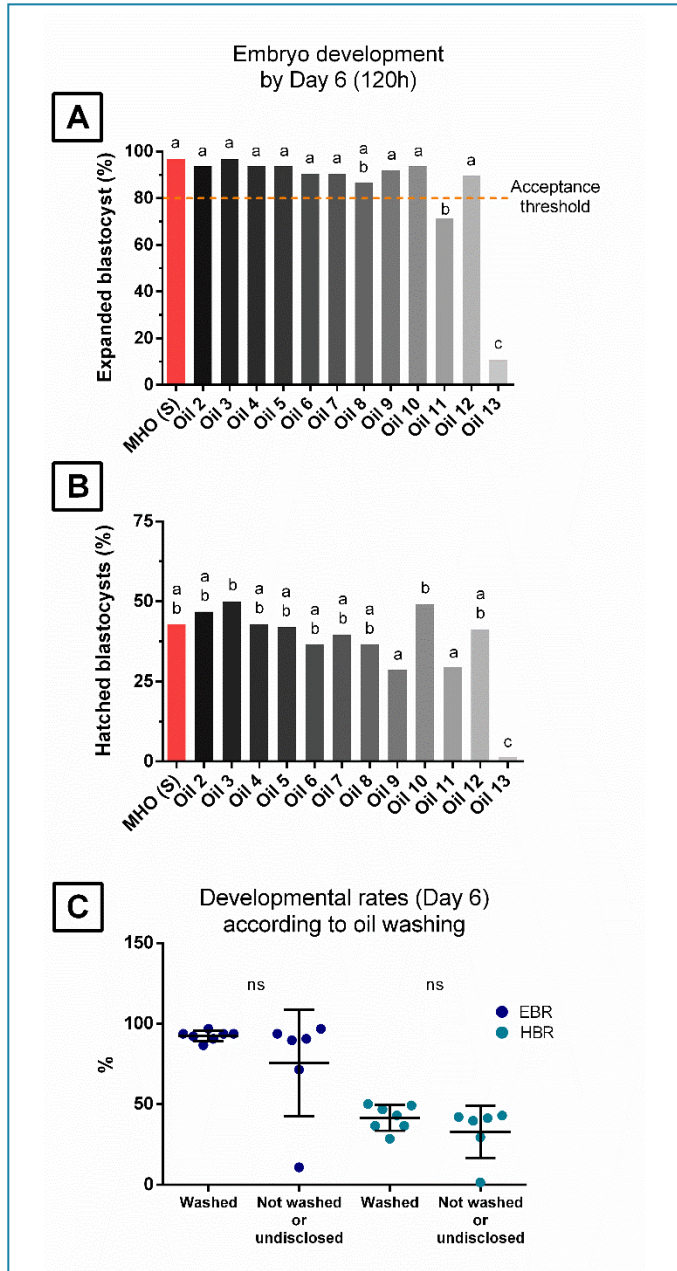
Discussion

The use of oil during manipulation and culture of human gametes and embryos creates a physical layer between the medium and the atmosphere, thus reducing the chances of contamination and also minimizing fluctuations of culture conditions, including temperature, pH and osmolality (Morbeck and Leonard, 2012). However, while many studies have typically focused in comparing and thoroughly characterizing commercial culture media (Morbeck *et al.*, 2014), oil remains an under-studied component of the culture system. Even

Figure 6 - Results after *in vitro* mouse embryo culture for 6 days with each of the studied oils. The majority of the oils surpassed the required blastocyst formation rate threshold (80%) by day 6 of culture; some toxicity was detected in oil 11, with a slightly under-the-threshold result (71% of the embryos reaching the expanded blastocyst stage), and major toxicity was detected in oil 13, with an 11% blastocyst formation rate (A). The proportion of completely hatched blastocysts at the end of culture was also compared between oils; besides oil 11 and 13, oil 9 also showed a lower hatched blastocyst rate than the rest of groups, which was statistically significant when compared to oils 3 and 10 (B). Finally, the expanded and hatched blastocyst rates were compared between the oils that had been pre-washed by the manufacturer and those that had not or for which this information was not disclosed, with no differences detected between them (C). A different superscript within the same graph indicates a statistically significant difference between groups; the p-values for all comparisons can be found in the supplementary tables VI and VII, respectively.

MHO (S) = Mineral Heavy Oil (Sigma); EBR = Expanded blastocyst rate; HBR = Hatched blastocyst rate; ns = not significant.

though the manufacturing processes to produce oil are quite constant, many differences exist between the commercial brands of oil marketed to be used in human IVF. Viscosity, a measure of a fluid's resistance to flow, and density, a measure of mass per unit of volume, are two factors that depend on the origin and chemical composition of the oil and, thus, are highly variable between brands (Sattarin *et al.*, 2007; Esteban *et al.*, 2012). While some publications have observed a direct correlation between viscosity and density of the oils (Alomair *et al.*, 2016), our results suggest that their correlation is far from perfect. Our data shows some variability especially in oils with low viscosities of around 20 cP at 30ºC, which present differing densities between 830 and 850 mg/ml at 30ºC and, consequently, both characteristics should not be inferred from one another. Unfortunately, information about the exact viscosity and density of the oils is typically not provided by manufacturers of oil, which rely on a dichotomic classification between light oils (low viscosity) and heavy oils (high/medium viscosity). While this classification seems to be on par with their quantified viscosity (Table II), the range within each category is still quite wide (12.0 – 22.4 and 43.1 – 110.7 cP, respectively), and embryologists are forced to choose which oil to use with scarce and insufficient information about the potential consequences of such a decision.

In an IVF lab, culture dishes must be prepared in advance to allow their equilibration, prior to be used. pH is the factor that requires a longer incubation time to change and stabilize, as

CO_2 must diffuse through the oil overlay to reach the medium and react with its bicarbonate buffer. Our results indicate that, while some oils can equilibrate fairly quickly, others may need at least an additional three hours just for pH to reach 7.4, and even up to 16 hours to completely stabilize. On the other hand, once a dish is taken out of the incubator for embryo grading or any manipulation technique, the low concentration of CO_2 in the atmosphere may result in a rise of pH. However, some oils present an increased capacity to maintain a stable pH for longer periods of time outside of the incubator, reducing the chances of stress being caused to the embryos. Our experiments suggest that oils with a high viscosity may take a longer time to equilibrate, but will later prevent fluctuations of pH much more efficiently than low viscosity oils. While a good non-linear correlation has been established between oil viscosity and the increase of pH, one of the studied oils, specifically the one with the highest viscosity, did not fit into this model. However, since this was the only oil not specifically produced for human IVF (Mineral heavy oil, Sigma), it is possible that some differences in the production and refinement steps have had an impact on the hydrocarbons and their structure that discerns it from the other oils. Thus, future studies that determine the exact chemical composition of the oils could help to better identify the role that the oil's viscosity and other factors play in the passive diffusion of CO_2 and, consequently, in the stability of pH or other factors.

An even greater difference was observed between the studied oils in their capacity to stabilize osmolality during culture or, in other words, prevent media evaporation. Osmolality is a critical factor of the culture system due to its direct impact on embryo development and viability (Brinster, 1965; Hay-Schmidt, 1993; Dawson and Baltz, 1997). In addition, osmolality is also prone
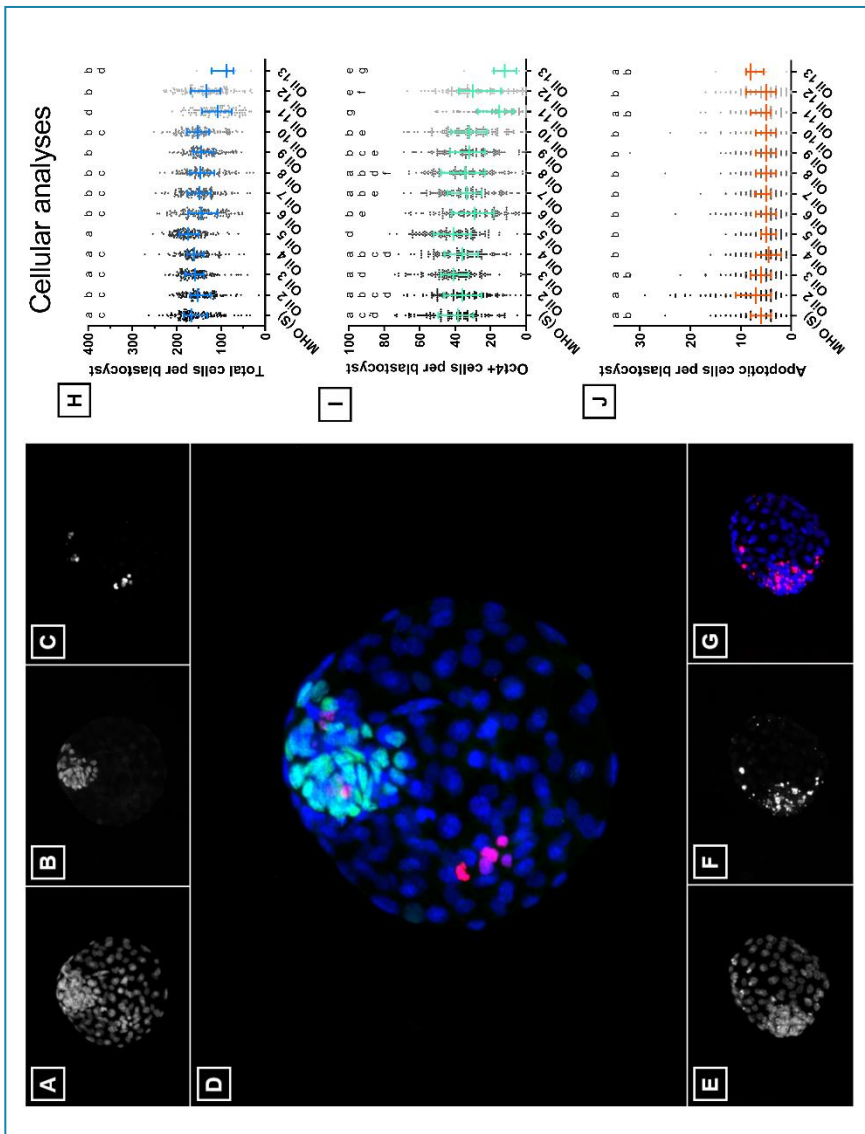
to become inadvertently altered during routine lab work (Swain *et al.*, 2012; Mestres *et al.*, 2021), especially during prolonged periods of culture in dry atmosphere incubators. Previous publications have already shown variations in the media's evaporation between "light" and "heavy" oils (Swain, 2018; Yumoto *et al.*, 2018, 2019) and, recently, Mullen described both a linear association between oil density and the rise of osmolality and a non-linear association between oil viscosity and osmolality increases (Mullen, 2021). Our results concur in establishing a non-linear correlation between the viscosity of the oils and the rise of osmolality, with high-viscosity oils presenting a bigger potential to minimize evaporation; however, in our case the linear model comparing the rise of osmolality with oil density was not a good fit. It is possible that

Figure 7 - **Fluorescence cellular analyses of the blastocysts obtained after 6-day culture.** Embryos were triple stained to assess under fluorescence microscopy the total number of cells (A, in blue), the number of Oct4+ pluripotent cells forming the inner cell mass (B, in green) and the number of apoptotic cells (C, in red). Picture (D) shows a composite image combining all three fluorescent markers. A positive control for the TUNEL kit, used as an apoptotic cell marker, was performed by briefly exposing blastocysts to UV light prior to staining; an increase in the proportion of apoptotic cells was clearly noted (E-G). The number of cells for each group and each of the markers is represented in graphs H-J, with the colored lines indicating the median ± IQR for each group. A different superscript within the same graph indicates a statistically significant difference between groups, and all p-values can be found in the supplementary tables VIII-X.
MHO (S) = Mineral Heavy Oil (Sigma).

the inclusion of a higher number of groups or the disparities in the methodology used to quantify the oils' viscosities may explain this difference. Mullen also described a triple model to predict changes in osmolality which identified the surface area between the medium droplet and the oil, the thickness of the oil overlay and the density of the oil as the three main factors affecting evaporation. Since the same methodology regarding the volume of oil and medium was maintained throughout our experiments, any potential impact on osmolality originated from these variables can be ruled out. Additionally, Yumoto recently described a small reduction in evaporation and an increased presence of water in the oil after washing it before culture (Yumoto *et al.*, 2019), whilst we have found no difference in the evaporation rate between washed and non-washed oils, in agreement with others (Swain *et al.*, 2016). Since Yumoto showed that permeation of water into the oil occurred overnight, we could hypothesize that evaporation of that water from the oil could also occur overtime after washing it. This would explain why no difference is observed when using oils pre-washed by the manufacturer, as time has elapsed since its washing and water may already have evaporated, but it could make a difference when oil is saturated with water just prior to the start of the culture.

Regarding temperature, we analyzed the dynamics of the in-drop temperature in a culture dish when taking it out of the incubator and found an initial 5-minute critical period in which temperature decreased the fastest. While some statistically

significant differences were noted in the cooling rate of various oils during this period of time, these were not related to the oils' viscosity nor density, and the raw difference in the cooling rate was small, less than 0.1°C/min. Similarly, no differences were observed between any of the oils regarding their warming rate once the dishes were returned to the incubator. As some introductory data seemed to show, it is likely that temperature dynamics is more directly linked to other factors, such as the design of the culture dishes and the volume of oil used, than to the oils' viscosity or density (Mestres *et al.*, 2016).

While oil plays an essential role on the stabilization of pH, osmolality and temperature, a special focus should be given to its potential role as a source of toxicity, since toxins present in oil may transfer to culture media and affect the embryos in culture (Otsuki *et al.*, 2009; Martinez *et al.*, 2017a). Oil may become contaminated with many elements during its production and, inconveniently, it may also spontaneously oxidize and become toxic later, over time and especially if exposed to sunlight or high temperatures (Gardner *et al.*, 2005; Otsuki *et al.*, 2007; Khan *et al.*, 2013). While not common, due to the strict regulations and testing applied to IVF consumables, the origin of the toxicity of some oil batches has been pinpointed to the contamination of the oil with elements such as zinc, Triton X-100 and, most commonly, peroxides (Otsuki *et al.*, 2007, 2009; Morbeck *et al.*, 2010; Turner, 2010; Morbeck, 2012). While some companies differentiate between mineral and paraffin oil, stating that the latter should be further

refined, it has been suggested that their molecular composition may not be that different and the disparity in their nomenclature could be due to regulatory and marketing strategies (Morbeck, 2018). Recently, the first synthetic oil, not derived from petroleum, for human IVF has been introduced (Ovoil heavy™, Vitrolife, Sweden). While it has not shown differences regarding viscosity and peroxidation compared to some of the other oils in this study, synthetic oils could prove to be an interesting strategy to better control the exact molecular composition of the oil, even though this would still not rule out the possibility of contamination and toxicity being found in the oil (Erbach *et al.*, 1995; Mellbin, 2021).

We determined the initial concentration of peroxides in the thirteen oils under study and all of them presented levels of <0.1 mE1/kg, which would not affect embryo viability. As expected, after a two-week exposure to sunlight, the POV increased in all the oil samples, with marked differences in the final POV among them. However, these had no association with the oils' viscosity or density, nor their mineral or synthetic origin or their pre-washing. It could be hypothesized that the resistance of the oils to oxidation may vary depending on the initial chemical structure of their hydrocarbons and their potential reactivity, which could be better determined in future studies through atomic fingerprinting. In addition, the elapsed storage time between production and testing of the oil could have been valuable data to better interpret these results but,

unfortunately, this information is not provided by all oil manufacturers at the moment.

Finally, considering the previously described variations in embryo culture associated with oil (Sifer *et al.*, 2009), we tested all the oil samples with an adapted protocol of the Mouse Embryo Assay described previously (Mestres *et al.*, 2019), aiming to detect even slight levels of embryo-toxicity. Using the standard MEA end-point solely based on the blastocyst formation rate, we were able to identify one sample with low toxicity (11) and one sample with high toxicity (13), which was unexpected considering that the selected oils included in this study were already on the human IVF market and, thus, should have been previously embryo-tested. While no significant differences in embryo development have been observed after comparing washed and non-washed/undisclosed oils, the fact that both the oils that showed toxicity in the standard MEA were part of the non-washed/undisclosed group is an interesting remark. By adding a secondary end-point to the assay, based on the number of blastocysts completing hatching by day 6, one more sample was identified that presented a lower proportion of hatched blastocysts than the rest of oils (9). Furthermore, several publications have shown that the total number of cells per embryo (Scott *et al.*, 1993), the number of cells in the ICM (Lane and Gardner, 1997), and the number of apoptotic cells (Hughes *et al.*, 2010), could be more sensitive detectors of embryo-toxicity and predictors of embryo viability than just embryo development data. In our results, assessing

the total cell number and the ICM cell number allowed to detect more statistically significant differences between groups than using uniquely the blastocyst formation or blastocyst hatching rates; the apoptotic index, however, was not a good marker to differentiate between our study groups. While the sensitivity of our testing methodology has been validated for the detection of peroxides, the detrimental effects of volatile organic compounds or other contaminants potentially present in the oils should not be ruled out (Martinez *et al.*, 2017b), and would be an interesting addition to in future studies.

In conclusion, there are currently several commercial oils for human IVF on the market, presenting big differences between them that go beyond just comfort and work habit. While the high or low viscosity of an oil will not have a negative impact per se, it may result in important variations in the culture system, which must be adjusted in order not to affect culture conditions, embryo viability and clinical outcomes. In addition, the detection of commercial oil samples of subpar quality showcases the importance of thorough, specialized and regulated oil testing prior to its release to the market, which embryologists should demand. Similarly, manufacturers should be encouraged to be as transparent and clear as possible in the provided information regarding their oils' characteristics and quality control testing.

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	0.0182	-	-	-	-	-	-	-	-	-	-	-
Oil 3	0.3047	0.9873	-	-	-	-	-	-	-	-	-	-
Oil 4	0.4566	0.9467	>0.9999	-	-	-	-	-	-	-	-	-
Oil 5	0.0191	>0.9999	0.9888	0.9511	-	-	-	-	-	-	-	-
Oil 6	0.9989	0.1748	0.8719	0.9552	0.1815	-	-	-	-	-	-	-
Oil 7	0.9662	0.3989	0.9857	0.9981	0.4102	>0.9999	-	-	-	-	-	-
Oil 8	0.9371	0.4805	0.9943	0.9995	0.4926	>0.9999	>0.9999	-	-	-	-	-
Oil 9	>0.9999	0.0157	0.2765	0.4216	0.0165	0.9981	0.9552	0.9206	-	-	-	-
Oil 10	>0.9999	0.0343	0.4448	0.6159	0.0360	>0.9999	0.9924	0.9820	>0.9999	-	-	-
Oil 11	0.9888	0.0005	0.0191	0.0377	0.0005	0.6159	0.3244	0.2587	0.9924	0.9552	-	-
Oil 12	0.9839	0.0004	0.0165	0.0327	0.0005	0.5788	0.2951	0.2335	0.9888	0.9420	>0.9999	-
Oil 13	0.0135	<0.0001	<0.0001	<0.0001	<0.0001	0.0009	0.0002	0.0001	0.0157	0.0069	0.2417	0.2675

Supplementary table I - Detailed p-values after group analysis (ANOVA) and multiple comparison correction (Tukey) for the pH of the culture medium after 15 minutes out of the incubator, using each of the oils under study. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	<0.0001	-	-	-	-	-	-	-	-	-	-	-
Oil 3	<0.0001	>0.9999	-	-	-	-	-	-	-	-	-	-
Oil 4	<0.0001	0.9689	0.9988	-	-	-	-	-	-	-	-	-
Oil 5	<0.0001	0.9922	>0.9999	>0.9999	-	-	-	-	-	-	-	-
Oil 6	0.8893	<0.0001	<0.0001	<0.0001	<0.0001	-	-	-	-	-	-	-
Oil 7	0.5329	<0.0001	<0.0001	0.0001	<0.0001	>0.9999	-	-	-	-	-	-
Oil 8	0.9875	<0.0001	<0.0001	<0.0001	<0.0001	>0.9999	0.9970	-	-	-	-	-
Oil 9	0.2042	<0.0001	<0.0001	0.0006	0.0003	0.9901	>0.9999	0.9022	-	-	-	-
Oil 10	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	0.4881	0.1725	0.7731	0.0445	-	-	-
Oil 11	0.9939	<0.0001	<0.0001	<0.0001	<0.0001	0.2326	0.0621	0.4771	0.0133	>0.9999	-	-
Oil 12	0.5329	<0.0001	<0.0001	<0.0001	<0.0001	0.0153	0.0027	0.0484	0.0004	0.9142	0.9931	-
Oil 13	0.0005	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0038	0.0140	0.2181

Supplementary table II - Detailed p-values after group analysis (ANOVA) and multiple comparison correction (Tukey) for the pH of the culture medium after 30 minutes out of the incubator, using each of the oils under study. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	0.5243	-	-	-	-	-	-	-	-	-	-	-
Oil 3	0.3289	0.6696	-	-	-	-	-	-	-	-	-	-
Oil 4	0.9147	0.4585	0.2844	-	-	-	-	-	-	-	-	-
Oil 5	0.7005	0.2424	0.1202	0.7951	-	-	-	-	-	-	-	-
Oil 6	0.7103	0.2496	0.1247	0.805	0.9889	-	-	-	-	-	-	-
Oil 7	0.07135	0.1323	0.2357	0.06205	0.01918	0.02002	-	-	-	-	-	-
Oil 8	0.3163	0.6394	0.9568	0.2738	0.1167	0.121	0.2631	-	-	-	-	-
Oil 9	0.6047	0.2248	0.1223	0.6878	0.8554	0.8459	0.02308	0.1184	-	-	-	-
Oil 10	0.5153	0.1286	0.05463	0.6063	0.777	0.7665	0.007908	0.0537	0.9492	-	-	-
Oil 11	0.7894	0.743	0.5049	0.7125	0.5005	0.5093	0.123	0.4854	0.4358	0.3438	-	-
Oil 12	0.1309	0.256	0.4353	0.1134	0.03856	0.04019	0.6693	0.4745	0.04388	0.01623	0.2181	-
Oil 13	0.09071	0.009227	0.003181	0.1217	0.1454	0.1426	0.0005287	0.003277	0.2529	0.2147	0.05034	0.001014

Supplementary table III - Detailed p-values after linear regression and comparison of the in-drop cooling rate during the first 5 minutes after taking a dish out of the incubator and placing it on a heated surface. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	0.9343	-	-	-	-	-	-	-	-	-	-	-
Oil 3	0.5094	0.5144	-	-	-	-	-	-	-	-	-	-
Oil 4	0.1964	0.2359	0.4826	-	-	-	-	-	-	-	-	-
Oil 5	0.3526	0.3765	0.7542	0.7132	-	-	-	-	-	-	-	-
Oil 6	0.78	0.7438	0.7128	0.3116	0.5163	-	-	-	-	-	-	-
Oil 7	0.3092	0.3406	0.6979	0.7453	0.9528	0.4661	-	-	-	-	-	-
Oil 8	0.07803	0.1166	0.2176	0.5992	0.3813	0.1361	0.3879	-	-	-	-	-
Oil 9	0.3085	0.3387	0.6891	0.7662	0.9385	0.4626	0.9841	0.4119	-	-	-	-
Oil 10	0.2861	0.3159	0.6369	0.8431	0.8737	0.4285	0.9142	0.4874	0.9311	-	-	-
Oil 11	0.4535	0.463	0.8921	0.6047	0.872	0.6352	0.8226	0.3168	0.8114	0.755	-	-
Oil 12	0.4575	0.4673	0.9048	0.5854	0.8557	0.6427	0.805	0.2984	0.794	0.7377	0.9861	-
Oil 13	0.2987	0.3213	0.6257	0.9079	0.8385	0.433	0.8738	0.5811	0.8894	0.9527	0.7318	0.7161

Supplementary table IV - Detailed p-values after linear regression and comparison of the in-drop warming rate during the first 4 minutes after returning a dish to the benchtop incubator. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	0.02334	-	-	-	-	-	-	-	-	-	-	-
Oil 3	0.04419	0.6895	-	-	-	-	-	-	-	-	-	-
Oil 4	0.2313	0.273	0.4481	-	-	-	-	-	-	-	-	-
Oil 5	0.1018	0.0001397	0.000234	0.005129	-	-	-	-	-	-	-	-
Oil 6	0.8792	0.006875	0.01355	0.1296	0.07596	-	-	-	-	-	-	-
Oil 7	0.0002774	<0.0001	<0.0001	<0.0001	0.02668	<0.0001	-	-	-	-	-	-
Oil 8	0.001142	<0.0001	<0.0001	<0.0001	0.06926	0.0002658	0.7162	-	-	-	-	-
Oil 9	<0.0001	<0.0001	<0.0001	<0.0001	0.00296	<0.0001	0.4589	0.2738	-	-	-	-
Oil 10	0.2853	0.003335	0.00602	0.04038	0.8017	0.288	0.0467	0.09317	0.009982	-	-	-
Oil 11	0.001014	<0.0001	<0.0001	<0.0001	0.05114	0.0003012	0.9451	0.7915	0.4573	0.06941	-	-
Oil 12	0.003161	<0.0001	<0.0001	<0.0001	0.137	0.001	0.4925	0.7497	0.1566	0.1552	0.5753	-
Oil 13	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002849	0.0001012	0.00252	<0.0001	0.000646	<0.0001

Supplementary table V - Detailed p-values after linear regression and comparison of the daily osmolality raise during culture in a dry benchtop incubator, using each of the 13 oils under study. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

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	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	>0.9999	-	-	-	-	-	-	-	-	-	-	-
Oil 3	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-	-
Oil 4	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-
Oil 5	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-	-
Oil 6	0.8788	>0.9999	0.8788	>0.9999	>0.9999	-	-	-	-	-	-	-
Oil 7	0.8788	>0.9999	0.8788	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-
Oil 8	0.0663	>0.9999	0.0663	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-
Oil 9	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-
Oil 10	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-
Oil 11	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	0.0026	0.0026	0.0663	<0.0013	<0.0013	-	-
Oil 12	0.5421	>0.9999	0.5421	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0052	-
Oil 13	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013

Supplementary table VI - Detailed p-values after the analysis of the expanded blastocyst formation rates obtained after 6 days of culture (120h) for each of the oils under study (Fisher's exact test) with Bonferroni correction for multiple comparison. Significance was set at $\alpha = 0.05$. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	>0.9999	-	-	-	-	-	-	-	-	-	-	-
Oil 3	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-	-
Oil 4	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-
Oil 5	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-	-
Oil 6	>0.9999	>0.9999	0.5421	>0.9999	>0.9999	-	-	-	-	-	-	-
Oil 7	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-
Oil 8	>0.9999	>0.9999	0.5421	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-
Oil 9	0.3276	0.0533	0.0104	0.3276	0.4511	>0.9999	>0.9999	>0.9999	-	-	-	-
Oil 10	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.728	>0.9999	0.728	0.0156	-	-	-
Oil 11	0.4628	0.0819	0.0156	0.4628	0.6279	>0.9999	>0.9999	>0.9999	>0.9999	0.0247	-	-
Oil 12	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.6136	>0.9999	0.8411	-
Oil 13	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013

Supplementary table VII - Detailed p-values after the analysis of the ratio of completely hatched blastocysts obtained after 120h of culture with each of the oils under study (Fisher's exact test) with Bonferroni correction for multiple comparison. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	>0.9999	-	-	-	-	-	-	-	-	-	-	-
Oil 3	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-	-
Oil 4	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-
Oil 5	0.7056	<0.0001	0.2870	0.2154	-	-	-	-	-	-	-	-
Oil 6	0.2318	>0.9999	0.5927	>0.9999	<0.0001	-	-	-	-	-	-	-
Oil 7	>0.9999	>0.9999	>0.9999	>0.9999	0.0003	>0.9999	-	-	-	-	-	-
Oil 8	0.1194	>0.9999	0.3201	0.7322	<0.0001	>0.9999	>0.9999	-	-	-	-	-
Oil 9	0.0035	>0.9999	0.0126	0.0416	<0.0001	>0.9999	>0.9999	>0.9999	-	-	-	-
Oil 10	>0.9999	>0.9999	>0.9999	>0.9999	0.0006	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-
Oil 11	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	0.0046	<0.0001	-	-
Oil 12	0.0023	>0.9999	0.0085	0.0287	<0.0001	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0088	-
Oil 13	0.0059	0.1480	0.0092	0.0129	<0.0001	0.3054	0.0998	0.3990	0.9553	0.0807	>0.9999	>0.9999

Supplementary table VIII - Detailed p-values after group analysis (Kruskal-Wallis) and multiple comparison correction (Dunn's) for the total cell number per blastocyst after 120h of culture with each of the oils under study. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	>0.9999	-	-	-	-	-	-	-	-	-	-	-
Oil 3	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-	-
Oil 4	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-
Oil 5	>0.9999	0.4668	>0.9999	>0.9999	-	-	-	-	-	-	-	-
Oil 6	0.0008	0.5103	<0.0001	0.2374	<0.0001	-	-	-	-	-	-	-
Oil 7	0.6096	>0.9999	0.0671	>0.9999	0.0162	>0.9999	-	-	-	-	-	-
Oil 8	>0.9999	>0.9999	0.3195	>0.9999	0.0918	>0.9999	>0.9999	-	-	-	-	-
Oil 9	0.0501	>0.9999	0.0034	>0.9999	0.0006	>0.9999	>0.9999	>0.9999	-	-	-	-
Oil 10	0.0109	>0.9999	0.0006	>0.9999	<0.0001	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-
Oil 11	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	-	-
Oil 12	<0.0001	0.0155	<0.0001	0.0064	<0.0001	>0.9999	0.4918	0.1497	>0.9999	>0.9999	0.0017	-
Oil 13	0.0017	0.0205	0.0005	0.0137	0.0003	0.6053	0.0766	0.0454	0.1879	0.2933	>0.9999	>0.9999

Supplementary table IX - Detailed p-values after group analysis (Kruskal-Wallis) and multiple comparison correction (Dunn's) for the number of Oct4+ cells per blastocyst after 120h of culture with each of the oils under study. Significance was set at $\alpha = 0.05$. MHO (S) = Mineral Heavy Oil (Sigma).

	MHO (S)	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10	Oil 11	Oil 12
Oil 2	>0.9999	-	-	-	-	-	-	-	-	-	-	-
Oil 3	>0.9999	>0.9999	-	-	-	-	-	-	-	-	-	-
Oil 4	0.1438	<0.0001	0.2022	-	-	-	-	-	-	-	-	-
Oil 5	0.1327	<0.0001	0.1889	>0.9999	-	-	-	-	-	-	-	-
Oil 6	>0.9999	0.0021	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-	-
Oil 7	>0.9999	0.0305	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-	-
Oil 8	>0.9999	0.0040	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-	-
Oil 9	>0.9999	0.0010	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-	-
Oil 10	>0.9999	0.0029	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-	-
Oil 11	>0.9999	0.3050	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-	-
Oil 12	>0.9999	0.0375	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	-
Oil 13	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999

Supplementary table X - Detailed p-values after group analysis (Kruskal-Wallis) and multiple comparison correction (Dunn's) for the number of apoptotic cells per blastocyst after 120h of culture with each of the oils under study. Significance was set at α = 0.05. MHO (S) = Mineral Heavy Oil (Sigma).

Author's roles

EM, NCB and GC designed the study. EM performed the density determination and pH experiments. QMA, AV and EM participated in the temperature measurements. EM, AC, AV, QMA, MA and MGJ participated in the osmolality determination experiments and performed the Mouse Embryo Assays with daily embryo grading. The fixation and triple staining of the blastocysts was carried out by EM, and the posterior counting of cells was done by EM, QMA, AC, AV and AMC. EM collected and analyzed the data, which was further discussed and interpreted with NCB and GC. EM drafted the manuscript and all authors participated in its revision.

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Conflict of interest

The authors have no conflicts of interest to declare in relation with the current work.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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DISCUSSION

The embryo culture system comprises many different elements which play a fundamental role on the stability of the *in vitro* culture conditions and, consequently, may potentially alter embryo development and embryo quality. Keeping some critical parameters, namely temperature, pH, osmolality and air quality, within an optimal and narrow range is a key factor to optimize the culture system, reduce embryonic stress, and achieve the best possible clinical outcomes. Equally important is to ensure the absence of embryo-toxicity in all products, materials or equipment that will have a direct or indirect contact with gametes or embryos during their *in vitro* culture. Quality Control testing and, in particular, the Mouse Embryo Assay, is an essential bioassay that uses the mouse model to screen for toxicity, ensuring that none of these products, used in the human IVF laboratory, will result detrimental to the gametes and embryos. In the present no regulation exists on how to perform a MEA, albeit the FDA has published a set of guidelines outlining some recommendations on testing ART devices, both during premarket approval processes and routine lot screening for already commercialized products [238]. However, there is still no consensus regarding testing protocols or methodologies used by QC testing laboratories, which vary widely between centers [237].

In this context, this thesis aimed, on the one hand, to explore the role of different parameters of the culture system and evaluate their importance on the stabilization of the culture conditions, and, on the other hand, to optimize the

methodology of the MEA to achieve the highest sensitivity when testing mineral oil, as this is often considered as a potential source of major embryo-toxicity in the culture system.

Stabilizing osmolality by reducing media evaporation

On the first study, we selected osmolality as the parameter with a widest room for improvement, since regular temperature and pH checks were already performed routinely in our laboratory. Osmolality is also a very variable factor which has a high relevance in the current IVF context. The development in the last decades of specialized consumables, materials and pieces of equipment specifically designed for human IVF has led to a substantial number of changes in clinical methodologies. Specifically, single-use culture media with modern re-formulations, better QC programs, and new benchtop and time-lapse incubators have allowed for many centers to adapt an "uninterrupted culture" approach, and prolong the embryo *in vitro* culture during six or even seven days, aiming to obtain high quality blastocysts and proceed with a single embryo transfer while increasing their implantation and pregnancy rates. However, while such a strategy presents some undeniable advantages, it can also magnify the detrimental effects of any weak links in the culture system.

One of the factors which may be easily affected during long-term culture is osmolality, as a consequence of the evaporation of the medium during the preparation and handling of the dishes, as well as during culture. While studies in human are

lacking, the mouse model has shown that the exposure to hyperosmotic media, above 300 mOsm, can trigger embryo stress responses and activate cell volume regulation mechanisms, which require the use of the limited embryonic energetic resources and may lead to reduced developmental outcomes [67–75,262,273,275,276]. In addition, medium evaporation induces the concentration of all its components and the modification of its original intended formula, including the concentration of bicarbonate with the subsequent and unintended rise in pH, ensuing the potential impairment of embryo development [77]. Thus, appropriate measures should be taken to minimize the evaporation of the media.

One of the variables with the highest impact on evaporation is the environmental humidity within the incubators used for embryo culture. Incubators on the market are typically referred to as "humid" or "dry" depending on their capacity to work with a high internal humidity, or lack thereof. The majority of modern time-lapse and benchtop incubators are "dry", meaning that they do not support a highly humidified atmosphere, a paradigm shift compared to traditional cell culture incubators. The rationale behind this change was that working in a dry atmosphere would help to reduce the chances of contamination associated with moisture in the incubators. However, by using small portable hygrometers we have detected significant differences not only between humid and dry incubators, which would be expected, but also between pairs of dry and pairs of humid incubators. These differing

humidity levels are probably caused by the specific humidification mechanism used by each incubator (e.g., a big water pan on the bottom of the incubator, a water bottle in the gas inlet, etc.) and, importantly, we have shown their direct impact on the evaporation rate of the medium. Accurate humidity measurements in each incubator could result much more informative than the current dichotomous dry/humid classification, and would also allow the publication of prospective randomized control trials in the human, that could correlate humidity levels with clinical outcomes, in addition to the currently available publications [89,265]. Importantly, these results do not necessary point towards inherently faulty conditions provided by dry incubators, but indicate that working with a dry atmosphere may require the application of other strategies to avoid the excessive evaporation of medium during culture. While some home-made systems have been proposed to increase the humidity in dry incubators, their reported efficiency is mediocre, with only an achieved relative humidity of around 40% [63]; moreover, the potential undesired effects of placing water in unprepared systems, such as the formation of condensation on the incubator's lids or the malfunction of its sensors, should be carefully considered before proceeding with such strategies.

Our data also showed a small but significant difference in the evaporation rate between a basic saline medium (HTF) and a complex-formulation medium (KSOM), which occurred faster in the latter regardless of their protein supplementation. While

the exact cause of this difference should be further studied in the future, our hypothesis is that the emulsifying properties of some of the amino acids present in KSOM's formulation could explain its slightly increased evaporation. Of higher importance is the marked difference in the initial osmolality between both media, approximately 285 and 255 mmol/kg in HTF and KSOM, respectively. In our model, it took only 72 hours of culture for osmolality to increase above the 300 mmol/kg threshold in the HTF groups, and it continued rising until reaching a final value of >325 mmol/kg. By contrast, the initially hypoosmotic levels of KSOM delayed the surpass of the same threshold until the 144-hour time-point, and reduced the final value down to 305 mmol/kg, barely above the 300 mmol/kg mark. In accordance with the literature, these results suggest that using a medium with an initially low osmolality could be a safer strategy to avoid reaching hypertonic osmolalities after long-term culture in dry incubators [65].

In addition to humidity and the culture medium, the design of the culture dish can also play a role in the evaporation rate. We selected six time-lapse dishes because of the inherent differences in each dish's unique design, such as the number of wells and the volume of oil and media they support, and also because their preparation protocol is typically detailed by the manufacturers themselves, thus avoiding many potential differences in dish preparation between laboratories. Firstly, we analyzed whether a significant evaporation of medium occurred during the dish setup, and found that this was indeed

the case in three out of the six studied dishes. In these three cases, osmolality was between 3 and 5 mmol/kg higher than in the fresh medium prior to the start of the culture, and thus the chances of reaching hyperosmotic levels by the end of culture were increased. Secondly, we were able to describe significantly different evaporation rates during the 7-day culture depending on the dish design, hence classifying three dishes with a low evaporation rate (<4 mmol/kg/day), two dishes with an intermediate one (4-5 mmol/kg/day) and one dish with a high evaporation rate (≥6 mmol/kg/day). As expected, this translated into dramatically different osmolalities by the end of culture, ranging between 278 mmol/kg and 300 mmol/kg. While in this case even the group with the fastest evaporation did not reach levels above the aforementioned 300 mmol/kg threshold, it should be noted that KSOM medium was used for these experiments and, had HTF or a similar medium with an initially higher osmolality been used instead, hyperosmotic levels could have been reached at a much earlier time-point. As discussed below, the volume of the media-filled wells and, specially, the thickness of the oil overlay allowed by each dish's design are the most probable cause for the observed differences in evaporation between dishes. This concurs with previously published data which also showcased differing osmolality alterations according to the design of the culture dishes [59,60]. In addition, in one of the studied time-lapse dishes, faster evaporation was detected when culture was carried out in its own time-lapse incubator, instead of a regular benchtop

incubator; we hypothesized that this disparity could be originated by the different heat transmission derived from the specific dish-incubator fit.

As exemplified in the non-published data in **Figure 5**, an essential last measure to reduce changes in osmolality is the use of oil to cover medium at all times during gamete/embryo manipulation and culture, which is also paramount to avoid changes in temperature in pH. In accordance with previous studies, our results also showed a higher power to avoid evaporation when bigger volumes of medium and/or oil were used and, especially, when increasing the thickness of the oil overlay placed above the medium [59,60,62,268]. This suggests that oil should be considered a crucial stabilizer of the culture system, which should be used at all times and in sufficient volumes as to maximize the protection of the medium and the gametes/embryos in culture. However, each laboratory should study their own culture system to analyze and choose the most appropriate approaches to stabilize its conditions.

In summary, we have shown that the volume of oil and the type of medium have a moderate impact on osmolality, while the most relevant aspect of the latter is its initial osmolality. In addition, the use of oil, the humidity in the incubators, and the design of the culture dishes all are pivotal in the stabilization of osmolality. The modification of any of these parameters could have a potential impact on the stability of osmolality within the culture system. This information is of the utmost importance to

embryologists, as well to manufacturers who must identify the strong and weak points of their products to improve them or design some new ones. The results published in this and other projects have already resulted relevant in the design of new dishes that provide reduced rates of medium evaporation [277].

Aiming to accelerate changes in evaporation and identify those factors related with evaporation, an oil with a low viscosity was used for all the aforementioned experiments, as recommended by the available literature [62,64,90]. However, no correlation had been established yet between the physical characteristics of different oils and their behavior in the culture system, which was set as our next objective.

Oil makes a difference: comparing the effects that several oils have on the culture system

Oil is a non-polar hydrophobic liquid that is used in IVF laboratories to create a physical barrier between the culture medium and the air in the lab or within the incubators. As seen before, using any oil during gamete/embryo culture and handling provides valuable stabilizing effects, reducing the fluctuations of temperature, pH and osmolality, as well as protecting against contamination and detrimental particles found in the air. Several manufacturers offer commercial oil for human IVF in the present day, and the differences among these are noteworthy. In particular, they show a wide range in their viscosity and density due to the diverse molecular length and structure of their hydrocarbons, although this information

is rarely disclosed by the manufacturers. Literature is scarce regarding the significance that these characteristics may have on the oils' protective capacities.

We selected twelve commercial oils used for human IVF plus an extra one not used for human clinical applications, but which could be useful due to its high viscosity. The dynamic viscosity was quantified in all the samples and varied between 12.0 and 110.7 cP at 30ºC; similarly, their density ranged between 835.4 and 868.1 mg/ml. These results were comparable to other studies analyzing viscosity and density in oil samples, with results between 8-66 cP for viscosity and between 825-862 mg/ml for density [62,64,268]; unfortunately, none of these studies seemed to include any of the oil brands with a considerable higher viscosity (>100 cP), which were included in our study. While some groups have reported that density in oils is directly proportional to their viscosity [62,272], our results concur with others that show a poor correlation between both variables [268], suggesting that they should be treated separately when assessing their effects on the following experiments.

The limited literature comparing different oil samples has typically focused on their capacity to prevent evaporation and hence reduce changes in the medium's osmolality, which we also analyzed as part of our study. Our setup to monitor osmolality on a daily basis consisted in 35 mm Petri dishes prepared with 4 ml of oil and 20 µl droplets of medium. The rationale behind this design was, on the one hand, to use a

setup as close as possible to the one used in human IVF, which tends to use small micro-droplets for culture, and, on the other hand, maximizing osmolality changes due to the reduced size of the droplets and their high surface:volume ratio (SA:vol), which has been associated with an increased medium evaporation [62,268]. This was only possible due to the vapor-pressure osmometer used in our experiments, which only requires 10 µl of sample to perform the analysis, contrarily to other osmometers on the market. Unfortunately, the differences in the design of the dishes and the volume of medium and oil used make it impossible to compare the raw evaporation rates with other publications which, for example, use 50 µl or even 200 µl of medium instead of the 20 µl micro-droplets used in our study [62]. However, the evaporation tendencies depending on the oils' characteristics should remain similar.

In agreement with our data, other groups have reported that a higher degree of evaporation is observed when using low-viscosity oils compared to those with a viscosity on the higher side [62,64,90]. We were able to identify a non-linear association between the oil's viscosity and the reduction of evaporation, coinciding with Mullen's recent publication [268]; whilst they also described a linear association between the oils' densities and changes in osmolality, such correlation could not be confirmed by our data. It is possible that the inclusion of additional test groups in our dataset, including those with a density above 860 mg/ml which were not included in Mullen's study, could explain

the difference in the linear model's fit. Interestingly, Mullen also described a complex osmolality model with an excellent prediction power, which accounted for the triple synergic effect of the oils' density, the thickness of the oil overlay and the SA:vol of the micro-droplets. Considering how our viscosity non-linear model seems to have a better correlation with osmolality changes than the density linear one, it would be interesting to assess a similar complex prediction model in future studies, which used viscosity data instead of density.

To analyze temperature stability, we measured the in-drop temperature in a 35 mm Petri dish prepared with 4 ml of oil and a central 20 µl droplet of medium. After overnight equilibration in a benchtop incubator, we firstly monitored temperature after taking the dish out of the incubator and placing it on the warm surface of a laminar flow hood. An initial 5-minute period with a fast-cooling rate (between -0.15 and -0.22°C/min, depending on the oil) was identified and, while there were some statistically significant differences between groups, these were minor (<0.1°C/min) and were not associated to the oils' viscosity nor density. Secondly, we monitored temperature after returning the dish to the benchtop incubator and, in this case, all the oil groups behaved similarly with a warming rate of approximately +0.3°C/min. Whilst not included in this study, it is probable that the warming rate could vary depending on the incubator used for temperature recovery and its heat-transmission mechanism. The minor differences observed

between oils in terms of temperature regulation are in accordance with previously published data [16].

Regarding the necessary pH equilibration prior to using culture dishes, our results using a 4-well dish show that complete equilibration can take between 6 and 16 hours depending on the oil used. We were able to describe a linear association between longer equilibration times and higher oil viscosity and density, with the former being a better predictor than the latter. Granted, these timings would probably vary with different settings (dish type, volume of oil, thickness of the oil overlay, etc.), which should be analyzed in further studies. Similarly, the viscosity of the oil was correlated with its capacity to prevent the rise of pH when using bicarbonate-buffered media outside of the incubator, following a non-linear model. This association was not observed, however, when applied to the oils' densities instead of their viscosities. Out of the thirteen studied oils, five presented a viscosity higher than 40 cP at 30°C. Four of these were the ones that were able to maintain pH below 7.4 even after 30 minutes out of the incubator. Interestingly, the oil with a higher viscosity was the only one which did not follow the proposed viscosity-pH association. Given that this was the only oil of the cohort which is not used in human clinical IVF (Sigma's Mineral Heavy Oil), further experiments could shed a light in which other distinctive characteristics of the oils, perhaps related with their refinement and their molecular composition, play a role on the diffusion of CO_2 through the oil overlay and the fluctuation of the medium's pH.

While the use of any oil, regardless of its physical characteristics, presents undeniable advantages regarding the stability of the culture conditions, its potential toxicity must be considered during manufacturing and excluded through sensitive QC testing, as bad quality oil could result harmful to the embryos or gametes in culture. Despite Mullen's and others' works, studying the physical characteristics of various oils and their relation to osmolality, no in-depth comparative screening had been performed assessing the quality of said commercial oils when actually used for embryo culture. With that objective in mind, we first set out to establish the most sensitive methodology for the Quality Control testing of oil.

Quality Control testing applied to oil samples

As with any other component of the embryo culture system, the oil used in IVF laboratories should pose no danger to the gametes and embryos in culture. Thus, it is imperative that oil gets thoroughly tested in specialized QC centers previous to its actual use on human samples. Unfortunately, oil is quite an heterogenous product, and is considered as one of the most common sources of embryo-toxicity in the culture system; there are three main reasons for that. The first one is the extensive refinement and saturation steps that oil goes through during its manufacture from crude oil, and the many potential contaminants derived from it, such as Triton X-100 (a powerful detergent used in the cleaning of industrial barrels) or peroxides, which are originated by the incomplete saturation of

the hydrocarbon molecules of the oil and represent the most common contaminant found in oil samples. The second one is that oxidation of the oil may occur spontaneously at any point during the oil's shelf life, meaning that even oil that was previously tested could become embryo-toxic at a later time. It has been shown that even an initially low presence of peroxides or reactive molecules in the oil can increase over time, especially if exposed to high temperatures or sunlight during transportation or storage. The third reason is that the specific testing protocols used in QC bioassays have a huge impact on their sensitivity, and hence suboptimal methodologies could result in embryo-toxic samples going undetected. All three explanations point to a shared solution: the development of an effective QC test that is able to detect embryo-toxicity in oil samples with the highest sensitivity possible.

In our study, we hypothesized that some key factors of the Mouse Embryo Assay could have a big impact on the assay's ability to detect low concentrations of peroxides. By irradiating a control oil sample with UV light, we were able to accelerate the formation of peroxides at several concentrations ranging from 0.128 to 0.756 mEq/kg. Only the oil with the lowest concentration of peroxides was used to optimize the MEA, since the rest resulted too embryo-toxic to study any further optimization, and because its POV was the closest to the quantification limit of the peroxide determination test (0.1 mEq/kg). All experiments were performed using embryos at the

1-cell stage collected from a hybrid mouse strain because, as mentioned previously, these have already been shown to be the most appropriate testing conditions for the MEA [227,241,242].

The first condition we studied was the protein supplementation of the medium. By comparing BSA with HSA-based supplements, we showed that BSA produced a significantly higher number of blastocysts or, in other words, it reduced the sensitivity to embryo-toxic conditions. Even though BSA is no longer used in human IVF, these results are still very relevant since some QC testing centers still use BSA to test products that will afterwards be utilized for clinical applications. In addition, we described a much-increased sensitivity when using HSA-based supplementations in a high concentration (5 mg/ml) instead than a lower one (0.5 mg/ml). Despite some reports about the chelating properties of protein, which could mask embryo-toxicity [260,261], our results suggested that, when testing mineral oil, protein could actually generate the opposite effect and increase the power to detect peroxides. Some years ago, Otsuki published interesting data that could explain this phenomenon, indicating that HSA might bind to the free radicals in the oil and facilitate their transfer to the medium and through the embryos' zona pellucida [220].

We next focused on the effect of embryo density by comparing two radically different conditions: culturing embryos in pairs in 50 μl micro-droplets (low embryo density, 25 μl per embryo) and culturing them in groups of seven in 21 μl micro-droplets

(high embryo density, 3 µl per embryo). Our data concurred with the extensive available literature showing that a high embryo density heavily reduces the sensitivity of the MEA and, in fact, may completely mask any embryo-toxic effects; it has been hypothesized that the accumulation of excreted autocrine and paracrine embryonic factors could explain the improved development associated to group culture [232,251,252]. Thus, whilst this strategy might result useful time and economic-wise, it should undoubtedly be avoided to obtain sensitive and reliable results in QC testing. Interestingly, in some cases a low embryo density may not necessarily translate into an improved toxicity detection. When using time-lapse dishes, which require embryos to be cultured individually in a micro-well (and, thus, in a very reduced embryo density), we observed that the developmental outcomes were significantly higher than using our regular culture settings, in micro-droplets of medium placed on a Petri dish. The culture in micro-wells also failed to detect the embryo-toxic oil sample in some cases, suggesting that time-lapse incubation should be applied cautiously to QC testing. This contradicts the findings of other groups, which recommend the use of individual culture and time-lapse technology to increase the MEA's sensitivity [184,187]. However, our results concur with the increased developmental rates observed in humans and other mammals when using a well-of-the-well culture device, as well as with the hypothesis that the micro-environment created in the reduced volume of the micro-well and the build-up of autocrine factors in it, could have very

similar effects to those seen in group culture [230]. Recently, another group has also reported differing sensitivities to toxic oil samples according to the type of dish used (4-well dish *vs.* Petri dish) and the consequent differences in the oil:medium ratio [278].

Lastly, we determined the effectivity of other end-points for the assay, different than the generally accepted blastocyst formation rate after 96 hours of culture. Some groups have proposed the prolongation of the culture period up to 120 or even 144 hours to exacerbate developmental differences between the test or control groups [231]. Our data, comparing the BFR between the 96 and 120-hour time-point, showed no benefit of the latter over the former, as the toxic oil sample was identified in all cases after 96 hours. While this strategy could still prove useful in other cases, the interpretation of the results should be done cautiously, as the extension of the culture period could allow those embryos with a slower development to reach the expanded blastocyst stage between day 5 and day 6 and, hence, sensitivity would be reduced [146]. Two potential solutions for this issue could be the use of a double end-point, accounting for the BFR at both time-points, or using a more advanced developmental stage as the cut-off at the 120-hour examination, such as the ratio of hatching or completely hatched blastocysts. Moreover, the fixation, staining and determination of the mean number of cells per blastocyst has also been proposed as a quantitative and more sensitive method to detect embryo-toxicity [227]. However, since no

specific cut-off value has been determined in the literature regarding the appropriate number of cells per embryo, cell counts results must rely on the statistical comparison between the test and control groups for each assay. Following this strategy, our data confirmed the incrementation of the assay's sensitivity when adding the analysis of the total cell counts to the regular morphological assessment of the blastocysts.

As a combination of our experimental sets, we determined that the most sensitive method to test oil samples and detect a potential contamination with peroxides was to culture embryos in Petri dishes (avoiding micro-wells), keeping a reduced embryo density and supplementing the culture medium with 5 mg/ml of HSA.

The optimized testing protocol was then applied to the thirteen commercial oil samples mentioned previously. Significant differences were detected in the BFR between some of the oils, agreeing with a previous publication that reported contrasting clinical outcomes in the human depending on the oil used during culture [215]. Surprisingly, in our case two of the oils did not reach the acceptance criteria for the MEA, one of them with a borderline 71.4% result (group 11), whilst the second one showed an alarmingly low 10.8% BFR (group 13). This was particularly unexpected considering that, being commercially available oils, all the samples included in the study had been previously MEA-tested by their own manufacturers and/or other external testing laboratories, and were being used at the

moment for human clinical IVF at various centers. In addition, by using the hatched blastocyst as the cut-off stage at the day 6 end-point, we were able to identify one more sample with a statistically lower hatching rate than the rest of groups (group 9).

We then proceeded with the fixation and staining of the obtained blastocysts. Instead of only determining the total blastomere number, we optimized a triple-staining protocol which, besides staining the nuclei of all the cells in the blastocyst, differentially marked the pluripotent Oct4+ cells forming the ICM and the cells undergoing apoptosis. In addition to the three suboptimal oils already singled out by the developmental rates analyses, a fourth group was identified with a statistically lower number of cells than some of the rest (group 12). Interestingly, the staining of the ICM also showed four groups with a lower number of pluripotent cells (groups 6, 10, 11 and 13), but two of them were not the same identified in the previous examinations (groups 6 and 10). Similarly, the determination of the cells with apoptotic-related DNA strand breaks only recognized one group which presented a statistically higher number of apoptotic cells, a group that had not been singled out by any of the previous markers (group 2). While all these markers have been proposed separately in the past [163,232,236], our results suggest that the information obtained by each of them might be unique and not be represented by the rest of them, indicating that the highest sensitivity would derive from the synergic information obtained from all three

fluoro-markers. In total, seven out of the thirteen studied oil samples performed poorly, or worse than other oils in the cohort, in at least one of the studied variables.

These results showcase an important variation in the quality and performance of the commercial oils used for human clinical IVF which, as has been shown, may have a detrimental impact on gametes, embryos and clinical outcomes. It is our goal that our findings may help to define a standardized protocol to test oil, and that the appropriate notified bodies strictly regulate the QC testing methodologies for oil and all other products, to guarantee that no embryo-toxic elements are introduced into the human embryo culture system.

CONCLUSIONS

The main conclusions of this thesis are:

- The embryo culture system is a multiplex structure constituted by multiple components with a direct impact on embryo development and human clinical outcomes.

- Only by optimizing the culture system and the culture conditions in the QC lab can the sensitivity of the MEA be maximized reliably, without risking a surge in the false positive error rate.

- Using oil at all times during the handling and culture of gametes and embryos is essential to reduce the fluctuations of temperature, pH and osmolality.

- Several factors are associated with the evaporation of medium and should be considered to avoid embryonic osmotic stress: the relative humidity level inside the incubators, the thickness and the viscosity of the oil overlay used to cover the medium, the type of culture medium and its initial osmolality, the design of the culture dish and its preparation protocol, the volume of medium used and its surface:volume ratio, and the heat transmission derived from the dish-incubator fit.

- Commercial oils present a wide range in their viscosity and density. Generally, oils with a high viscosity are more effective in reducing media evaporation and preventing changes in pH than low-viscosity oils, whilst their modulation of temperature is similar.

- The optimization of the Mouse Embryo Assay applied to oil samples and the maximization of its sensitivity to detect peroxides can be achieved by supplementing culture medium with a high concentration of HSA, culturing embryos in a low-embryo-density environment that avoids micro-wells, and the inclusion of quantitative cell-number analyses as part of the QC bioassay.

- Striking differences in quality exist between commercial oils used for clinical IVF, regarding their ability to support the *in vitro* development of mouse embryos until the expanded and hatched blastocyst stages, and the mean total number of cells, number of pluripotent Oct4+ cells in the ICM, and number of apoptotic cells found per blastocyst.

- The standardization and regulation of QC testing protocols will result crucial to reduce the differences in quality between commercial oil brands in the future.

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