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EFFECTS OF THE VOLATILE ORGANIC COMPOUNDS OVER THE HUMAN PRE-EMBRYO DEVELOPMENT

Doctoral report by

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INFORMA:

Que el trabajo titulado:

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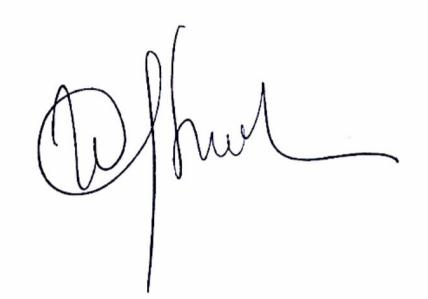
Ha sido realizado íntegramente por Dña. **Sandra Viviana Vásquez Cubillos,** bajo mi dirección y supervisión. Dicho trabajo está concluido y reúne todos los requisitos para su presentación y defensa como Tesis Doctoral ante un tribunal.

Y para que así conste a los efectos oportunos, se expide la presente certificación, en Valencia a 30 de Mayo de 2017.



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RESÚMEN

La contaminación ambiental produce una gran variedad de efectos negativos en la salud humana, dentro de los cuales se incluyen diferentes problemas reproductivos. En lo que respecta a la reproducción asistida existen aún muchos vacíos en cuanto a las interacciones que los compuestos puedan tener con diferentes tipos de células, gametos y embriones, los cuales son manipulados a diario través de diferentes técnicas dentro de los laboratorios de fecundación *in vitro* (FIV). Así mismo, la calidad del aire de estos laboratorios depende de las personas, actividades y elementos presentes dentro y fuera del laboratorio, por lo cual existe un riesgo constante de contaminación que puede ocasionar daños a corto y largo plazo.

Gracias a la experiencia empírica, estudios en modelos animales y estudios retrospectivos, se han podido identificar y describir algunos de los contaminantes presentes dentro del laboratorio de FIV y así mismo, proponer mecanismos con los que se ha logrado contrarrestar los efectos que estos producen sobre el desarrollo embrionario y los resultados clínicos (tasas de implantación, embarazo y natalidad, entre otros), a través del desarrollo de sistemas de filtración que pueden ser muy eficientes. Muchos de los estudios también enfatizan en lo difícil que es eliminar ciertos contaminantes, ya que algunos de ellos se producen dentro del mismo laboratorio de FIV. Sin embargo, se necesitan más estudios prospectivos sobre las interacciones específicas entre compuestos y gametos o embriones humanos como la Inducción de estrés oxidativo, efectos negativos sobre las membranas y organelos, y también efectos deletéreos sobre el ADN, entre otros.

Durante el desarrollo de esta tesis se ha llevado a cabo un plan de ensayos enfocado en determinar los efectos específicos de dos de los compuestos orgánicos volátiles (COVs) más frecuentemente encontrados dentro del laboratorio de FIV, sobre el desarrollo de embriones humanos. El plan de estudio de dividió en tres partes: la primera se centró en analizar las caracterizaciones de calidad de aire de las clínicas IVI en las que se valoró cuáles compuestos eran encontrados con mayor frecuencia dentro del laboratorio de FIV. La segunda parte se enfocó en un estudio comparativo de las tasas de desarrollo embriones de un grupo control y embriones expuestos a uno de los dos contaminantes (Benceno y Limoneno), evaluando además la tasa de expansión y la morfología de los mismos. Finalmente, en la tercera parte del estudio se determinó el cariotipo y el contenido de ADN mitochondrial de los embriones que se desarrollaron.

Los resultados permitieron determinar que la presencia de Benceno y Limoneno, directamente diluídos en el medio de cultivo embrionario, sí producen efectos deletéreos sobre el desarrollo de los embriones posiblemente por mecanismos

relacionados a su metabolismo y por estrés oxidativo, que también puede influir en los errores cromosómicos encontrados principalmente tras la exposición a Benceno.

Estos resultados son de particular importancia ya que hasta el momento éste es el único estudio que aborda, a través de parámetros morfológicos y cromosómicos, las implicaciones de la presencia de compuestos orgánicos volátiles en el aire ambiental de los embriones humanos

RESUM

La contaminació ambiental produeix una gran varietat d'efectes negatius en la salut humana, dins dels quals s'inclouen diferents problemes reproductius. Pel que fa a la reproducció assistida existeixen encara molts buits pel que fa a les interaccions que els compostos puguin tenir amb diferents tipus de cèl·lules, gàmetes i embrions, els quals són manipulats diàriament través de diferents tècniques dins dels laboratoris de fecundació in vitro (FIV). Així mateix, la qualitat de l'aire d'aquests laboratoris depèn de les persones, activitats i elements presents dins i fora del laboratori, per la qual cosa hi ha un risc constant de contaminació que pot ocasionar danys a curt i llarg termini.

Gràcies a l'experiència empírica, estudis en models animals i estudis retrospectius, s'han pogut identificar i descriure alguns dels contaminants presents dins el laboratori de FIV i així mateix, proposar mecanismes amb els quals s'ha aconseguit contrarestar els efectes que aquests produeixen sobre el desenvolupament embrionari i els resultats clínics (taxes d'implantació, embaràs i natalitat, entre d'altres), a través del desenvolupament de sistemes de filtració que poden ser molt eficients. Molts dels estudis també emfatitzen en la dificultat a l'hora d'eliminar certs contaminants, ja que alguns d'ells es produeixen dins del mateix laboratori de FIV. No obstant això, es necessiten més estudis prospectius sobre les interaccions específiques entre compostos i gàmetes o embrions humans com la inducció d'estrès oxidatiu, efectes negatius sobre les membranes i orgànuls, i també efectes deleteris sobre l'ADN, entre d'altres.

Durant el desenvolupament d'aquesta tesi s'ha dut a terme un pla d'assajos enfocat a determinar els efectes específics de dos dels compostos orgànics volàtils (COV) més freqüentment trobats dins del laboratori de FIV, sobre el desenvolupament d'embrions humans. El pla d'estudi es va dividir en tres parts: la primera es va centrar en analitzar les caracteritzacions de qualitat d'aire de les clíniques IVI en què es va valorar quins compostos eren trobats amb més freqüència dins del laboratori de FIV. La segona part es va enfocar en un estudi comparatiu de les taxes de desenvolupament embrions d'un grup de control i embrions exposats a un dels dos contaminants (benzè i limonè), avaluant a més la taxa d'expansió i la morfologia dels mateixos. Finalment, en la

tercera part de l'estudi es va determinar el cariotip i el contingut d'ADN mitocondrial dels embrions que es van desenvolupar.

Els resultats van permetre determinar que la presència de benzè i limonè, directament diluïts en el medi de cultiu embrionari, sí produeixen efectes deleteris sobre el desenvolupament dels embrions possiblement per mecanismes relacionats amb el seu metabolisme i per estrès oxidatiu, que també pot influir en els errors cromosòmics trobats principalment després de l'exposició a benzè.

Aquests resultats són de particular importància ja que fins al moment aquest és l'únic estudi que aborda, a través de paràmetres morfològics i cromosòmics, les implicacions de la presència de compostos orgànics volàtils en l'aire ambiental dels embrions humans

SUMMARY

Environmental pollution produces a wide range of negative effects on human health, including different reproductive problems. As far as assisted reproduction is concerned, there are still many gaps in the interactions that the compounds may have with different types of cells, gametes and embryos, which are manipulated daily through different techniques within the *in vitro* fertilization laboratories (IVF). Likewise, the air quality of these laboratories depends on the people, activities and elements present inside and outside the laboratory, for which there is a constant risk of contamination that can cause short and long term embryo development failures.

Thanks to the empirical experience, studies in animal models and retrospective studies, it has been possible to identify and describe some of the pollutants present in the IVF laboratory and also, to propose mechanisms to counteract the effects they produce on the embryo development and clinical outcomes (rates of implantation, pregnancy and birth, among others), mainly through the development of filtration systems that can be very efficient. However, many of the studies also emphasize on how difficult it is to eliminate certain contaminants, since some of them are produced within the same IVF laboratory. Despite the known information available, further prospective studies are needed in order to evaluate specific interactions between compounds and human gametes or embryos such as induction of oxidative stress, negative effects on membranes and organelles, as well as deleterious effects on DNA, among others.

During the development of this thesis, a test plan has been carried out focused on determining the specific effects of two of the most frequently encountered volatile organic compounds (VOCs) within the IVF laboratory. The study complied three parts: the first focused on analyzing the air quality characterizations of the IVI clinics, to assess

which compounds were most frequently found within the IVF laboratory. The second part focused on a comparative study of the developmental rates of embryos of a control group and embryos exposed to one of the two contaminants (Benzene and Limonene), also evaluating the rate of expansion and the morphology of the groups. Finally, in the third part of the study, the karyotype and the mitochondrial DNA content of the embryos were determined.

The results allowed to determine that, the presence of benzene and limonene directly diluted in the embryonic culture medium can produce deleterious effects on the development of the embryos, possibly by mechanisms related to its metabolism and by oxidative stress, which can also influence the chromosomes errors, found mainly after exposure to benzene.

These results are of particular interest since, to date, this is the only study that addresses through morphological and chromosomal parameters, the implications of the presence of volatile organic compounds in the environmental air of human embryos.

“We want more than this world's got to offer,
we want more than the wars of our fathers
and everything inside, screams for second life”

Chapter 1

1 INTRODUCTION

1.1 ASSISTED REPRODUCTION AND THE ENVIRONMENTAL CONDITIONS

Infertility, as a clinical definition, is a reproductive system disease defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009) . However, for women aged 35 and older, infertility is generally considered as the inability to conceive after 6 months. If couples don´t conceive within a year of trying, their chance of conceiving gets lower each month, and this happens faster as the women get older (American Society for Reproductive Medicine, 2014a)

In general, the infertility or the state of subfertility can be manifested as the inability to become pregnant, the inability to maintain a pregnancy or the inability to carry a pregnancy to a live birth. It has become an increasingly common health problem in the contemporary reproductive medicine and it has been estimated that it can affect around 10- 15% of the couples in reproductive age (20 to 45 years old), being also considered by the world health organization (WHO) as a social disease due to its high prevalence. However, it is a unique health problem because it always concerns two people simultaneously (American Society for Reproductive Medicine, 2014a; Szamatowicz, 2016; World Health Organization, 2017a) . The most common problems that cause infertility are ovulation problems (polycystic ovary syndrome, thyroid disease and other hormonal disorders, etc.) or damage or blocked fallopian tubes that can cause ectopic pregnancies as well (woman: 20—35%) and male factor (20- 30%). 25-40% of cases are because of a problem in both partners. In 10- 20% no cause is found. Infertility is also associated with lifestyle factors such as smoking, body-weight and stress and the polluted environment (American Society for Reproductive Medicine, 2014a; European Society of Human Reproduction and Embryology, 2017) .

Environment risks have been also addressed by official organizations all over the world and they have found many correlations to all type of health problems, including reproductive ones, but in terms of assisted reproduction there are many gaps in the relationship of the most common pollutants and the interactions with gametes and embryos, which are daily handled inside the clinical facilities, the in vitro fertilization (IVF) laboratories and with different assisted reproductive technologies (ART). Mammal studies have helped to unmask several deleterious effects in cells, such as damage over cells structures (organelles, membranes and cytoplasm), the intracellular communication, oxidative stress and even epigenetic effects, etc., but because of the

variety of environmental biological and chemical compounds and the differences between species it is difficult to specify in detail the damaging mechanisms that occur in the human early development.

So far, empirical experience about how the in vitro environment can produce deleterious effects on the daily practice or over clinical results, has been part of the inspiration to spur preventive measures inside laboratories and the worst is that few literature can be found establishing the relationships between pollutants and the early human development in the normal clinical setting. Yet, environmental pollution affects both male and female gametes or both embryos and fetuses, through all stages of development and through different ways of exposure (outdoor, indoor and even inside cleanrooms).

This study is going to focus on the environmental influence over the culture systems and its impact over the early embryo development inside the IVF, specifically from day (D+) 3 to D+6, which is a period of high vulnerability for embryos, because there are many un-natural factors and events that will influence them both on a short- and long-term basis. This research work shows that the presence of very low doses, below 1% of the official occupational limit thresholds (OLT), of the volatile organic compounds (VOCs), very common pollutants inside the IVF, have a possible relationship with the embryo culture system and the developmental potential of D+3 thawed embryos. The morphologic categorization and the survival results will reflect this as well. Likewise, because the chromosomal pattern of embryos in the early cleavage stage does not represent the final pattern in a later stage of blastulation, the analysis of the cultured blastocysts was performed. And despite no significant associations were obtained between negative results of the chromosome profiles, the embryos exposed to VOCs presented more aneuploidies as well.

Chapter 2

2 BACKGROUND

The WHO created in 1965 a unit dedicated to human reproduction that evolutioned later into a scientific group on the biological components of the human reproduction (1969), to understand the fertility problems and to understand the global burden of the infertility, and then in 1972, the Human Reproduction Programme (HRP) was established for the research, development and training in human reproduction, addressing not only infertility but also contraception. In 1975 an epidemiology scientific group started to coordinate the etiological research and to compare the results between different countries worldwide (Van der Poel, 2012). Since then, definitions and recommendations were design to work on diagnosis, treatments definitions, reproductive rights, techniques until it became a primary health care issue. If fertility problems arise, interventions can be attempted from simple fertility awareness methods to more advance methods associated with in vitro fertilization (IVF). The interventions developed to assist and resolve involuntary infertility have not only become medically and scientifically innovative, but they also have revolutionized the concepts of generational identity, family, and human reproductive potential because the reproduction is the choice of each individual, within their own sense of conscience, to determine the size of their family unit and the timing of when to have children (World Health Organization 2017b).

Nowadays, the current development of guidelines and manuals through international clinical research continue to support the future success in parental, child and general reproductive health (Van der Poel, 2012). However, there are many factors that have to be considered to reach that success.

2.1 THE ASSISTED REPRODUCTION IN THE WORLD

Infertility affects as many as 186 million people worldwide, with male infertility contributing to over half of all cases. At present, there are three main therapeutic strategies to prevent and treat the fertility problems: pharmacological therapy, surgical therapy (Szamatowicz, 2016).

In the last decade the world has witnessed changes in the number of new treatments integrated into the routine ART practice. According to the literature report of Kushnir et al., 2017, in more than 10 years, the ART cycles volume have gradually increased in most regions (Australia/ New Zealand, Europe, Latin America, North America, United Kingdom), although some regions were able to have a rapid increase in cycle numbers (Japan) compared to the rest. Some other regions are difficult to include in these types of reports (Middle East, Africa and large parts of Asia). The use of cryopreserved embryos was very common in the past ART cycles performed in Australia and New

Zealand, but now it has also increased in most regions. At the same time, the low live birth rates of fresh cycles are correlated with the increasing use of the single embryo transfer (SET) at blastocyst stage, which has become a new strong characteristic of the IVF laboratories, mostly in Australia, New Zealand, Canada, and in Northern Europe. While there have been live birth rates improvements in most of the regions, some showed no change and others have their rates declined, but the increasing use of SET is also correlated to this fact. SET policies have been able to lower multiple delivery rates as well (Kushnir et al., 2017), which are related to other health risks for mothers and babies; actually the frozen-thawed SET approach is used in good prognosis patients (under 35 years old) because after a good embryo selection and a successful cryopreservation, it produces similar chances of cumulative pregnancy as the double embryo transfer (DET), (Kissin et al., 2014; Practice Committee of the Society for Assisted Reproductive Technology and Practice Committee of the American Society for Reproductive Medicine, 2012). The neonatal health rates have been also proposed to measure the improvements in ARTs and their outcomes (Kushnir et al., 2017) .

Another important issue is that there are considerable disparities worldwide in terms of improving ART practices because these require coordinated and multidisciplinary efforts by the health care professionals, the scientific community, the society, insurance providers, governments, etc., and because there are many differences and restrictions of every region in terms of patient demographics, social norms, legislations, economics, etc., many ART´s practices could be also locally affected (Kissin et al., 2015; Kushnir et al., 2017; Okhovati et al., 2015). However, the number of research publications on IVF and ARTs has increased dramatically since 1998. Okhovati et al., described that the global shift in ART-related literature is not only directed to developing new infertility interventions but it is more focused towards more in-depth analysis of the health implications for mothers and their children (Okhovati et al., 2015).

2.2 Assisted reproductive technologies

Since, the first three human babies were born by ARTs (Louise Brown, the first baby, born in 1978, Courtney Cross in 1978 and Alastair MacDonald, the first male baby, in 1979), significant development of techniques have been seen in the last three decades, resulting in higher implantation and take home healthy baby rates (Kamel, 2013; Wale and Gardner, 2016a) . Now, there have been records of more than 5,4 million births worldwide related to ART procedures, helping infertile people to have their own genetic babies. Costs and complexities of the treatments have been reduced to alleviate the stress and social troubles as well (Sallam, 2011) , which is making treatments more accessible and advance research can be performed at the same time. ARTs are performed to achieve pregnancies through procedures such as fertility

medication, IVF and surrogacy (which is a limited option because of its legal implications).

It is important to point out that ARTs include the fertility treatments in which both oocytes and embryos are handled, such as the IVF and the intracytoplasmic sperm injection (ICSI). Individual or joint ART techniques can be used in one patient, or couples, depending on their specific needs; that it is why ART is a multidisciplinary activity in which many factors have to be considered and it will also require a specific coordination of different departments (Asociación Española de Normalización y Certificación, 2013)

There are many ART treatments but the most common, related to the management of oocytes and embryos such as the ones mentioned on the following sections.

2.2.1 Ovarian stimulation

Despite the fact that the first successful IVF cycle resulted from a natural, non-stimulated cycle, the clinical pregnancy rates are higher in stimulated cycles (Sallam, 2011). It is possible to induce ovulation by the administration of gonadotropins during the menstrual cycle in order to obtain a higher number of oocytes. This ART treatments are used in women that don´t ovulate regularly or when women aren´t able to get pregnant being regular. There are different stimulation protocols designed to produce the ovarian hyper-stimulation of patients that are undergoing IVF treatments, using follicle- stimulating hormone (FSH) in combination with gonadotropin releasing hormone (agonist or antagonist GnRH). These drugs are used to stimulate the development and recruitment of multiple mature follicles and oocytes. There are also other combined protocols that have been proved to be more efficient on increasing the proportion of mature metaphase II oocytes, good grade embryos and increasing delivery rates, but the efficacy of some of them needs to be confirmed clinically (Pacchiarotti et al., 2016). Since ultrasonography techniques allow evaluating the ovarian follicles and the oocyte retrieval, a close follow up of the patients permits to lower any risk of ovarian hyper-stimulation syndrome (OHS). The count and measurement of the ovarian follicles by ultrasonography, and/ or hormonal assessment of the estradiol concentration are frequently used to prevent and control OHS (Martins et al., 2014).

2.2.2 Intrauterine insemination (IVI)

Because the cervix naturally limits the number of sperm that could enter into the uterus, only a small percent of sperm of the ejaculate will make their way into the fallopian tubes. The IUI is an ART technique that places the sperm into a specific area of the female reproductive tract, around the time of the ovulation, to increase the

amount of sperm that will encounter the ovulated oocyte into the fallopian tubes (American Society for Reproductive Medicine, 2014b) .

This technique was first introduced in 1962 and it is one of the first lines of infertility treatments and ART because its simplicity; it has been used in many cases of unexplained subfertility and it is indicated in male and non-male factor infertility and in minimal or mild endometriosis, cervical factor, OHS, etc., being a relatively inexpensive procedure as well. IUI allow the patients as well as donor semen samples to be analyzed and prepared inside the laboratory, and then they will be placed in the best anatomical area (cervix, uterus, fallopian tubes) to assure a short and easy journey to the oocyte. IUI can be performed in natural or in ovarian-stimulated cycles but the main concern of this technique is the risk of increased multiple pregnancy rates because of the latter type of cycles (Kim et al., 2014; Veltman-Verhulst et al., 2016) .

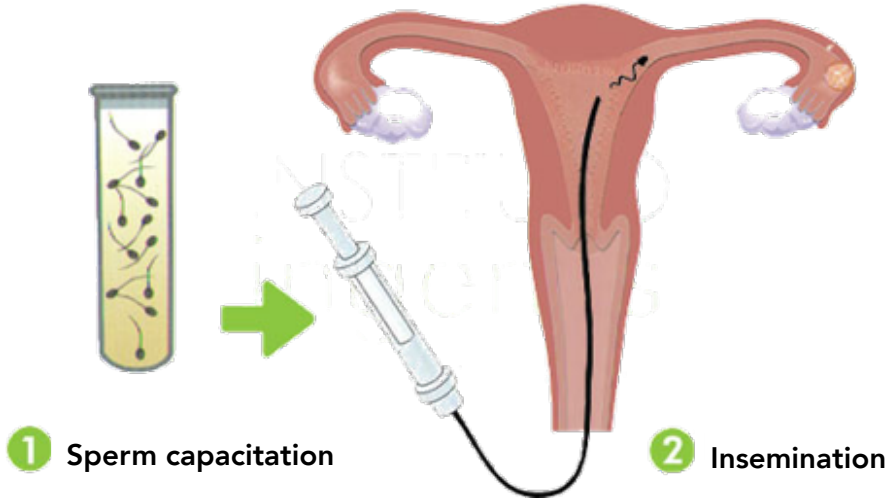


Figure 2-1. . General steps for an Intra-uterine insemination (IUI).

2.2.3 In-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)

When managing the ovarian reserve, more than 1 mature oocyte will be obtained so the embryologists can manage them through culture systems inside the IVF laboratory. Their tasks (Rienzi and Ubaldi, 2009) depend on the oocyte selection prior to the insemination, which will be potentially very useful during the IVF and ICSI programs. After aspiration and through the entire laboratory procedures, important information can be obtained about the quality of the oocytes retrieved, allowing to reduce their number and thus the amount of supernumerary embryos, avoiding potential risks of chromosome abnormalities and choosing the appropriate amount for egg donation programs (Rienzi and Ubaldi, 2009) . The fertilization outcomes depend on the techniques used, technical factors, the indication for ART, sperm motility and maturity and the pre-treatment (medical or surgical therapy) (Merchant et al., 2011) . The oocyte quality must be evaluated by assessing the aspect of cumulus-corona cells and

then by the morphology of the cytoplasm, organelles and on the aspect of the extra-cytoplasmic structures such as the zona pellucida (ZP), the periviteline space (PVS), and the presence of the 1st polar body for the maturation assessment, so on (Rienzi and Ubaldi, 2009) . But the genetic role of the sperm is very important as well because sperm cells are artificially selected for ICSI on the basis of their morphological appearance only, in contrast to the natural sperm selection in conventional IVF (Van Landuyt et al., 2005) , without considering other factors that can be affecting them at the same time (physiological, psychological, environmental, etc.).

IVF and ICSI are designed methods to help the encounter between the sperm and the oocyte: IVF allows this procedure in microdoplets of culture medium in a dish and ICSI is through injecting one single sperm into the oocyte.

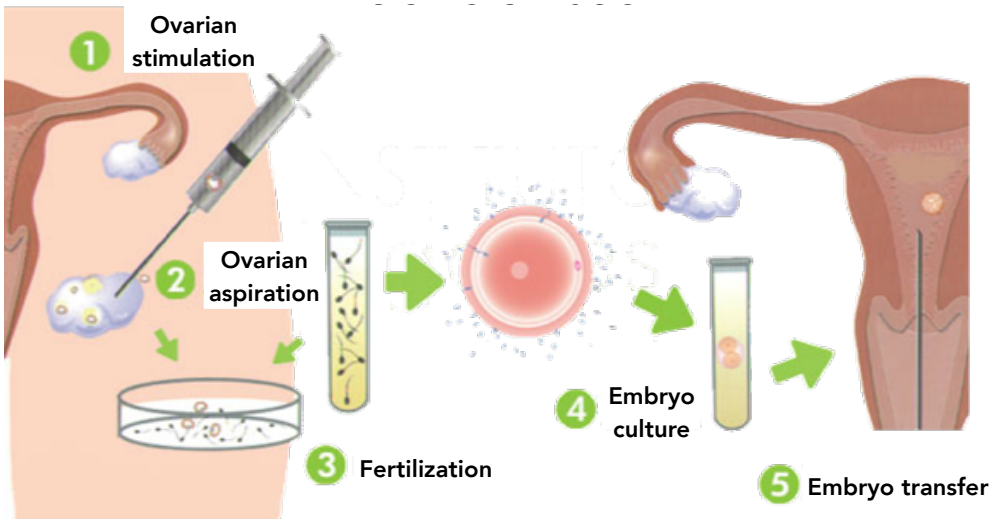


Figure 2-2. General steps for an in Vitro Fertilization (IVF).

IVF and ICSI bypass the fallopian tubes and is usually the treatment of choice for women who have badly damaged or absent tubes, but over the last two decades there have been other indications (premature ovarian failure and mostly woman of advance reproductive age) that have led to perform oocyte donation as well. Both techniques are also especially indicated for older patients as pregnancy rates decline precipitously after 36 years of age, mostly due to the age associated decline in normal oocytes. Older women`s infertility is a trend caused by social factors (career, divorce, late marriages, effective contraception, etc.) that are delaying childbearing. In summary, IVF and ICSI both may be indicated in male or non-male factor (Merchant et al., 2011; Wang and Sauer, 2006) .

Since its clinical introduction in 1991, ICSI has become the most common fertilization treatment technique. Overall, ICSI accounts for two-thirds of all treatments worldwide, and conventional IVF around one-third. However, these proportions vary greatly between countries, even though outcome rates with each technique are comparable (European Society of Human Reproduction and Embryology, 2017). It has been

suggested that performing IVF and mostly ICSI on sibling oocytes for patients with non-male factor infertility could improve the reproductive outcomes (Sallam, 2011; Van der Westerlaken et al., 2006) . On the other hand, there are several reasons to assume that the ICSI procedure itself may be detrimental to the oocyte and to the further development of the embryo. Technical factors such as the imprecise positioning of the injection needle with regard to the second meiotic spindle location can produce an accidental damage of the spindle and can cause errors in the first cleavage divisions leading to aneuploidization. Besides, it has been postulated that the injection procedure can physically disrupt the cytoplasmic organization of the oocyte. The ICSI is of course an expensive and time-consuming process, requiring highly skilled laboratory personnel (Van Landuyt et al., 2005) . The insemination/injection time should be decided based on the number of hours elapsed from ovulation trigger and/or oocyte retrieval, also keeping in mind that fertilization will need to be checked 16-18 h later (ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016) .

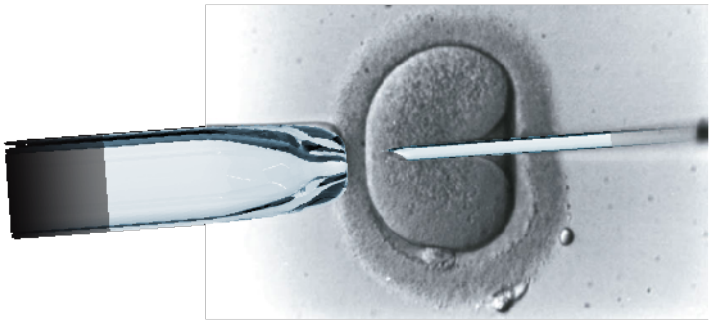


Figure 2-3. Moment of an intracytoplasmic sperm injection.

2.2.4 Cryopreservation

Advances in assisted reproduction have led to an increase of cryopreserve embryos which have been accomplished due to the improvements in the laboratory conditions, which is reflected in the improved embryo quality, the optimized embryo selection criteria and the reduced number of transferred embryos over time. Through a good cryopreservation program, an increase of the pregnancy rates per stimulated cycle and a decrease in the rates of multiple pregnancies can be achieved. Actually, the cryopreserved-pregnancy rates are now closer to those obtained with fresh embryos, making this technique very useful to maximize the ovarian stimulation cycles as well. In the last Cochrane review, reported that there are not clear differences between the freeze all strategy and the conventional IVF/ ICSI strategy in clinical pregnancy, ongoing pregnancy and cumulative live birth rates per woman, but the prevalence of ovarian hyper-stimulation syndrome appears to be lower after the freeze-all strategy. Likewise, the miscarriage rate is lower and singletons seem to have higher birth weight in the freeze-all strategy (Wong et al., 2017) .

The success of the cryopreservation program is not only due to the proper selection of biological material to be preserved (embryos or oocytes of suboptimal quality do not support cryopreservation processes as well as those with good viability), but to the application of processes that allow the maximum viability in the freezing and thawing procedures. A good knowledge of the physicochemical fundamentals that occur during the freezing of aqueous solutions and the response of living cells have helped to determine the key points of the process and to prevent possible adverse effects. There are basically two types of cryopreservation of specimens generated inside the IVF laboratories: slow freezing protocols and vitrification protocols. Not so long ago, the preferred protocol was the slow freezing but with time the vitrification is being used more often, because it has obtained spectacular results, and the cryopreservation procedures are relatively simple and easy to execute due to the availability of commercial mediums and equipment; the mediums can be prepared in the laboratory but this is discourage by European regulations (Buendía, 2015; Rienzi et al., 2017) .

Slow freezing is known as equilibrium freezing due to the exchange of fluids between the extra- and intracellular spaces, being a safe freezing technique without serious osmotic and deformation effects to cells. It uses relatively low concentration of cryoprotectants that might not cause serious toxic and osmotic damage; however, as low concentrations of cryoprotectants may be insufficient for avoiding ice crystal formation within the cells, the slow freezing is more time-consuming and requires an expensive programmable freezing machine. On the other hand, the vitrification has better satisfied embryologists because the ice crystal formation is totally eliminated. Vitrification is a non-equilibrium method that requires an extremely high cooling rate along side much higher concentrations of cryoprotectants when compared with slow freezing. This method does not require expensive equipment and is not time-consuming. Since 1990, when the technique was reported in human cleavage stage embryo with a successful delivery, different types of devices have been developed for this technique, such as the electron microscope grids, open pulled and hemi-straws, the Flexipet, the Cryotop and the CryoLoop (Rezazadeh Valojerdi et al., 2009) . Chen and Yang, reported that survival, implantation and pregnancy rates using the slow-freezing method vs. vitrification, did not seem significantly different between different studies reviewed. However, rates were higher with the vitrification method. Other reports also have described that vitrification of oocytes and embryos improve the cumulative pregnancy rates and the post-warmed good embryo morphology, which also helps to reduce the number of transferred embryos (Elnahas et al., 2010; Rezazadeh Valojerdi et al., 2009) .

Vitrification has been used from immature oocytes to blastocysts preservation. The substantial improvement in the viability of oocytes after the application of cryopreservation protocols is allowing to use more and more this technique in oocytes

instead of embryos, with the consequent benefits of reducing the oocyte retrieval procedures and the surplus of embryos left over from IVF cycles, and allowing women to have more options to postpone the maternity. Likewise, the oocyte cryopreservation has wider clinical implications than embryo freezing: women that will lose their ovarian function due to surgery, chemotherapy or radiotherapy, when ethical issues and legal restrictions exist in different countries and, therefore, to improve oocyte donation programs. In immature oocytes it has been reported to have a higher rate of spindle and chromosomal abnormalities but still they can survive to the process. In mature oocytes it is important to take into account that they have lower permeability to cryoprotective additives (CPAs) compared to embryos, which makes them quite sensitive to physical and chemical aggressions, especially because of the vulnerable spindle apparatus, so it is important to control the amount of CPAs in the vitrification solution, to reduce toxicity and osmotic injuries and even, adding macromolecules and stabilizers to improve their viability post-thawing (Chen and Yang, 2009; Elnahas et al., 2010) .

On the other hand embryo cryopreservation allows several pregnancies to be achieved in a single controlled ovarian stimulation cycle, thus contributing to an increase in cumulative outcome. In zygotes and cleavage embryos there have been reported high survival rates after freezing and warming procedures, which can be due to the hardening of the zona pellucida after the cortical reaction (fertilization) and the high stability given to the ooplasmic membrane in front of low temperature and osmotic changes. The vitrification of blastocysts has the advantage to have passed the genomic activation and it has a high developmental potential because the loss of some cells during the vitrification process is probably less harmful for further embryo development (Chen and Yang, 2009; Elnahas et al., 2010) .

But the most important fact is that many variables have to be manage during the vitrification process to influence its effectiveness and the potential to improve survival rates of vitrified cells: the type of cryoprotectant, the temperature of the vitrification solution, the duration of exposure to the final cryoprotectant (before the liquid nitrogen step), the type of device used for vitrification and the embryo quality. In IVI Valencia there is an extensive experience over the Cryotop® method in oocyte vitrification (Cobo et al., 2010; Cobo et al., 2008; Cobo et al., 2012) and the IVF program permits its routinely usability in early developing embryos and blastocysts, allowing the analysis of the impact over the embryo development and quality. It has been observed a good quality and high post-thawing expansion rates (Buendía, 2015). Cobo et al, reported that 95% embryos in cleavage state had 100% intact blastomeres and that there is a high rate of re-expansion for the blastocyst stage, as well as a normal morphologic appearance after vitrification (Cobo et al., 2012) It has been also

demonstrated the high efficiency of the vitrification method on blastocysts, even after a biopsy (Schoolcraft et al., 2010) .

2.2.5 Pre-implantation genetic diagnosis

PGD was introduced at the beginning of the 1990s as an alternative to prenatal diagnosis, to prevent a possible termination of pregnancy in couples with high risk of offspring affected by genetic diseases. The initial idea was to establish a genetic diagnosis of monogenic diseases on IVF embryos and only transfer those who showed to be free of disease in the hope to achieve a healthy pregnancy. However, with the development of the PGD technologies, it was noticed that embryos have a great deal of acquired numerical and structural chromosomal abnormalities that were also related to the low success rate of IVF. Three technologies allowed this progress: the IVF, which gave access to early human embryos between fertilization and D+5 or D+6 of development and allowed the improvement of embryo manipulation methods and biopsy; the second, is the polymerase chain reaction (PCR), which was able to amplify exponentially a small piece of the DNA of a single cell for mutation analysis; and the third, is the fluorescent in situ hybridization (FISH) that was developed and applied to detect the chromosomal abnormalities in early human embryos. With further evolution on the IVF methods, it became a standard clinical practice to grow more embryos in vitro up to D+5 or more, at which point they have reached the blastocyst stage, which is the first stage of differentiation into two distinct cell types: the inner cell mass (ICM) that will develop further into the embryo and the fetus, and the trophectoderm (TE) that will become the placenta (Sermon, 2017) . Since multiple embryos are created in IVF, PGD has a distinct numerical advantage over testing of a single ongoing pregnancy: "the greater the number of embryos created, the greater the chance that genetically normal embryos can be identified" (Stern, 2014) . PGD can improve IVF success by increasing the implantation rates and reducing pregnancy losses

There are several groups of patients for whom PGD is essential: patients that have already experienced prenatal diagnosis and the termination of an affected fetus, those who have moral or religious objections to termination of pregnancy, those who are carrying a translocation or other chromosomal abnormality and have experienced repeated miscarriages or infertility, and those who are at risk of transmitting a genetic disease and are also infertile. Additionally, patients that may have a late onset disease, such as predisposition to inherited cancer, and want a healthy child can also use PGD, or for tissue typing of the cord blood of a PGD baby to use it as treatment for an already existing ill child (Harper and Delhanty, 2009; Stern, 2014) . However, PGD in also being highly used in the IVF practice without clinical reasons because it has been reported that the complementary ploidy diagnosis can increase the implantation by selecting the embryos with best morphology and with normal chromosomic profiles.

PGD is especifically used to select euploid embryos from "bad prognosis" patients' allowing a high effectiveness of the IVF procedure, after screening for embryonic aneuploidy. The biopsy can be performed in three different stages:

- Polar body screening: It is a less harmful analysis for the embryo because it is done in the early IVF process. The biopsy is performed prior the fertilization (first polar body) and after fertilization (second polar body). The basis of polar body screening is that any abnormality identified is associated with a corresponding error in the oocytes. The first polar body is present before fertilization, and therefore, with its biopsy, one can obtain pre-conceptual information on the oocyte, allowing embryologists to choose the oocytes that should undergo fertilization.

  However, errors on the mitotic nondisjunction will not be detected, as well as other errors in the oocyte, and there is no information about the paternal DNA, which is important to determine embryonic chromosomal anomalies. So the first and second polar body need to be evaluated, which doubles the cost of the analysis. The optimal time for biopsy seems to be 6-9 hours post-fertilization.
- Cleavage state screening: Biopsies performed at early stages (4 cells) may alter the ratio of ICM- TE, which may be detrimental for the embryo development. However, D+3 cleavage cell biopsy involves blastomere removal at the 6–10 cell embryonic stage, after opening the zona pellucida with laser, mechanical dissection or exposure to Tyrode´s solution. The blastomere is removed after the introduction of the biopsy pipette by aspiration or by extrusion of the cell with pressure on the outside of the zona. This technique is able to detect maternally and paternally derived chromosome defects as well as some mitotic defects, and it also allows 2- 3 days for the PGD analysis to have results from fresh embryos that clinicians could transfer.

  The presence of embryo mosaicism is a major limiting factor in the interpretation of PGD results in cleavage-stage embryos because it affects around 15%–80% of embryos at D+3, and significant mosaicism remains present at the D+5 blastocyst stage. This raises the question as to whether a biopsied blastomere is an accurate representation of the embryo as a whole. In addition, it appears that the ability of the early embryo to undergo self-correction by either selective apoptosis or allocation of abnormal cells to the trophectoderm is limited.
- Blastocyst stage screening: The human blastocyst contains 130- 150 cells approximately between ICM and TE, from which 5- 10 TE cells can be biopsied. After laser assisted hatching on Day 3, a 25- 30 mm opening is created in the ZP allowing the herniation of the TE; the cells are stretched out with the biopsy pipette and removed with a laser. These cells are obtained in D+5 or D+6 of

development. Because ICM cells are avoided, this technique seems to produce less harm to the embryos. Despite its advantages compared to the other two techniques (it avoids the chromosomal mosaicism at D+3) and since only about 50% of the fertilized embryos will progress to blastocysts, fewer embryos can be biopsied compared to the cleavage biopsy (Harper and Delhanty, 2009; Sermon, 2017; Stern, 2014) .

In the last 20 years, the chromosomic analysis has change through the development of new molecular genetic technologies that allow the copy number analysis of 23 pairs of chromosomes, 22 pairs of autosomes, and the sex chromosomes, or "24 chromosomes", in a single cell or small number of cells. Several methods have been applied clinically to analyze the embryo(s), such as PCR, which has been the preferred method of diagnosis for single gene disorders, or FISH, which was first performed for sexing to avoid the transfer of embryos with X-linked genetic disease, but later, it was used for most of the chromosome studies performed in cleavage embryos prior to 2007 until it was replaced by molecular technologies for aneuploidy testing, including array comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) arrays, quantitative PCR (qPCR) and most recently, next generation sequencing (NGS)- based protocols. Since 2008, aCGH was the first technology to be widely available for reliable, accurate, and relatively fast 24-chromosome copy number analysis for aneuploidies and is now used extensively around the world despite the relatively high cost of testing multiple samples (Handyside, 2013; Stern, 2014). It also detects all mitotic and meiotic abnormalities present in one cell or group of cells, and its able to diagnose some translocations, inversions and other abnormalities, such as gains or losses of chromatin.

On the other hand, since 2010, NGS is being considered as a high efficient sequencing technology, because of its flexibility and preciseness. Depending on the number of investigated chromosome locuses, it was possible to change the precision and purpose of analysis: from aneuploidy to medium size deletions or insertions in chromosomes. Dozens of samples could be evaluated in the same run at the same time that it reduced the cost of investigation compared to aCGH. However, both techniques have shown to obtain concordant results. Aleksandrova et al, found 94,8% of coincidence between the results of aCGH and NGS cases. The group reported that a low level in the fluorescent signal, according to the aCGH technique, could produce differences and that there could be differences in the signal level produced by the different genome amplifications kits, which could influence the measurements (Aleksandrova et al., 2016) .

2.3 STRATEGIES OF EMBRYO CULTURE

The human embryo exhibits a considerable degree of plasticity, enabling it to develop under a wide variety of circumstances and culture conditions. It could be said that the human embryo is one of the most resilient of all mammalian species, and it is because its ability to adapt to its surroundings and not to our ability to culture it perfectly. This is evident when the viability of the embryos is compromised after a suboptimal collection or culture conditions, and in consequence, the pregnancy outcomes will be affected, not being able to give rise to a healthy term baby. However, the embryo adaptability depends the cellular regulations, either through metabolic or molecular adaptations. The laboratory should ensure that the employed systems could reduce these embryos adaptations and thereby maintain their normal development at a cellular level and their viability (Gardner and Lane, 2009) .

The embryo quality depends on many biological factors such as the individual genetics, the mothers´ age, hormones, the uterus environment, etc., but in ARTs, everything depends on a display of fundamental technical requirements such as: the human resources and their training, the laboratory´s technical equipment, the in vitro environment, the quality monitoring and the in vitro culture conditions that will support the quality and survival of the embryos during its passage through the all the laboratory procedures, until they are transferred to the maternal environment. Until then, the in vitro culture needs to be performed into a cleanroom and the success that has been achieved until now is by controlling and understanding the requirements of gametes and embryos. Culture media is one of the most important factors in IVF because it can affect live birth, pregnancy, implantation and fertilization rates, and the number of good quality embryos. Embryos in vitro are constantly exposed to constant stress as well because of suboptimal culture conditions that force the embryo to undergo the previously mentioned adaptations, and thus cause lower pregnancy and higher miscarriage rates (Bronet and Agudo, 2017; Cohen et al., 1998; De los Santos, 2001) .

2.3.1 Environment requirements

The concentrations of metabolites to which the human oocytes and embryos are exposed in vivo differ along the reproductive tract because the pre-implantation human embryo is a highly dynamic entity with its needs changing as the development proceeds; it goes from being one of the most quiescent tissues (oocyte) to one of the most metabolically active within 4 days. The development *in vivo*, is exposed to an environment composed of several gradients of nutrients, hormones, cytokines and growth factors as it progresses through the fallopian tube to the uterus. Within the lumen of the female tract, the embryo resides in a few hundred nanolitres of a complex viscous fluid characterized by high levels of mucins, albumin and glycosaminoglycan, and by reduced levels of oxygen (2 to 8%). Pyruvate and lactate concentrations are

significantly higher in the oviduct fluid compared to the uterine fluid, while the glucose concentration will be in its highest concentration in the uterus, which means that the preferred source of energy for the cleavage stages is pyruvate and the glucose uptake will remain low, until the blastocyst stage. The embryo is in constant motion, moved by gentle ciliated and muscular action of the female tract, and metabolites produced by the embryos are removed from its immediate vicinity due to its proximity to the epithelia of the female tract and hence maternal circulation (Gardner et al., 1996, Wale and Gardner, 2016b) .

In contrast, the in vitro culture requires proactive quality control and quality assurance programs where highly trained embryologists are needed, due to all the artificial circumstances to which the embryos will be exposed. Early events of life are happening outside of the human body and just for this reason, the embryo culture is more than just appropriate culture media formulations, which already vary in ingredients concentrations and not all of them can be determined by the information provided by the manufacturers. The culture has to assure a stable environment because gametes and embryos remain static during this complex system, resting inside of relatively large volumes of culture medium (up to 100-µl per embryo or up to 1000-µl for groups of embryos) and on a polystyrene substrate, where the end products of metabolism concentrate and nutrients become rate limiting. But at the end, it is the interactions between the embryo, its surroundings and the interactions between all parameters within the laboratory that will maintain the viability of gametes and healthy embryos (Bolton et al., 2014; Gardner and Lane, 2009; Wale and Gardner, 2016b). The composition of the mediums also depends on the requirements of specific stages of development: from zygotes to pre-compaction embryos and from post-compaction to blastocyst so the fluctuations of any of the artificial culture conditions should be minimized as well, to protect embryo homeostasis during culture and handling (ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016) .

The culture system consists on several factors such as the gas phase, type of medium overlay, the culture vessel, the incubation chamber and the ambient air quality (Gardner and Lane, 2009) . The main chemical and physical factors that can also affect the in vitro development are: oxygen (O2), ammonium, VOCs, albumin, temperature, pH (7,3- 7,4), osmolarity, oil overlay, incubation volume/ embryo density, pipetting, the static nature of culture and light (Wale and Gardner, 2016b) . Incubators should have a controlled temperature, being at 37 °C, the CO2 concentration of 5% and humidity saturation. The ideal concentration of O2 in the incubator is 5% to prevent the free radicals' formation, which could damage the cellular membranes and produce perturbations in the global pattern of gene expression because of oxidative stress. O2 regulates all the cellular events and functions during the pre-implantation period but the medium interactions with O2 are also very important: the differences on the

medium composition can compromise the blastocyst development, especially with the higher concentrations of octanoate, a stabilizer used in proteins in HSA preparations, and that has been recognized to produce cellular energy metabolism disruptions and oxidative stress in similar embryo culture concentrations. (Hong et al., 2014; Leonard et al., 2013; Morbeck et al., 2017; Wale and Gardner, 2016b) . In the presence of aminoacids, more cells are allocated in the inner cell mass of the resulted blastocysts, the energy metabolism is better supported especially in cleavage stages; there is reduced apoptosis and more embryo viability (Gardner, 2008) .

Embryos are currently cultured in small drops of culture medium placed on culture dishes that are then overlaid with oil, a system that will be referred to as the droplet-oil-dish culture system. These culture plates are then stored in incubators that maintain a constant temperature and gaseous environment. This method of culture has remained unchanged for a long time now and that means that there are many advantages; for example, the fact that many small drops can be placed on standard tissue culture plates makes it easy to culture many embryos in a single dish, and embryos can be easily evaluated and accessed. The relatively large volumes of culture medium used provide both a vast supply of nutrients at relatively steady concentrations and a large reservoir for diluting out waste products. The overlay of oil should protect against evaporative losses and also serves as a thermal and gaseous sink, temporarily protecting the embryos from fluctuations of this importance (Johnson and Gardner, 2011) .

2.3.2 One step vs. sequential media

The culture media designed to support embryo development to the blastocyst stage have been based on two distinct approaches: the "back to nature" sequential approach and the "let the embryo choose" single medium step approach (Sfontouris et al., 2016) .

The Sequential media have been designed to mimic the environment for metabolic and nutritional changes that occur in vivo when the embryo progresses in the oviduct to the uterus (from D+1 to D+5 or D+6). Embryos are grown from D+1 to D+3 in a growth medium and then, in cleavage stage on D+3, they are moved to a second medium or "blastocyst medium". With this, the embryo will be inside mediums designed to reflect the changes in concentrations of pyruvate, lactate and glucose, similarly to what happens inside of the fallopian tube versus to what happens inside the uterus. In this technique, culture medium is renewed at regular intervals (24–48 h), not only to alleviates the accumulation of potentially harmful by-products of the medium, but to enable the exposition of embryo to gradients of nutrients in order to better maintain cell function and reduce intracellular metabolic stress (Salvaing et al., 2017; Sfontouris et al., 2016) .

The single-step media, on the other hand, are designed to contain all what the embryo would need for all stages of pre-implantation development, arguing that brutal changes of culture environment on D+3 are detrimental to the embryo, which needs to adapt. Additionally, it is compatible with the use of time-lapse technology. The single-step culture can be done with refreshment, without changing the media composition or, even, without any refreshment, especially for uninterrupted embryo culture such as the time-lapse application, or even limiting the embryo evaluation only to fertilization and blastocyst stage. However, one potential concern with not renewing the medium on D+3 is the possible built-up of ammonium caused by breakdown of amino acids, particularly glutamine. For now, the available data suggest that both types of media seem to provide adequate support for the embryo development. The uninterrupted protocol may be more susceptible to the VOCs and to environmental culture conditions, as well, so its applications should be limited to certain IVF laboratories (Bronet and Agudo, 2017; Salvaing et al., 2017; Sfontouris et al., 2016) .

2.3.3 Co-culture and group culture

Another culture method used to improve implantation and pregnancy rates is by culturing human embryos in the presence of feeder cell layers, such as the Vero cell lines (heterologous) or the granullosa cells as well as autologous cryopreserved endometrial cells, because the culture environment will include trophic factors such as nutrients substrates, growth factors, cytokines, and the removal of potentially toxic substances by these cells (Rubio et al., 2000; Simon and Laufer, 2012; Simon et al., 1999) . The criteria to choose the co- culture instead of regular systems has been the history of successive previous implantation failures after several cycles of IVF (3 as a general agreement between professionals), and there have been cases of advance age of the female partner or to reduce the risk of multiple pregnancies (Guerin and Nicollet, 1997; Simon and Laufer, 2012) .

There have been reported improvements in cleavage rates, embryo morphology, and blastocyst formation rate, and some even reported improvements in the clinical pregnancy rates and the ongoing pregnancy rates, but because the studies are not considered homogeneous there are still many doubts about the outcomes. For example, there have been concerns regarding the transmission of disease from non-human cells lines to the developing embryo or the intended parent, and for this reason official organizations such as the US Food and Drug Administration (FDA) have recommended that non-human co-culture cell lines not be used in human IVF (Sallam, 2011; Simon and Laufer, 2012). Granullosa cell culture is probably the most simple, time-efficient, and inexpensive co-culture method; its advantages include that the cells are easily collected during oocyte retrieval and are ready for co-culture 1 day later, and that it is unnecessary the screening for infections. The monolayer remains epitheloid and metabolically active and synthesizing protein for a maximum of seven to 10 days.

Additionally, there is no need to collect the sample in previous cycles such as with endometrial co-culture. Granullosa cells have demonstrated to be beneficial for in vitro maturation of oocytes and to improve embryo quality (McKenzie, 2011) .

On the other hand, group culture has been proposed like an option to let embryos reach blastocyst and then evaluate each one at this stage. Group culture of embryos in a small volume could improve development, potentially through secretion of autocrine/paracrine factors. Embryos can communicate through paracrine biomolecules that have been suggested to affect embryo homeostasis and growth. Actually, some factors derived from poor quality embryos may affect negatively the development of the surrounding embryos and, on contraire; results can be improved when creating groups of only good quality embryos. Group culture has also demonstrated to be positive for compaction, blastulation, blastocyst quality, implantation and pregnancy rates as well (Bronet and Agudo, 2017; Ebner et al., 2010; Tao et al., 2013) .

2.3.4 Culture up to blastocyst stage

Despite there are no benefits in terms of cumulative pregnancy rate per initiated cycle, there is a trend toward to perform blastocyst transfer rather than cleavage embryo transfer, due to the fact that after fresh blastocyst transfer there are higher rates on live births and clinical pregnancies. Despite no benefits in terms of cumulative pregnancy rate per initiated cycle, there is a trend toward to blastocyst transfer rather than cleavage embryo transfers because it may shorten time to pregnancy (Bronet and Agudo, 2017; Glujovsky et al., 2016) .

The prolonged culture is a method that allows a better selection of embryos with the best potential for survival and hence for implantation. Increased live birth rates have been reported after blastocyst transfer on D+5 or D+6 compared to cleavage stage transfer on D+2 or D+3, adding as a real advantage the reduction of multiple pregnancies, as one or two embryos only can be selected for having the best potential for implantation. However, sometimes the embryo development up to blastocyst stage does not necessarily equate to the development of a viable embryo: viability is defined as the ability of the embryo to implant successfully and give rise to a healthy baby. It has also been shown that combining the morphological examination through time-lapse imaging and oxygen consumption measurements, embryologists are able to have more knowledge about the developmental competence of the embryo for a better selection before transfer, giving relevance to the morpho-kinetic parameters, which take place in the early stages of embryo development. Moreover, good quality blastocysts could be aneuploid and end in an implant failure or in miscarriage (Bronet and Agudo, 2017; Sallam, 2011) .

2.3.5 Evaluation of the embryo quality

Despite many changes that have occurred in the IVF field, the observation of the oocyte and embryo morphology remains a valuable and efficient tool in clinical embryology. However, one of the most important needs is the standardization of the times of assessment from oocyte retrieval and during the successive days of culture. Systems for selection have been performed by assessing the pre-embryo on D+2 or D+3 of culture but now, it is mostly about assessing the blastocyst stage on D+5 or D+6; however, there are studies that have confirmed that the early valuation (oocyte on D+0, zygote, first cellular division on D+1 and even morula stage on D+4) can be also useful in the morphologic categorizations as well (Asociación para el Estudio de la Biología de la Reproducción, 2015; Bronet and Agudo, 2017; De los Santos et al., 2014; Magli et al., 2012) .

The efficiency of an IVF treatment however, can be poor due to the low probability of an individual embryo successfully implanting in the uterus, and then having an ongoing pregnancy and a child, so to achieve this, many treatments should include the transfer of more than 1 embryo, which leads to increasing probabilities of multiple pregnancies that are associated with several health risks for mothers and infants (low birth weight, preterm delivery, higher incidence of perinatal, medical or neonatal complications such as risk of cerebral palsy). Transferring fewer embryos to the mother´s uterus, each cycle, is the ideal strategy for a single embryo transfer (SET). So, to achieve this, the embryologists have to identify the most viable embryo for transfer by using non-invasive microscopy observation, because a good morphologic assessment permits to observe the transformations that occur from the fertilization and during the early embryo development. However, the available grading systems rely mostly on visual information obtained and are thus subject to inter-observer variability (Filho et al., 2010) .

After performing IVF or ICSI, there must be an enhance follow-up of zygotes as well as the selection of the embryos with the best implantation potential, up to the blastocyst stage. For this, several scoring systems based on morphological criteria have been developed, using some key morphological features that are relevant for the embryo viability as the following:

- Cell number and degree of symmetry: if all cells are similar in size and if an appropriate number of cells are present, it will indicate that the embryo has a good chance of being viable.
- Fragmentation of cells: a low proportion of embryo volume composed of cell fragments is an indicator of high viability. Thus, an embryo containing many fragmented cells is considered to have a reduced viability potential.

- Characteristics of the zona pellucida (ZP): embryos with a thinner ZP and higher variation in ZP thickness have a greater likelihood of producing a pregnancy (Filho et al., 2010) .

All the specific morphological parameters will allow the embryo grading and they might indicate the implantation potential; for example, the blastomere number and size in relation to the time of development, the presence of fragments in the perivitelline space and the appearance of the cytoplasm are the most important non-invasive parameters that guide embryo selection for transfer or cryopreservation (Magli et al., 2012) .

Other parameters can help to choose between different embryos of the same category and there are other options that could be useful but need more studies to determine their efficacy. Non-invasive approaches do not impact the integrity of the gamete or embryo. Most non-invasive approaches to date have focused on either imaging of the embryo or evaluation of culture media following exposure to the embryo. Also, as these new technologies arise, such as the Time-lapse imaging, the embryologists are being able to get more objective information about the morphology and metabolism of the embryos. The imaging approach has principally focused on the embryo at specific developmental time-points (Asociación para el Estudio de la Biología de la Reproducción, 2015; Castello et al., 2016; Sallam, 2011) .

Unfortunately, it has also been shown that many normal-appearing embryos have chromosomal abnormalities that preclude the possibility of producing a healthy live born child. As mentioned before, for the health and developmental potential, the gametes and embryos, they can be further analyzed by other non- invasive methods. On the other hand, invasive approaches encompass all procedures that disrupt the integrity of the gamete or embryo by either removing a part of or inserting a foreign object into the cell(s) to obtain more objective information. The most common invasive approach is the removal of one or more cells of the embryo for performing genetic analysis, commonly referred to as preimplantation genetic diagnostics (PGD), previously described.

2.4 Environmental pollution

We know now that the earth is contaminated and we know that it is negative in several aspects for the world and the ones who live in it, and despite the efforts of official and private institutions to control and reduce the pollution, the final impact is still uncertain in many areas.

Awareness about how the environmental pollution affects the animal and mostly the human health has increased over time, but at the same time the industrial development constantly creates new or greater risks because the human activities are

altering the earth´s climate, and that, can impact all kinds of areas as well. The environmental pollution increasingly affects the air or atmosphere, the water and the soil of both urban and rural areas that makes populations at risk through different routes of exposure (biological, chemical, radioactive, thermal, visual light, noise, etc). The total exposure assessment also includes time and activity patterns and health effects which may vary significantly among the exposure routes (National Research Council (US) Commission on Engineering and Technical Systems and National Research Council (US) Commission on Life Sciences, 2000) . All the types of pollution have produced an increase in the morbidity and mortality rates in animals and humans according to the epidemiological studies which also point out that health effects are not only physical but they could be psychological and social as well (Hasegawa et al., 2015) .

One of the most common routes of exposure to pollution is through the air and in less proportion food and water. Air pollution is the contamination of the indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere (World Health Organization, 2017c). In urban areas, primarily within industrialized and developed countries, the contaminated ambient air has become a significant public health problem due to household combustion devices, motor vehicles, industrial facilities and forest fires, which are common sources of air pollution, and the concentration of toxic compounds has increased significantly, both on the outside and inside of all type of facilities. Actually, more than 80% of people living in urban areas are exposed to air pollutant levels that exceed the World Health Organization (WHO) limits, but 100% of the population could be affected from womb to death (Frutos et al., 2015; World Health Organization, 2016a) .

The pollutants of major public health concern include particulate matter, carbon monoxide, ozone, nitrogen and sulfur dioxide, but there are many more substances that can produce negative effects such as lead, ground-level ozone and sulfur oxides (Centers for Disease Control and Prevention, 2014; World Health Organization, 2017c). Actually, atmospheric air pollutants can be classified in many ways. They can be categorized based on whether they are directly emitted from their sources into the atmosphere, which are the primary pollutants, or if they are formed from photochemical reactions from primary pollutants, compounds that are called secondary pollutants. They can also be classified according to chemical composition (organic or inorganic), type of source (natural or anthropogenic), degradation properties (degradable or non-degradable), or place of generation (indoor or outdoor), etc. Ultimately, they can be easily classified based on state of matter (solid, liquid such as particulate matter, or gaseous such as VOCs); this specific phase of a pollutant (vapor or condensed) is the additional factor that influence the health effects that can be caused from inhalation (Daly and Zannetti, 2007). And the pollutants that are known

or suspected to cause irreversible illnesses such as cancer, cardiovascular and pulmonary problems, reproductive, or birth defects are classified as toxic hazardous air pollutants (HAP) (U.S. Environmental Protection Agency, 2016a) .

2.4.1 Health effects produced by the environmental pollution

The outdoor and indoor exposure risk to critical pollutants such as particulate matter (PM) or VOCs is always a subject of research and regulatory action. However, the indoor exposure as a potential source of adverse health effects has been less studied to date. For example, some official organizations such as the Environmental Protection Agency (EPA) do not regulate indoor air quality (IAQ) because their priority are the biological or chemical pollutants that are high public health risks, but still they promote non- regulatory activities of voluntary interventions to reduce the indoor exposure. IAQ can be addressed by focusing on the design, construction, operations and maintenance of facilities which helps to prevent the exposure, specially to PM, VOCs and microorganisms (Board on Population Health and Public Health Practice et al., 2016; Carbonell, 2015) .

All types of air pollution at low (chronic) and high (acute) concentrations produce adverse effects. The environmental risk factors contribute to more than 100 diseases and injuries related to neurological, respiratory, cardiovascular outcomes in adults or neurological and respiratory outcomes in children, and reproductive adverse effects (World Health Organization, 2017d). Inhalation is the most rapid way of air pollution uptake, followed by dermal contact and ingestion causing respiratory and other diseases such as cerebrovascular accidents, lung cancers, acute and chronic lung diseases, etc., all related to increase hospital admissions and mortality (Kampa and Castanas, 2008; McKone and Hammond, 2000) .

2.4.1.1 Health effects by type of pollutant

- Particulate matter: The major components of PM are sulfate, nitrates, ammonia, sodium chloride, black carbon, mineral dust and water. Chronic exposure contributes to the risk of developing cardiovascular and respiratory diseases such as emphysema, as well as of lung cancer. Small particulate pollution has health impacts even at very low concentrations.
- Ozone (O3): Ozone is a major factor in asthma morbidity and mortality. It can cause breathing problems, trigger asthma, reduce lung function and cause lung diseases.
- Nitrogen dioxide (NO2): there can be symptoms of bronchitis in asthmatic children with long-term exposure to NO2, as well as other bronchial symptoms, lung inflammation and reduced lung function growth. It can also increase the susceptibility to respiratory infections or there can be seen emphysema- like lessions.

- Sulfur dioxide (SO2): it can affect the respiratory system and the functions of the lungs, and causes irritation of the eyes. Inflammation of the respiratory tract causes coughing, mucus secretion, aggravation of asthma and chronic bronchitis and makes people more prone to infections of the respiratory tract. Hospital admissions for cardiac disease and mortality increase on days with higher SO2 levels (Kampa and Castanas, 2008; World Health Organization, 2016b) .

2.4.1.2 Health effects by system affected

The reported pollutants effects have been primarily related to acute and chronic cardiopulmonary affections through the activation of local and systemic inflammatory pathways which promote systemic oxidative stress and inflammatory responses (releasing cytokines and other pro-inflammatory mediators), thrombosis and coagulation, vascular dysfunction, epigenetic changes and genotoxicity (suppression of DNA repair and more DNA errors) (Chin, 2015; Kannan et al., 2006; Nemmar et al., 2013). Alveoli and vasculature are affected producing declined pulmonary function, respiratory diseases, ischemic heart disease, heart failure, cerebrovascular disease, deep venous thrombosis, hypertension, cardiac arrhythmias and mortality (Chin, 2015; Shah et al., 2015) .

On the respiratory system, there are symptoms such as nose and throat irritiation, followed by bronchoconstriction and dyspnea, especially in asthmatic individuals. There could be also wheezing and allergies, and in severe cases chronic obstructive pulmonary disease (COPD) and respiratory mortality. Particulate matter penetrates the alveolar ephitelium and ozone produces lung inflammation, which will worsen the state of patients with lungs previous lesions or diseases (Kampa and Castanas, 2008) .

On the cardiovascular system have been reported in several epidemiological studies, mostly about chronic exposure, but both, acute and chronic exposure are responsible for over a million of premature deaths worldwide every year due to direct and indirect effects on the vascular tone, endothelial function, thrombosis and myocardial ischaemia. Long-term exposures to particulate matter (especially PM2,5) and gaseous pollutants in low concentrations are responsible for several adverse effects. Gaseous pollutants, except for O3, have also an adverse relation with heart failure. Acute events associated with short-term exposures can be underestimated because their effects are likely to be greater in patients with pre-existing heart failure (Shah et al., 2013; Shah et al., 2015) .

The nervous system is mainly affected by heavy metals and dioxins. Long term exposure to ambient air pollution and coronary or cerebrovascular events have a positive association but there is no certainty that the acute exposure can trigger a cerebrovascular disease. Air pollution can affect the vascular endothelium and increase activity of the nervous system, resulting in vasoconstriction, increases in blood

pressure, ischemia and risk of thrombosis. Minor increases in PM2.5 concentrations are associated with changes in cerebrovascular hemodynamics, including increased cerebrovascular resistance and reduced cerebral blood flow. The neuropathies have symptoms such as memory disturbances; sleep disorders, anger, fatigue, hand tremors, slurred speech, affected sensatory functions and neurological cancers. Dioxins can decrease the nerve conduction velocity and impaired mental development of children (Kampa and Castanas, 2008; Shah et al., 2015).

In the urinary system there can be kidney damage. It has been reported tubular dysfunction evidenced by an increased excretion of low molecular weight proteins, which can progress to decrease glomerular filtration rate; stones formation, nephrocalcinosis and even kidney cancer. In the digestive system there have been described liver cell damage, as well as gastrointestinal and liver cancer (Kampa and Castanas, 2008).

2.4.1.3 Reproductive health effects produced by the environmental pollution

When speaking about the reproductive system, there are many targets on which pollutants can produce reactions or adverse effects. Either parent's exposure at any point in life will affect not only their own health, but also their progeny´s health even in a long-term basis. The adverse effects over human reproduction can vary widely and are not well understood; there are many hypotheses, some of them weak, especially about the embryonic development that is based on precarious data; the preliminary information was obtained through non-published anecdotal exchange of information (Boone et al., 1999; Cohen et al., 1997; Hall et al., 1998).

Maternal active and passive smoking, for example, could impair the reproductive outcomes, thus the prenatal exposure to environmental contaminants can produce similar effects and can also lead to some adverse pregnancy outcomes. Very common environmental pollutants such as particulate matter, nitric oxide (NO), nitrogen dioxide (NO2), sulfide dioxide (SO2) or carbon monoxide (CO) have been correlated to different type of negative reproductive outcomes (Xu et al., 2011). Studies and reviews had focused mainly in the relationship between pollutants and birth defects (Tanner et al., 2015), intrauterine growth retardation or low birth weight (LBW) and preterm births (almost 60% of LBW) which have been related mostly to pregnant women exposed during their 1st trimester (Bell et al., 2007; Diaz et al., 2016; Medeiros and Gouveia, 2005; Santos V de P et al., 2016), lack of fetal immune development (Herr et al., 2010) or menstrual disorders and their possible relationship with higher incidence rates of spontaneous abortion (Huang, 1991), etc. that can also lead to intrauterine and infant mortality (Pereira et al., 1998; Racowsky et al., 1999; Sram et al., 2005) .

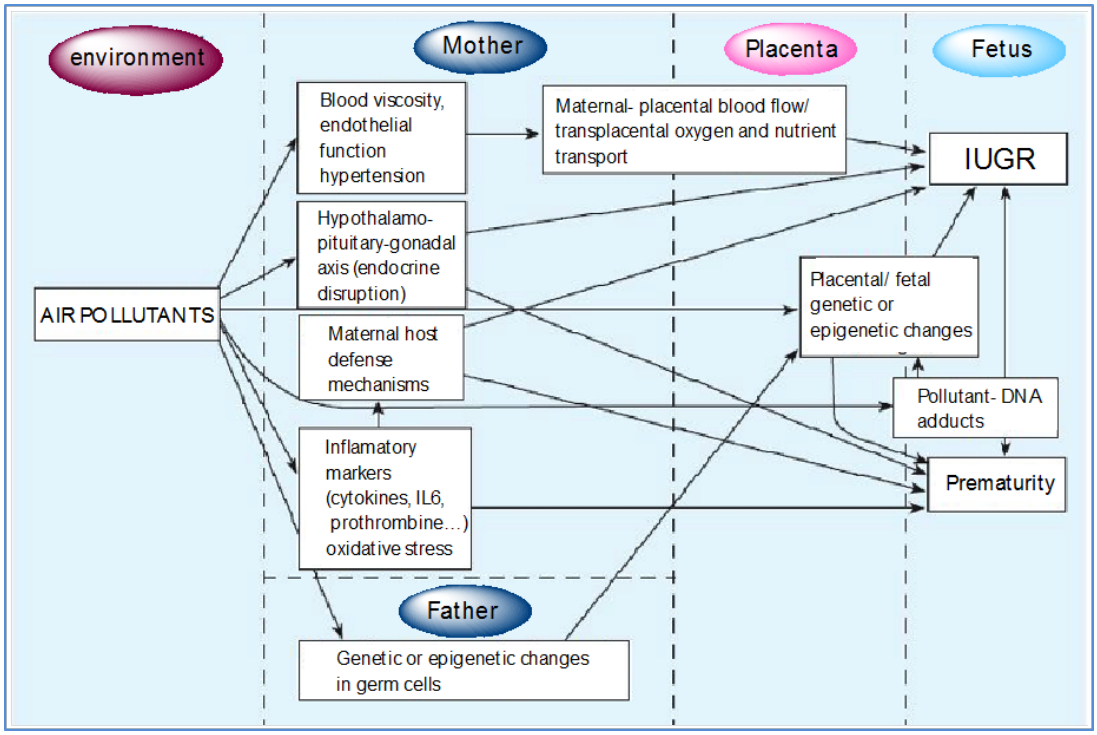


Figure 2-4.Possible biological mechanisms by which air pollutants may affect birth outcomes (Xu et al. 2011) .

2.4.2 Air quality and pollution inside the IVF

Despite the empirical experience and scientific evidence about how environmental toxins and pollutants are detrimental to reproduction and development, the studies are still unable to reveal the extension of the damage produced by these pollutants, especially within the in vitro fertilization (IVF). The majority of the assisted reproduction technology (ART) laboratories are exposed to the pollutants found in the outdoor environment. However, daily routine activities and equipment within ART laboratories may also contribute to indoor pollution

There are few studies correlating the presence of IVF known pollutants with deleterious effects over human gametes and embryos. Additionally, facilities manage occupational limit values (OLV) inside the IVF laboratories to daily cover exposed workers without adverse health effects, but specific quality standards and specific threshold levels, at which most of the common pollutants in the IVF`s ambient air can cause harm to cultured human embryos, have not been officially established.

Embryo development in the IVF laboratory it is definitively influenced by its environment. Types, sources, environmental control and pollutants effects have been compiled for a better understanding of the positive evolution on the IVF environment and the path that remains ahead for waging against known pollution because information is still too dispersed and unofficial. Research results confirm the stress of gametes or since early stages of human development, in addition to the damage that

has already been done during other reproductive stages. Hazardous air pollutants have deleterious effects over cells structures and at a molecular level affecting their viability.

The majority of assisted reproduction technology (ART) laboratories are exposed to the pollutants found in the external environment. However, daily routine activities and equipment within ART laboratories may also contribute to local pollution (De los Santos, 2001; Esteves and Bento, 2016; The National Institute for Occupational Safety and Health, 2015).

Ironically, in some cases the air progressively diminishes in quality from the outside of the building throughout the laboratory, reaching the highest toxicity inside incubators that receive most of their ambient air from within the laboratory (95-96%) (Cohen et al., 1997). Contaminants are frequently detected inside ART facilities; indeed, a 5- to 6-fold increase in volatile organic compounds (VOCs) has been reported inside laboratory equipment (incubators) (Hall et al., 1998). If any of these compounds is capable of diffusing into the culture media, they will adversely affect gametes and embryos at a sensitive stage, with teratogenic effects and devastating outcomes (Cohen et al., 1998; Fabro, 1978). Strategies have been developed to prevent negative outcomes (Hall et al., 1998), though there remains a lack of data regarding the types of pollutants detectable inside IVF laboratories and what concentrations represent a danger to embryos and future offspring. The air quality is a major determinant of IVF success.

2.4.3 Types of pollution

Atmospheric air pollutants can be classified in many ways. They can be categorized based on whether they are directly emitted from their sources into the atmosphere (primary pollutants) or if they are formed from photochemical reactions from primary pollutants (secondary pollutants). They can also be classified according to chemical composition (organic or inorganic), type of source (natural or anthropogenic), degradation properties (degradable or non-degradable), or place of generation (indoor or outdoor), and finally they can be easily classified based on state of matter as well (solid, liquid such as particulate matter (PM), or gaseous such as VOCs) (Daly and Zannetti, 2007). In spite of this, it is very important to bear in mind that each pollutant has its very own chemical composition, reaction properties, emission, and persistence in the environment, ability to be transported in long and short distances and their specific impact on human and/ or animal health. Additionally, the pollutants that are known or suspected to cause irreversible illnesses because of their toxicity, such as cancer or reproductive and birth defects, are classified as hazardous air pollutants (HAP) (Kampa and Castanas, 2008; U.S. Environmental Protection Agency, 2016a) .

2.4.3.1 Particulate matter (PM)

PM is a complex mixture of extremely small solid and liquid particles (droplets) that can contain a wide range of inorganic and organic components (U.S. Environmental

Protection Agency, 2016b). These are the most common atmospheric pollutants and their mass and composition are strongly influenced by climatic and meteorological conditions. There are two principal categories: coarse particles mostly larger than 2.5 µm in aerodynamic diameter, and fine particles mostly smaller than 2.5 µm in aerodynamic diameter (PM2.5) (World Health Organization, 2000a). However, for a proper identification, PM can be categorized as follows:

Particles according to size	Origin
Inhalable coarse particles: larger than 2.5 µm and smaller than 10 µm in diameter	Crust materials and fugitive dust found near roadways and dusty industries.
Fine particles or $PM_{2.5}$: (2.5 µm or smaller) and black carbon; emissions are based on speciation of $PM_{2.5}$	Aerosols formed from gas to particle conversion. Emitted from industries, automobiles, or forest fires.
$PM_{1.0}$: Less than 1.0 µm in diameter	Largest number of particles and the most hazardous in terms of mortality and cardiovascular and respiratory evidence.
Ultra-fine particles $PM_{0.1}$: Less than 0.1 µm of diameter	Nanoparticles: (Vaccines, personalized cancer therapy, drug delivery, and diagnostic methods)

Table 2-1.Classification of particulate matter (PM) according to size and origin (Centre Interprofessionnel Technique d'Etudes de la Pollution Atmosphérique 2016, U.S. Environmental Protection Agency, 2016b) .

Most PM can pass through the throat and nose and enter the lungs, most of all, the smallest fractions. Once they are inhaled, particles can affect the heart and lungs, causing serious health problems due to their ability to penetrate deep into the blood stream and through different mechanisms, depending also if there is an acute or chronic exposure (Shah et al., 2013; Shah et al., 2015; U.S. Environmental Protection Agency, 2016b).

Animals and humans are exposed to a great variety of PM, which depends on the exposition place because of the differences between outdoor and indoor pollutants, or the individuals' characteristics that will influence as well. The PM10 and PM2,5 are usually related to the outdoor ambient air, regular indoor spaces (tobacco smoke) or occupational exposition, while inside a cleanroom, because of the air has to be in high quality status, PM10 it is not that relevant. On the other hand, small particles (<PM2,5) are more related to personal exposure, also related to gaseous air pollution, and the health of the cells and an individual will be compromised due to different types of interactions (World Health Organization 2003).

The PM that is suspended in the air may be composed of acids or salts (nitrates and sulfates, carbonates and chlorides), organic chemicals (polycyclic aromatic

hydrocarbons (PAHs), condensable organic compounds or organic carbons originated by the oxidation of VOCs, trace elements such as heavy metals (HM), soil, or dust particles, and black carbon (Centre Interprofessionnel Technique d´Etudes de la Pollution Atmosphérique, 2016). However, according to the WHO the most commonly PM associated with human health problems comprises HM, PAHs or other organic components, endotoxins (not discuss in this review), and nanoparticles, etc., (World Health Organization, 2003).

HM particles are responsible of toxic effects when they are involved in the biochemical reactions of living organisms. Typical toxic responses include growth inhibition, suppression of oxygen consumption, and impairment of reproduction and tissue repair. Lead (Pb), cadmium (Cd), and mercury (Hg) are non-essential HM emitted through industrial activities and they are some of the most common toxic heavy metals to which humans are exposed due to their persistent, accumulative, and toxic nature (Suvarapu and Baek, 2016). These non-essential heavy metals are emitted through industrial activities and they can be found in almost every region. Cd, for example, is a compound that can be found in almost everything that we breathe, eat and drink, and it can travel long distances from its source via natural and anthropogenic atmospheric transports. Cd can be found attached to 0.1–1-μm size particles, which have an atmospheric lifetime of a few days, depending on the particle density and meteorological parameters (World Health Organization, 2007) .

Incomplete combustion processes tend to originate PM with carbonaceous cores that adsorb different organic toxicants, like PAHs, contributing to the strong toxic potential of submicron-sized particles. Moreover, atmospheric organic compounds (both in gas and particulate phase) generate oxidized derivatives by photochemical oxidation processes (Kim et al., 2013a; Mesquita et al., 2015). There are many PAHs, mostly considered PM but a few of them could be found in both, particulate and volatile phase because they can be vaporized when exposed to above room temperatures; for this reason, they can be called semi VOCs as well. In the ambient air the carcinogenic 4- to 7-7-ring PAHs (high molecular weight compounds) are preferentially attached to other particles or absorbed by particles because of their low pressure in the air; but because of the impact of the indoor characteristics and activities, the low molecular weight PAHs can be or become, volatile: naphthalene (most volatile: 98%), fluorene, acenaphtene, acenaphtylene, phenanthrene, anthracene, fluoroanthene, pyrene.(less volatile: 55%).

Some of the diesel exhaust particles (DEP) are composed from both particle PAHs and semi volatile PAHs, including benzo [b] fluoranthene, benzo [k] fluoranthene (Januário et al., 2009). The small size particles also interact with pollutants such as ozone (O3), NO (nitrogen oxide) and SO2 (Sulfur dioxide), and they can be degraded by microorganisms (Kim et al., 2013a; Mesquita et al., 2014; World Health Organization,

2000b) . So because the PAHs are complex mixtures of chemicals some are well-known carcinogens, mutagens, and teratogens. Some of the most potent PAH carcinogens that have been identified include: 7, 12– dimethylbenz(a)anthracene, benzo[a] anthracene, benzo[a]pyrene, and dibenz[ah]anthracene, which have shown clear evidence of mutagenicity/genotoxicity in somatic cells (Detmar et al., 2006; Kim et al., 2013a; Urbancova et al., 2016).

Ultrafine particles or nanoparticles (NPs) are synthesized from materials such as cadmium selenide (CdSe), gold (Au), silver (Ag), perylene (C20H12), polystyrene $(C8H8)_n$, carbon (C), iron oxide (Fe2O3), silica (SiO2), titanium dioxide (TiO2), and organics such as latex, polylactic acid, polyglycolic acid, and polyalkylcyanoacrylate. As a result, a large number of products exist in any clinical setting that can release NPs into the environment. They have been widely applied in biomedicine for different purposes in human and veterinary medicine, during preclinical or clinical phases (in the development of vaccines, personalized cancer therapy, drug delivery, and diagnostic methods, among others) (Bosman et al., 2005) .

2.4.3.2 Volatile Organic Compounds (VOCs)

VOCs are gaseous emissions of organic compounds that participate in forming O3 and have health implications such as cancer and reproductive toxicity (Webb et al., 2014). VOCs are chemicals that contain carbon (C) along with other elements (hydrogen, oxygen, fluorine, chlorine, bromine, sulfur, or nitrogen). However, carbon monoxide, carbon dioxide, carbonic acid, metallic carbides, or carbonates and ammonium carbonate are not VOCs. VOCs are volatized into the air during the manufacture or use of everyday products and are released into the indoor air from many products, materials, and people. Their composition makes it possible for them to evaporate under normal indoor atmospheric conditions of temperature and pressure (U.S. Environmental Protection Agency, 2016c).

VOCs are formed as intermediate compounds during the combustion, decomposition, or breakdown of longer-chain carbon compounds, as well as during the photosynthesis process in vegetation (Dutta et al., 2016; Eller et al., 2016; Pinto et al., 2010). VOCs have an initial boiling point less than or equal to 250°C, measured at a standard atmospheric pressure of 101.3 kPa. The higher the volatility (lower boiling point) the more likely the compounds will be emitted into the air due to weakened intermolecular forces. The United States Environmental Protection Agency (EPA) has technically categorized these compounds depending on the ease with which they are emitted (U.S. Environmental Protection Agency, 2016d).

SVOCs can be transported with by the wind, transformed by direct or indirect photolysis during the transport, and removed from the atmosphere by wet and dry deposition; gaseous SVOCs are more mobile and liable to photolysis (Wei and Li, 2010). This group comprises a very wide range of individual substances including

hydrocarbons, halocarbons, and oxygenates, which is why many hundreds of them are present in the atmosphere.

Type of VOCs	Examples of substance
Very Volatile Organic Compounds (VVOCs): Found almost entirely as gases.	Propane, butane, methyl chloride. They can be difficult to measure.
Volatile Organic Compounds (VOCs): Boiling point below 150 °C and vapor pressure greater than 0.1 mmHg)	Limonene, toluene, acetone, ethanol, isopropyl alcohol, hexanal, etc.
Semi-Volatile Organic Compounds (SVOCs): Higher boiling point and lower vapor pressure than VOCs. Present in both gas and particle phases in the air.	Pesticides (dichlorodiphenyltrichloroethane or DDT, chlordane, plasticizers (phthalates), fire retardants (polychlorinated biphenyl or PCB, polybrominated biphenyl or PBB). Highly related to Fine Particles.

Table 2-2Classification of indoor VOCs according to volatile properties by the WHO (U.S. Environmental Protection Agency, 2016d; Wei and Li, 2010) .

2.4.4 Sources of pollution

Outdoor pollution contributes to indoor air quality through the type of ventilation in each facility (natural or forced), the ventilation rate (air changes per hour), and the nature of the contaminants. Some of the most significant outdoor air pollutant emissions are associated with industry and traffic emissions (Daly and Zannetti, 2007; Jones, 1999a; Perin et al., 2010a). The indoor environment depends on exposure to chemicals and odors that are recognized as hazards to human and animal health. Metropolitan areas are intermittently exposed to PM and VOCs through different types of products that interact with environmental factors that influence their reactions (Perin et al., 2010a). PM and VOC emissions are generated from numerous sources (Table 2-3).

Emissions	General sources
Outdoor and Soil Emissions	Vehicle exhaust, gasoline or diesel powered vehicles (CO, NOx, PM), VOCs from dumpsters, buildings or construction activities, chemical spills, fire damage, radon; soil, road and agricultural dust; pesticides, pollen, biomass burning
Indoor Emissions	Bio and microbial aerosols, VOCs and O_3 from equipment, solvents, toners, ammonia, chlorine of stored supplies, construction activities. Emissions from laboratories and facilities mechanical systems, sewer odors, improper bathroom ventilation, emissions from housekeeping/ cleaning activities, pesticides, trash and chemical spills, fire damage (soot, polychlorinated biphenyls from electrical equipment, odors), smoking, cooking, fabric materials, scented products

Table 2-3. General sources of hazardous air PM and VOCs, taken from general description given by NIOSH (The National Institute for Occupational Safety and Health, 2015) .

The physicochemical properties of PAHs make them highly mobile in the environment as well, allowing them to distribute across air, soil, and water bodies where their presence is ubiquitous (Kim et al., 2013a). The major route of exposure to PAHs in the general population is from breathing ambient (and indoor) air, eating food containing PAHs (non-smokers), smoking cigarettes, or breathing smoke from open fireplaces (Abdel-Shafy and Mansour, 2016). Processing (such as drying and smoking) and cooking of foods at high temperatures (grilling, roasting, and frying) are major sources of PAHs (Ciecierska and Obiedzinski, 2013). Some crops (such as wheat, rye, and lentils) may synthesize PAHs or absorb them via water, air, or, soil (Abdel-Shafy and Mansour 2016). So, the intake may occur via soil ingestion, inhalation or dermal exposure, or from inhalation of PAH vapors (Srogi, 2007; Wang et al., 2012). Occupational exposure occurs mostly by breathing exhaust fumes (such as mechanics, street vendors, or motor vehicle drivers) and those involved in mining, metal working, or oil refining. Some exposures may simultaneously involve multiple routes such as dermal and inhalation exposures from contaminated air, affecting the total dose of absorption (Kim et al., 2013a).

As far ART laboratories are concerned, VOCs are one of the most worrisome airborne pollutants for embryos. They are not only introduced from outside air, but they are very difficult to remove from IVF's ambient air and from the incubators, and they can interact with PM as well (Khoudja et al., 2013; Wale and Gardner, 2016a). VOCs are constantly released inside reproductive facilities as different types of unsaturated volatiles and can accumulate through the oxidation of air and light over materials routinely used in ART facilities. Although nowadays laboratories use appropriate materials in construction, painting, flooring and furniture to accomplish cleanroom standards on minimizing pollutants, and therefore embryo toxicity, the clinical setting environment may, in fact, be two to five times or even more toxic than the outdoor

environment (ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016; Hall et al., 1998; Jones, 1999b; Khoudja et al., 2013; Wale and Gardner, 2016a).

VOCs and PM are present inside IVF laboratories through various vectors, such as heating, ventilation, and air conditioning (HVAC) systems; diffusion of volatiles from adjacent rooms and hallways; produced inside the incubators; off-gassing materials; equipment; people inside the laboratory (perfumes and other personal odors); and medical and anesthetic gases, among others. Indeed, additional sources include potable water, dust particles, glass fragments, alcohol burners, plastics and disposable plastic ware and their shavings (petri dishes, culture flasks and test tubes), markers, disinfectants, microscopes, television monitors, and furniture. Alcohol for example, should not be used while embryos or gametes are being manipulated; any activity related to them has to wait at least 30 minutes until the vapors can be removed; but if gametes and embryos have to stay inside incubators they risk becoming contaminated by benzene or other VOCs, because these have been found inside the CO2 gas cylinders (Cohen et al., 1997; De los Santos, 2001; Lawrence et al., 2007; Mehta, 2013; Thomas, 2012).

Due to empiric findings and studies focusing on this issue, it is well established that laboratories have many measurable substances being continuously released and produced. Official research on this matter is very scarce on peer review and the majority of related investigations were carried out years ago (Gilligan et al., 1997; Nijs et al., 2009). Gilligan et al. determined that air chemicals were emitted from the plastic ware used for culturing; all the tested batches released styrene, ethyl-benzene, and benzaldehyde at low levels, and a total of 33 VOCs were detected in two types of manufacturers' plastic ware. Despite the fact that one type had a lower number of VOCs, some flasks had significantly higher levels of chemical contaminants. High levels of C8-C10 branched hydrocarbons were also detected. Further, the levels of styrene, ethyl-benzene, and benzaldehyde were often proportional to the total loading of petri dishes per incubator, indicating that their source was derived from the disposables used during gamete and embryo culture (Gilligan et al., 1997).

2.4.5 Control of pollution

A good understanding on how pollutants can infiltrate or be produced inside the clinics, laboratories, and incubators is necessary to improve the design and the management strategies of the laboratory so as to minimize contamination. To optimize human in vitro development, more must be done, than simply improving the culture media formulations to minimize exposures during early embryo development (Hyslop et al., 2012; Wale and Gardner, 2016a). The first improvement concepts aimed at transforming other cleanroom designs, minimizing pollutants like toxic vapors and particles to accomplish its specific permitted values and reduce their concentrations inside laboratories, mostly inside incubators, and the impact over gametes and

embryos (Lawrence et al., 2007; Thomas, 2012). Now, we know that quality control should involve culture media, all contact supplies and gases used in an IVF procedure to ensure excellence from oocyte collection through blastocyst development and to accomplish a viable pregnancy (Cohen et al., 2009; ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016) .

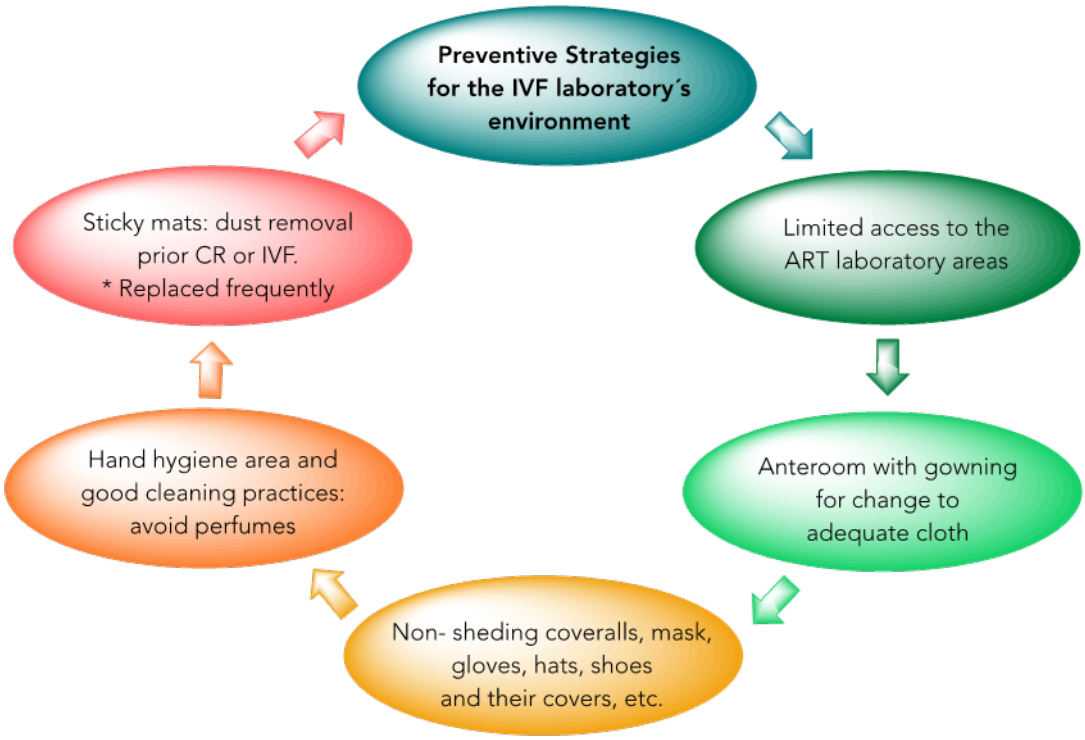


Table 2-4. Cleanroom preventive strategies against contamination (Boone et al., 1999; De los Santos, 2001; ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016; Esteves and Agarwal, 2013;, Esteves and Bento, 2016) .

The design and adaptability of the laboratory is very important as well, especially to future improvements that could lead to decrease air pollutants and therefore increase good clinical outcomes (Forman et al., 2014; Hyslop et al.; 2012; Khoudja et al., 2013). The isolation of the IVF lab, retrieval room, and transfer room is essential (Dickey et al., 2010). For a clean access of personnel and materials, the anterooms between the operating room (OR) and the IVF lab (cleanroom) should be equipped with double doors with a window to allow personnel to pass oocytes and embryos through while minimizing the mixing of air, specially the medical gases. A separate laboratory with a safety fume hood should be provided for analyses using fixatives and other toxic reagents. The area for cleaning and sterilization of materials, if present, should be separated from the laboratory as well. On the inside, there should be laminar flow cabinets, positive pressure and air filtration systems, among other procedures. The task of eliminating pollutants must be ongoing, considering that they are also related to the presence of microorganisms: for example, it has been calculated that dust particles of <0.5 um in diameter often carry bacteria and/or fungi (Boone et al., 1999; De los

Santos, 2001; Dickey et al., 2010; ESHRE Guideline Group on Good Practice in IVF Labs et al., 2016; Esteves and Agarwal, 2013) .

One of the first reports describing changes in air quality and the influence on the laboratory environment was performed also by Cohen et al., in newly designed laboratories with pollution handling systems and with an incubator with control devices for VOCs in one of the laboratories. The group was able to identify possible aldehyde threshold values to use as the reference to evaluate the laboratory practices and determined the solubility of the other compounds found in air samples (Cohen et al., 1997). In a follow-up report, they determined the total-VOCs or TVOCs (sum of the ambient air organics found in the ambient air), finding that they are valid quality indicators for laboratories and the incubators air and to determine the gametes and embryos risk of exposure to pollutants (Cohen et al., 1998). More recently, Khoudja et al. confirmed that after two periods of improvements of the air filtration system, the TVOCs levels outside, inside the laboratory, and inside the incubators had a positive evolution: 18 of 49 different types of volatile pollutants decreased significantly. But in spite of this, there were pollutants that were difficult to remove from the incubators and minimal or no changes were seen in others like propylene, propane, heptane, xylene, cyclopentane, ethylbenzene and styrene, etc. It was also noticed that the outside VOC levels depended on the period of time they were being measured: they could be three to four times higher in winter than in summer, which suggests the seasonal influence (Khoudja et al. 2013).

As to prevent and control the IVF pollutants, various parameters could be taken into account to be measured, such as compound concentration and composition, solubility and vapor pressure (specially for VOCs), particle size, shape, surface modification, and degree of agglomeration of particles, as well as the ambient temperature and the surface area from which they could be released (Celá et al., 2014; Thomas, 2012). Eg., the degree of solubility of VOCs or particle compounds is necessary to determine if the compound might penetrate the culture medium, or whether if it is able to penetrate the mineral oil layer. When the latter is used as a protective overlay for the IVF culture medium it will hinder the penetration of a pollutant, but if it is soluble in both oil and water the risk of molecules penetration can be estimated by partition coefficients from air to oil (with vegetable oil) and oil to water (with octanol). The latter technique was also used by Cohen et al. to evaluate the risk of absorption of several compounds into the culture media; the octanol-water partition coefficients ranged within -0.01 to 6.25, with negative values indicating that a compound is most likely to be absorbed by the media (eg. acrolein, -0.01 and methanol, -0.74) (Cohen et al., 2001; Thomas 2012). This has also been tested to determine semi-VOCs such as PCBs and PAHs (Finizio et al., 1997).

It is equally important that culture systems supply a healthy environment for the embryos so they can develop and be capable of develop and implant properly; for this reason, quality control systems should be able to screen all products to detect embryotoxic substances. Washing mineral oil with a medium with albumin may lower both toxic lipophilic and toxic hydrophilic contaminants (Otsuki et al., 2009). Also, manufacturers and laboratories have different types of bioassays [the 1-cell or 2-cell mouse embryo assays (MEA) or the human sperm motility assays, HSMA], that can be used to indicate the toxicity and functionality of the reproductive, transport and cryopreservation media (among others), and lab-ware or materials that can be in contact with gametes and embryos (pipettes and petri dishes, etc.) but it is still unclear whether the sensitivity of these assays is enough to detect the toxins that are relevant to the human IVF laboratory, especially in the early stages of development (Wolff et al., 2013).

Mineral oil, used for embryo culture and embryo research applications, can act as a sink for toxins as well as a source because is not inert (Morbeck et al., 2010). It can vary widely in quality in each lot because it is derived from crude oil which is the same starting material used for chemicals like benzene, which is an IVF-related chemical used for polystyrene to make petri dishes. It is a mixture of straight chain, cyclic and aromatic hydrocarbons, mostly saturated but also low levels of unsaturated hydrocarbons, which include the more reactive PAHs (Khan et al., 2013; Morbeck and Leonard, 2012), and compounds like peroxides, aldehydes, alkenals can be present as well (Morbeck et al., 2010). It can also be affected by oxygen exposition (peroxidation and free radicals formation) and storage conditions (temperature) varying widely its quality (Ainsworth et al., 2017). Mineral oils have passed the manufacturers bioassays because of the lack of sensitivity of the tests, and have reached the laboratories affecting the embryos. Nonetheless, mineral oil can improve results after being washed, regardless of the wash solution (HTF, HTF-HSA, water) or the washing temperature (Morbeck et al., 2010) and likewise, the modification of the assays (HSMA + 1-cell MEA, instead of the 2-cell MEA) could be used in conjunction to obtain reliable results when screening for toxins (Hughes et al., 2010; Morbeck et al., 2010). Experimental improvements on the assays have shown that the mouse strain used is a factor; when testing different proven embryo stressors (cumene, peroxide and triton- X) inside culture media, the CF1 outbred mice strain was more sensitive than inbred or hybrid strains (Khan et al., 2013). Other study described that the MEA used in conjunction with time-lapse imaging was more sensitive to the toxicity of the mineral oil, because the timing of the cells cleavage could provide quantitative and sensitive markers of stress (Wolff et al., 2013); however, this assessment requires costly equipment, which may not be available or realistically used for QC testing. Recently, Ainsworth et al., described a modified MEA that could be more suitable for many laboratories: the extended MEA (eMEA) is more simple and sensitive when assessing

the cells number and blastocyst formation rate was at 144h (instead of the 96h assessment as the regular technique) of individually cultured embryos, because group culture can stabilize the embryo environment and mask toxicity (Ainsworth et al., 2017).

The ambient air quality awareness has enabled the laboratories to control against detrimental factors and emphasize on implementing good control practices. Facilities now have an improved environment after the installation of air management systems such as high efficiency particulate air filtration systems (HEPA), which removes almost 99.9 % of particles larger than approximately 0.3 μm (Cohen et al., 1997; Esteves et al., 2004; Higdon et al., 2003) , ultra-low penetration air (ULPA) (Boone et al., 1999; Dickey et al., 2010), activated carbon filters, potassium-permanganate filters (Esteves et al. 2004; Munch et al., 2015; Sene et al., 2009), photo-catalytic units (Lawrence et al., 2007), , UV radiation (Gea Izquierdo et al., 2009), and incubator control devices such as the CodaÒ system: incubator filtration units within the incubators and chambers, the CodaÒ CO2 and Tri-Gas InlineÒ filters in the incoming gas lines, and the Coda tower that filters the air of the laboratory, procedure rooms and working environment. The amount and form of activated carbon and oxidizing media (KMn4O4) can also vary between filters (Forman et al., 2004; Khoudja et al., 2013; Merton et al., 2007a; Racowsky et al., 1999). Activated carbon absorbs the higher molecular weight hydrocarbons (PAHs) because of the pores of varying size and a field of molecular attraction that captures large flat electron-rich molecules. Low molecular weight organics, alcohols, ketones and aldehydes can be oxidized and degraded by potassium permanganate. Photocatalytic oxidation (PCO) technologies is also used to fights against VOCs (Munch et al., 2015; Worrilow, 2017).

However, despite these, the risk of gamete and embryo exposure to pollutant compounds is never completely removed. Additionally, the specific requirements for IVF laboratories can also be different due to variations in regulations among countries and regions (Morbeck, 2015). New proposals such as the isolated IVF and engineered molecular media and genomically modeled biological inactivation are also in development and have shown significant increase in blastocyst conversion rates (Forman et al., 2014; Hyslop et al., 2012).

Environmental and work health institutions (WHO, Occupational Safety and Health Administration (OSHA) and Instituto Nacional de Seguridad e Higiene en el Trabajo (INSHT), spanish for National Institute of Work Security and Hygiene), have chemical standards for evaluating industrial hygiene and health. These were only designed to cover workers that could be exposed every day without adverse effects, but they are not designed for cultured and largely unprotected cells such as the embryos and gametes as they lack physical barriers (epithelial surfaces), immunological defense or detoxifying mechanisms (Cohen et al., 1997; European Commission, 2010; Instituto Nacional de Seguridad e Higiene en el Trabajo, 2016; Thomas, 2012; Worrilow, 2017).

For general contamination, threshold limit values can be obtained and registered in concentrations of milligrams (mg/m3), parts per million (ppm), or micromoles (μm). To measure and evaluate the negative effects in cultured cells inside laboratories, limit values need to be in much lower concentrations, like μg/m3, ppm, or ppb. Unfortunately, specific quality standards and specific threshold levels at which contaminants cause harm to embryos have not been determined (Thomas,, 2012). Further, the measured composition of air pollutants, such as VOCs, can vary significantly depending on the methods and recognized terminology, lending to confusion (U.S. Environmental Protection Agency, 2016c).

2.5 Effects of pollution over the embryo health

It is important to take into account that patients undergoing reproductive treatments, who are already considered sub-fertile, are more susceptible to environmental influences, and that gametes and embryos are going to be even more vulnerable because they lack the physiological maturity of a differentiated mammal to protect themselves (Cohen et al., 1997; Hall et al. 1998 and Legro, et al., 2010). The pattern of substance distribution among the placenta, ovarian follicular fluid, uterine luminal fluid, embryonic stages, and plasma also varies with each compound and is related to molecular weight, solubility, and degree of ionization at a physiological pH. These individual mechanisms can be toxic and produce adverse effects on fertilization, implantation, and on further development. Pregnancy may also modify the degree to which drugs are distributed (Fabro, 1978). Perin et al. reported that infertile women undergoing reproductive treatments, during the follicular phase of the conception cycle, were at an increased risk of early pregnancy loss on account of short-term exposure to increasing levels of atmospheric PM10 (Perin et al.; 2010b).

The first human pre-implantation toxicology research known was able to find 300 environmental toxins in samples of unfiltered outdoors, filtered and incubator's air, and they were able to establish a correlation between lower pregnancy (PR) and implantation rates (IR) linked to laboratory overhauls such as constructions, refurbishments (installation of filters), punctual events such as fumigations (Cohen et al.; 1997) or, in other studies, because laboratory's inner activities, certain pollutants have increasing concentrations on specific laboratory's spots: inside incubators (Cohen et al., 1998; Hall et al., 1998; Racowsky et al., 1999) or minihoods used for oocyte retrieval (Boone et al., 1999). In the same period, Cohen et al, demonstrated that the construction of a laboratory produced gaseous emissions, that arrested 90% of the two-cell stage mouse embryos despite of the use of freestanding ionization units and HEPA. Blastocyst development rates (BDR) were higher before and after the construction period (Cohen et al, 1997) .

Since then, the development of the cleanrooms and filtration systems has change the organic chemistry of the ambient air within the laboratories and incubators, by reducing VOCs and PM levels very effectively, improving embryo development, IR, PR and other outcomes (Boone et al., 1999; Dickey et al., 2010; Legro et al., 2010). The first study to demonstrate this, reported that the four-cell stage embryos rates increased significantly (p value= 0.003) after the ULPA filters were functioning properly (Boone et al., 1999). A comparison between cleanrooms for micromanipulation and embryo culture, found out that inside a Class 100 (ISO 5) laboratory, higher rates of fertilization, cleavage, good quality embryo formation (p= 0.001) and pregnancy were obtained, compared to Class 1000 (ISO 6). This laboratory was equipped with HEPA and carbon filters inside of the IVF and adjacent rooms, from the gas cylinders to incubators, and intra-incubator filtration units were also utilized. Not all the clinical outcomes were significant but embryos cultured with VOC filtration had a higher likelihood of reaching the blastocyst stage (Esteves et al., 2004) .

However, despite of the improvements, pollutants are difficult to eradicate and IVF daily activities will always influence the outcomes. Otsuki et al, found elevated peroxidation values (POVs) in the mineral oil used on a failed human embryo culture because it was exposed to oxygen and a bad storage. Then evaluated mouse oocytes allocated in different POV treatments resulting in a significant decrease in fertilization, cleavage and BDRs. When POV value was 0.5 mEq/kg, all of the embryos died by day (D) 3 and when the POV was 1.0 mEq/kg, all embryos died by D2 (Otsuki et al., 2007). Morbeck et al. obtained improved blastocyst development outcomes and reduced toxicity in the one-cell MEA after washing mineral oil that previously was analyzed and confirmed with toxins (Morbeck et al., 2010). Just by comparing 4 different commercial oils, other study observed significant differences between in the embryo development and quality on D3 (Sifer et al., 2009). Recently, Munch et al. retrospectively reviewed the embryo development and PRs of embryos cultured before the unintentional removal of the incubator´s carbon filter and after they restored it. Fertilization, cleavage and BDRs from fresh cycles all dropped during the "absent" period, specially the ones that resulted from ICSI, probably due to combination of environmental and mechanical stressors, and recovered significantly during and after placing the carbon filter. However, embryos that were fertilized and cryopreserved prior to the "absent" period, but were thawed and cultured during it, showed no decrease in cleavage rate or blastocyst formation rate. So, this lack of carbon filtration affects embryos preferentially in the peri-fertilization period (Munch et al., 2015). Similar results were obtained in a previous study, when the laboratory`s carbon filter was inadvertently removed (Kresowik et al., 2012) .

Meanwhile, detailed information about the relationship between pollution and embryo parameters, such as morphology and quality, cleavage rate, symmetry, fragmentation,

multi-nucleation, embryo development rate inside incubators, BDRs, pattern, hatching process and the defense mechanisms require more attention (Esteves et al., 2004; Legro et al., 2010; Maluf et al., 2009; Thomas, 2012). These pre-implantation processes are not be able to be followed in vivo, hence the importance of their evaluation during IVF culture to observe unique reproductive and developmental events (Kresowik et al., 2012; Maluf et al., 2009). Hyslop et al, reported an accelerated progression of development from early embryos up to blastocysts stage by D7 ($p<0.001$) inside an enclosed system that protect oocytes and embryos throughout the IVF process up to their transfer. A higher proportion of expanded and hatched blastocysts was obtained by D6 and significantly higher cells number on D6 and D7 were obtained ($p=0.05$) as well, by the nuclear counts of fixed blastocysts of the enclosed system compared with the open system (Hyslop et al., 2012) .

The main mechanisms related to exposure to air pollution have been studied for cardiopulmonary diseases and few studies have related the oxidative stress in early human development with clinical outcomes in pregnant women (Mohorovic, 2004), however there is still a long way to go. Oxidative damage produced by pollutants has showed time- dependent cumulative effects and it can affect the membranes potential of mitochondrias or produces apoptosis. Embryonic stem cells can have different responses compared to somatic cells when exposed to pollutants or antioxidant treatments (pyruvate in higher doses into the culture media) (Ramos-Ibeas et al., 2017). Oxidative stress-related genes and pancreatic and eye-lens gene markers appear de-regulated in embryos exposed to urban pollution, whereas exposure to rural extracts affected genes implicated in basic cellular functions (Mesquita et al. 2015; Nemmar et al., 2013) .

It is necessary to understand the mechanisms of pollutant action on the developing embryo while researching ways to prevent contamination in the future. Research on specific pollutants will provide insight as to how to prevent exposure. Deleterious effects of specific pollutants inside the IVF laboratory and their effects on mammal and human IVF results are described below.

Recently, growing evidence is indicating that mitochondria can be targeted organelles of pollutants

2.5.1 Effects of general PM on IVF

Specific effects of airborne PM have been described mainly in animal models. Maluf et al. evaluated different patterns of ovarian response in mice and the interactions with different types of air: pre-postnatal exposure to filtered air (FA), FA-ambient air (AA), and AA-AA. A significant impairment in fertilization, zygotes, embryo development up to blastocyst stage and hatching process was observed in the high-response subgroup. There was also a defective lineage of specification in blastocysts that impacted embryo survival and the post-implantation developmental potential after being exposed to

PM2.5. These results showed a significant decrease in the inner cell mass (ICM) cell count (20%) and the ICM/Trophectoderm (TE) ratio (25%), especially in the AA-AA group, because ICM and TE cell lines seem to have a differential susceptibility to embryotoxic agents (Maluf et al., 2009).

The mixture of pollutants can be more dangerous if unprotected embryos are exposed, but to obtain good embryo quality it is necessary to have a proper folliculogenesis as well. Despite that this process does not happen inside the laboratory, authors have found correlations between IVF clinical results and the most common pollutants: PM2.5, PM10, Nitrogen dioxide (NO2) and O3; all of them can interfere with the IVF process, after chronic or acute exposure. Chronic exposure to PM10 seems to impact the follicular growth and can decrease live birth rates (Legro et al., 2010; Perin et al., 2010b) . However, the main effects over the ovarian response and the top embryos can be obtained after acute exposure (Carre et al., 2016) . There can be adverse effects on conception and intrauterine pregnancy due to increasing PM2.5 levels as well. Legro et al, described that the presence of varying diameter particles can induce harmful effects throughout the period from retrieval to transfer, which is when mothers and embryos are separated, without significant associations to pregnancy or live births (Legro et al., 2010) . In addition, Legro et al., reported that the presence of NO2 had the majority of negative effects on live birth rates, mainly in the period from embryo transfer to pregnancy confirmation. Carré et al., reported lower number of top embryos and decreased implantation rates after acute more than after a chronic exposure to NO2. In contrast, when NO2 exposure is limited and O3 effects seem to diminish and authors have not found the association with IVF failure. Legro et al, reported two different phases: 1) O3 increasing levels during oocyte maturation, associated with an increased live birth rates, and 2) O3 increasing levels after embryo transfer, associated with significantly decreased live births odds (P= 0.002). Carré et al, also found positive effects on the ovarian response as well as in the number of top embryos, even though high levels of NO2 and PM10 were measured, indicating that O3 could have an effect of its own. The reason for this has yet to be elucidated (Carre et al., 2016; Legro et al., 2010) .

2.5.1.1 Effects of heavy metals on IVF

Some heavy metals (HM) have physiological functions. However, pathologies may develop with deficiencies and/or excesses of essential metals, and more so with non-essential metals (those with no physiological functions), because of noxious effects caused by binding to macromolecules or by activating or inactivating cellular processes that can be controlled by the essential metals (Vinken et al., 2010). Some of its health implications in humans are unknown and data describing reproductive health effects are sometimes inconclusive (World Health Organization, 2007).

While the reproductive and developmental effects of cadmium (Cd) or other HM are not considered critical in humans by official organizations such as the WHO, specific investigations have provided evidence of their negative effects on reproductive tissues and developing embryos in several species. Buffalo oocytes and embryos showed dose-dependent negative effects on the viability, maturation, morphological abnormalities, cleavage, morula/ blastocyst yield, and blastocyst hatching when exposed to Mercury (Hg) and Cd; however, the applied doses were higher than those typically found in body fluids or the environment (Instituto Nacional de Seguridad e Higiene en el Trabajo, 2016; Nandi et al., 2010). When exposing oocytes to increasing concentrations of HM, abnormalities have been seen as a process: 1) absence of the 1st polar body in the perivitelline space, 2) degenerated ooplasm, and 3) abnormal perivitelline space, followed by 4) presence of multiple abnormalities (with higher doses). When exposing embryos to concentrations starting at 0.05 µg/mL of Cd and 0.5 µg/mL of Pb, a reduction in the morula/ blastocyst yield and blastocyst hatching was found. Higher concentrations (0.5 µg/mL Cd and 1.0 µg/mL Pb) caused the arrest of four- to eight-cell embryos and increased degeneration and/or asynchronous division. Control embryos were also cultured and exposed to increasing concentrations of metals during the two- to four-cell stages of development and the total cell count and ICM values declined (Nandi et al., 2010). Similar results were reported when exposing two-cell mouse embryos (D1) to Cd, which yielded few effects on D8 of development, compared to the effects after exposing D4 embryos to the same conditions. In the D1 group, implantation was delayed temporarily and 62% of implantation sites were absent on D5, but it was not embryo-lethal. On the contrary, in the D4 exposure group, pregnancy failure was reported for all mice on D8, with no implantation sites reported on D5, and the few blastocysts recovered were degenerated. Two-cell embryos seem to be resistant because the lack of Cd uptake at this stage but toxicity increases with development, as the morula stage showed degeneration due to the rapid accumulation of Cd in blastocysts (De et al., 1993). In rats, after the incubation of eight-cell embryos and morula for 24 h with cadmium chloride (CdCl2), development before the blastocyst stage was interrupted with clear evidence of apoptosis such as shrunken cells and pyknotic nuclei. In addition, morula appeared smaller in size with fewer cells and the early blastocyst ICM were damaged with cell death, pyknosis, and debris accumulation (Abraham et al., 1986).

Cd is also known to inhibit gap junction intercellular communication (GJIC) and connexin phosphorylation, both of which are essential processes in the progression from the four-cell stage in humans (eight-cell stage in mice) through compaction (Hardy et al., 1996). There have been mechanisms suggested for Cd toxicity including ionic and molecular mimicry, interference with cell adhesion and signaling, oxidative stress, apoptosis, genotoxicity, and cell cycle disturbance due to either synergism or just one mechanism predominating in a cell specific manner (Thompson and Bannigan,

2008). Wang et al. studied some mechanisms that could affect the zonula occludens (ZO-1): a protein that regulates tight junction formation between cells. This protein is first expressed during the compaction of eight-cell mouse embryos and it has been suggested as a necessary mechanism for blastocyst formation, helping in the differentiation of the trophectoderm (TE) and ICM. When its function is altered, the number of formed blastocysts can decrease significantly and produce degeneration as it affects the number of embryonic cells, the bi-functional barrier that limits the diffusion of solutes, and the epithelial cell polarity (Wang et al., 2008). Although this study was not correlated with a specific substance, a follow up study found negative effects associated with the interaction between different types of PM and pulmonary cells; the protein their degradation was evident as proteins were relocated from the cell periphery, disrupting the epithelial barrier (Wang et al., 2012). Additional in vitro embryo development research should be performed to correlate these

2.5.1.2 Effects of nanoparticles on IVF

Nanoparticles (NPs) can be a source of different developmental malformations with fatal impacts on exposed animals and their offspring. Reduced fetal growth and genetic abnormalities in infants are known to be associated with NP exposure past the threshold dose of toxicity during vulnerable times of embryonic and fetal development. However, there is little information regarding the specific effects of NPs during the early stages of human development (Bosman et al., 2005; Celá et al., 2014); Bosman et al. found a numerical trend toward fewer hatched mouse embryos previously exposed to mixed sized polystyrene (Ps) NPs at the two-cell stage, but there were no significant differences in BDR between exposed (89,4%) and control embryos (96,8%). Rates of embryos exposed at the two-cell and at blastocyst stages did not differ in developmental capacity neither in terms of hatching and implantation "on dish" and/or blastocyst degeneration. There is also evidence that smaller NPs can be internalized by the trophoblast cells (by endocytosis or pinocytosis), but no negative effects on cellular processes or expression of factors needed for development were seen. A few NPs were internalized in the ICM as well. This group suggested that the trophoblasts have a larger dimension to absorb NPs and that the ICM can expel the NPs during the compaction phase (Bosman et al. 2005).

Different particles derived from nanomaterials, or in higher doses, might behave differently. After tagging embryos through intracytoplasmic injection or by co-incubation with Ps-NPs and polyacrylonitrile (Pa-NPs), it was noticed that on D2 there was an evident reduction when co-culturing with Pa-NPs but on D6 the percentage of embryos co-cultured with either type of NPs had lower hatching percentages. When assessing the injected embryos, D2 development was lower in both types of NPs and on D6, Pa-NP embryos did not develop and the hatching percentage was lower with both types of NPs. The Pa-NPs had effects on the eight-cell embryo which is a cell

stage characterized by compaction, blastomere membrane fusion, and GJIC (Fynewever et al. 2007).

Other studies have demonstrated in different species, that different NP doses cause several embryonic developmental effects. Celá et al. reviewed some of the most relevant results in different animal models, highlighting the fact that the negative effects of NPs in other mammalian models can show similarities with possible deleterious effects in human oocytes and embryos. Some of the effects described in mouse oocytes were after a 24-hour exposure to cadmium selenide (CdSe-NPs) that resulted in decreased cell numbers, induced apoptosis, and inhibited post-implantation development, possibly due to a teratogenic effect. Blastocysts exposed to CdSe-NPs had induced apoptosis, inhibited cell proliferation, retarded post-implantation blastocyst development, and increased early-stage blastocyst death in vitro and in vivo; cytotoxicity was significantly reduced by the addition of a zinc sulfide (ZnS) coating. Silver (Ag-NPs) exposition caused TE and ICM apoptosis and significantly inhibited cell proliferation. Exposing mouse morulas to chitosan (CS-NPs) induced defects in blastocysts such as small or no blastocoel cavity, lower expression of TE associated genes and pluripotent marker genes and a reduction in total cell numbers with enhanced apoptosis at this stage. The IR was reduced and embryos that implanted were subsequently reabsorbed (Celá et al., 2014). A recent study reported that CS-NPs can be internalized into the zona pellucida, periviteline space and cytoplasm of mouse blastocysts, due to their high aqueous solubility, initiating an intercellular oxidative stress reaction in which they reported swollen mitochondrias, mitophagosomes, lipophagy, lysosomes, degenerated organelles and early signs of apoptosis. The expression levels of the endoplasmic reticulum stress-related genes were also increased and epigenetic reprogramming was affected (Choi et al., 2016).

2.5.1.3 Effects of polycyclic aromatic hydrocarbons on IVF

Most of the reproductive negative health effects related to PAHs, have been documented in animals or humans that were exposed to cigarette smoke (Cinar et al., 2014; Dechanet et al, 2011), but despite that it can affect the reproductive status of an ART patient, it seems that there are not IVF-related reports.

PAHs can alter embryo and fetal development at the molecular level, reducing the allocation of embryonic and placental cell lineages and inducing apoptosis. Detmar et al, exposed mouse embryos to 7, 12– dimethylbenz(a)anthracene (DMBA) finding that some were smaller, the number of cells was significantly lower and that there was a significant difference in the grade of apoptosis between the non-exposed and the exposed embryos: higher cellular death in the ICM and TE cells with characteristics such as nuclear condensation and fragmentation. Despite this, the progression of the embryo from the eight-cell stage to the blastocyst stage was not interrupted. There was also a significant increase in Bax levels (a Bcl-2 group family member) and

therefore the activation of caspase 3, which is known to induce cellular death and consequently could affect the developmental potential of the embryos, increasing their resorption potential (Detmar et al., 2006). Later, the authors described that high levels of PAHs have immunosuppressive effects and suggesting that pregnancy resorption could be an immune-mediated mechanism on the part of the mother after being chronically exposed to low doses (Detmar and Jurisicova, 2010).

Januario et al. exposed mouse embryos to different doses of diesel exhaust particles (DEP) finding that all doses led to the disruption of the normal segregation pattern (cell lineage specification): there were fewer ICM cells not affecting the total cell count and the ICM/TE ratio were both significantly affected. Lower doses had low rates of apoptosis with a higher percentage of live cells but with a 2 µg/cm2 dose the ICM morphological integrity was significantly impaired, the hatching status and the developmental potential of the blastocysts were significantly affected. Significant negative effects in all aspects were produced with higher doses. On a molecular level, a reduction in Oct-4 expression was observed in the embryos, which can be associated with impaired formation of a proper ICM with fewer cells. There was also an over-expression of Cdx-2, which it could trigger the differentiation toward TE as a compensatory mechanism to preserve blastocyst cells number (Januário et al., 2009). When comparing the heritable mutations after exposing a group of mice to filtered and non-filtered urban-industrial ambient air (UFA and Non-UFA), they found that mice inherited extensive polymorphism at expanded-simple-tandem-repeat (ESTR) loci Ms6-hm and HM-2 of parental origin, at a rate 1.5 to 2.0 times higher than the offspring of the rural-air (RA) treatment group. Additionally, despite that UFA group had 52% lower rates of paternal mutations, when the urban-industrial exposed males mated with unexposed females, the offspring inherited the paternal origin mutations, 2.8 times as frequently as the RA group. Mean total suspended particulate was up to 10 times higher and the weighted-average daily PAHs were 33 fold higher in the Urb-Ind ambient air. Twenty-six PAHs were quantified, of which seven were carcinogens and all of these were correlated with increasing evidence of genetic damage in germ cells (Somers et al., 2004) . Brevik et al, demonstrated that the parental origin of mutations happen even after an acute exposition to PAHs, and this may lead to affect early embryonic transcription, the activation of the embryonic genome and may lead to transgenerational effects in humans (Brevik et al., 2012) .

2.5.2 Effects of VOCs on IVF

In routine ART laboratory audits, it is recommended to evaluate the concentrations of VOCs in the air among other pollutants to prevent occupational hazards (Asociación Española de Normalización y Certificación, 2013) however, while these substances do not often surpass the OLV due to current environment management strategies, there is still no certainty that they will not interact with gametes or embryos during culture. Past

studies have demonstrated lethal effects on early mouse embryos by gaseous emissions produced after the construction of a new laboratory: 90% of the two-cell stage embryos were arrested despite of the use of freestanding ionization units and HEPA filtration. BDR were higher before and after the construction period. Other studies have been able to corroborate the same pattern of an increase and decrease in VOC measurement before and after the redesign of clinics and laboratories or the construction of new ones (Cohen et al., 1997; Hall et al., 1998). Khoudja et al. stated that more than 1 ppm of VOCs can be considered high and directly toxic to animal embryos and that VOC levels around 0.5 ppm allow for acceptable blastocyst development and reasonable pregnancy rates, although there could be high percentage of miscarriages and that VOC levels should be below 0.2 ppm to have an ideal IVF environment (Khoudja et al., 2013) .

The seasonal influence of the IVF laboratory`s ambient air over the embryo development has been found to be related to the outside temperature (OT) and humidity (OH). Two studies by Worrilow et al. observed negative effects on IR by evaluating different testing quarters (TQ). In both studies they found that when toluene was in higher levels IR decreased, and in one study the fertilization rates, zygote quality, and embryo morphology scoring decreased significantly as well. The toluene presence was also related to high OT and OH (Worrilow et al., 2001; Worrilow et al., 2002). Other groups have correlated the presence of the VOCs filtration systems with higher rates of blastocyst development and higher number of blastomeres and one of these groups also suggested that higher embryo fragmentation could be a mechanism that may provide means through which embryos improve developmental competency and for that there was a reduction in spontaneous abortion (Higdon et al.,2003; Merton et al., 2007b; Racowsky et al., 1999). Koudhja et al. established that morphological and intrinsic embryo parameters: fertilization, cleavage, D5 blastocyst formation, implantation, and pregnancy rates significantly improved when reducing the presence of VOCs after the installation of an air filtration system. The concentrations of formaldehyde, ethylene, acetylene, ethane, propylene, SO2, NOx, isobutene, cis-butene, cyclopentane, benzene, CFC-11, chloroform, carbon tetrachloride, halon-1211, and alcohol, were reduced with the new air filtration system (Khoudja et al., 2013) .

A well-known VOC is Toluene, which is an organic solvent (an aromatic hydrocarbon) that can be easily found in commercial products such as paints, varnishes, glue, and gasoline. Toluene and other organic solvents have a high affinity for lipid-rich tissues and can readily cross the placenta allowing direct exposure of the fetus. Significant degenerative changes were found in preimplantation embryos exposed to toluene in vitro. Higher doses decreased the fertilization rate of exposed eggs and enhanced embryo degeneration resulting in increased embryonic lethality (Yelian and Dukelow 1992). Other studies in animal models have demonstrated that morphological

anomalies and congenital defects, such as reduced placental weight, fetal weight and growth, reduced skeletal ossification, negative neurobehavioral effects as well as fetal resorptions and death, are produced specially in early female fetuses (gestational D8 to D20) or during other stages of pregnancy after exposing mothers to inhaled toluene (Bowen et al., 2009; Callan et al., 2015; Callan et al., 2016). It has been described also that toluene cytotoxicity effects on human embryonic stem cells are comparable to 1-octen-3-ol and its enantiomers (a major fungal VOC associated to indoor mold and odors) (Inamdar et al., 2012). When exposing murine bone marrow stem cells to two fungal VOCs [(E)-2-octenal and oct-1-en-3-ol], a shift to unsaturated fatty acids and lower cholesterol levels in the cells membrane was produced, which means increased the membrane fluidity, and this could be related to malfunction of the immune system (Hokeness et al., 2014).

Acrolein is an airborne VOC with widespread environmental prevalence produced during lipid peroxidation and by burning tobacco or liquid fuels. Preliminary studies have already demonstrated negative effects on cleavage, cell number, and blastocyst development when exposed to acrolein (Hall et al., 1998; Little and Mirkes, 1990). However, a recent study of short-term exposure demonstrated that the negative effects depend on the protein concentration in the medium and the quality of the oil. Mouse embryos incubated with 500 ppb of acrolein inside the embryoscope were found to develop to the blastocyst stage at a normal rate (80%) when also exposed to 5 mg/mL of human serum albumin (HAS) in the culture medium. Embryos incubated with low-protein medium were arrested at the four-cell stage and the remainder arrested at the morula stage. The embryos of the protein-free group were also exposed to mineral oil that contained peroxides, and were arrested and lysed at the one-cell stage within 24 h of culture. The protein concentration in the groups that contained HSA did not affect the timing of the cell cycles (Karaouga et al., 2014).

Trichloroethylene (TCE), is a highly volatile inhalation anesthetic used mainly in short surgical procedures where light anesthesia with good analgesia is required or as an industrial solvent. Significant anomalies in skeletal and soft tissues, indicative of delay in the development, have been observed in groups exposed to TCE during pregnancy in rats (Dorfmueller et al., 1979). TCE effects over the cardiac tissue, function and development (National Center for Biotechnology Information, 2017a) have been studied in animal models describing embryonic genotoxicity as well, causing cardiac valvular and septal malformations or it can disrupts calcium (Ca**) flux regulation in embryonic myocites (Boyer et al., 2000; Caldwell et al., 2010; Johnson et al., 1998). Caldwell et al., determined that TCE produces gene expression errors of the calcium homeostasis and that, could be the mechanism by which cardiac malformations can occur. The research was conducted in mice embryonic hearts obtained from pregnant females that had received folate supplementation in 3 different doses: high folate

levels in combination with TCE induced over-expression of many genes of the ion channel pathway. The majority encodes for potassium (K), Ca**, and other cation transportation channels indicating that TCE may alter the permeability of the cell membrane causing an electrolyte imbalance. TCE caused great changes in gene expression during critical phases of the heart development, but folate may have a dual effect on cardiogenesis too, depending on the nutritional variations and the presence of environmental toxins (Caldwell et al., 2010).

2.5.2.1 Benzene

Benzene (C6H6) is one of the most common VOC air pollutants found inside laboratories. It is a colorless, clear liquid that is fairly stable and highly volatile and has a general ambient concentration between 1 and 50 ppb. The EPA estimates that exposure over 0.4 ppb in air over a lifetime pose a significant risk of cancer. OSHA allows 1 ppm as the maximum allowable amount of benzene in workroom air during an eight-hour workday over the course of a 40-hour workweek. NIOSH recommends that all workers wear special breathing equipment when they are likely to be exposed to benzene at levels exceeding the recommended (eight hour) exposure limit of 0.1 ppm (Agency for Toxic Substances and Disease Registry, 2007). There are not threshold levels for this substance inside laboratories and there is little evidence linking benzene to IVF reproductive outcomes. Through a pilot study Alviggi et al., evaluated if the levels of benzene in the follicular fluid could influence the response of controlled ovarian stimulation and the outcome of IVF/ ICSI cycles. Two groups of exposure were established: low intra-follicular concentration (A) and high intra-follicular concentration (B) (>0.54 ng/mL<). Benzene levels were significantly related to a positively trend in baseline FSH levels and negatively trend of E2 peak levels, average number of oocytes retrived and average number of embryos transfered in the B-group. The A-group had a higher number of embryos transferred. The intra-ovarian levels of benzene are associated with hypo- sensitivity of follicles to endogenous and exogenous gonadotropin, leading to an unknown mechanism of resistance (Alviggi et al., 2014).

Studies on different cell types have evaluated the intrinsic damage that benzene produces inside laboratories. Meiotic delay of MI oocytes and frequencies of aneuploidies in MII mouse oocytes were observed after a dose-dependent inhalation of benzene, especially with higher doses in a "multiple inhalations" group (Zeng et al., 2001). Tsutsui et al. demonstrated a marked dose-dependent genotoxicity on Syrian hamster embryo cells when exposed to benzene and its metabolites. Some of the effects seen were disturbed cell growth, cells transformation, increased frequency of chromosomal aberrations (gaps and breaks), alterations in chromosome numbers, and genetic mutations. Catechol is the most harmful metabolite for cells at lower concentrations, but hydroquinone and phenol also have negative effects (Tsutsui et al. 1997a). Benzene or its metabolites can produce diverse effects in individuals of

different ages and genders as well. Corti et al. exposed 16-day-old male and female, adult males, females, and pregnant females mouse fetuses colony forming units, erythroid or CFU-e (precursor cells), finding that hydroquinone and benzoquinone were the most cytotoxic, producing a dose-dependent decrease in all cell types growth. The adult male cells showed more susceptibility to these metabolites while fetal cells were more resistant to catechol but showed more deleterious effects when exposed to binary mixtures containing catechol. Phenol was found to affect fetal male cells at 40 μM (Corti and Snyder, 1998). The possible confounding factors (individual genetic susceptibility or previous exposure of the parents) can cause genotoxicity, as well as individual cell and tissue responses.

Because benzene is a carcinogenic substance, in utero exposure studies have suggested that predisposition of the embryo and/or fetal tissues to carcinogenesis as well, caused by alterations in the redox signaling pathways, excess of production of reactive oxygen species (ROS) and therefore oxidative stress, affecting the regulation of gene expression, cell growth, and cell death. But there are a lot of intrinsic differences in the susceptibility of the target cells (type, age, gender) as well. In utero benzene can cause alterations in critical cell signaling pathways necessary for normal hematopoiesis; male fetuses have been found to be more susceptible to benzene-induced ROS production after two hours of exposure. Benzene was observed to be very deleterious for unprotected embryos given their rapid growth and developmental changes and reliance on the cellular signaling that occurs during embryonic development (Badham et al., 2010).

2.5.2.2 Limonene

Limonene it is another well-known VOC that can be present inside of the IVF laboratory, because it is used as an additive in cleaning products. Despite of this, few studies have been performed evaluating possible early life deleterious effects, perhaps because it is known as a low toxicity VOC and it is considered that it does not have strong mutagenic, carcinogenic or nephrotoxic effects. However, A couple of studies have reported that even though it is not highly toxic or carcinogenic it does induce a few cell transformations in Syrian hamster embryo cells (Rivedal et al., 2000), or that influences mechanisms of increase intracellular Ca++ pathways and Ca++ activated potassium (BKCa) channels, which could be correlated to increasing myometrium contractions (Hajagos-Tóth et al., 2015).

“hey! Have you ever felt that way?

If not you will cause we're the same.,

stars in the sky, we're are all connected”

Chapter 3

3 JUSTIFICATION – HYPOTHESIS

It is well known in *in vitro* fertilization that embryonic developmental capacity is affected by maternal and paternal intrinsic factors such as age, body mass index, infertility condition etc. However, other external factors such as mother diet, health habits or inadequate embryo in vitro culture conditions such as changes in pH, osmolarity, temperature, oxygen tension, so on, have a negative impact on embryonic competence.

In the contest of the environment, the chemical compounds are part of these external factors. Their presence within the assisted reproduction facilities and especially, within IVF laboratories, has been widely and officially recognized. But despite that the risk of exposure to most substances is controlled in the professional scope for humans, the great unknown about which are the deleterious specific effects that can cause human embryonic development failure still remains.

Because of this, the hypothesis we set out in this project is that the presence of chemical compounds, specifically volatile organic compounds (VOCs), in early stages of human embryos produces appreciable effects at embryo developmental capacity, morphology of inner cells mass and trophectoderm, chromosome abnormalities and mitochondrial content as a marker of cellular stress.

Chapter 4

4 OBJECTIVES

4.1 GENERAL OBJECTIVE

To evaluate the human embryo development since D+3 up to the blastocyst stage on D+6, after the direct exposure to Benzene and Limonene, two volatile organic compounds (VOCs) recognized for being present inside the IVF laboratory, as well as the chromosomal alterations that can cause.

4.2 SPECIFIC OBJECTIVES

- To analyze the measurements of VOCs concentrations found by an air quality consultant (MON SOLAR) in order to identify which are the most common compounds inside the IVF laboratories belonging to the IVI group in Spain.
- To establish comparisons between the VOCs concentrations found on the outside and the inter-clinical area, specifically inside the IVF laboratory, and to analyze the variability of their presence inside the IVI clinics through a 5-year period (2010- 2014).
- To study the specific effects produced by VOCs on human embryo developmental potential till blastocyst stage.
- To determine the effect produced by VOCs over the chromosome content of the embryos that were able to develop up to blastocyst stage on D+6.
- To determine the effect produced by VOCs over the mitochondrial DNA of the embryos that were able to develop up to blastocyst stage on D+6.
- To suggest limit values (OLV) for "culture" exposure for human pre-implantation embryos.

Chapter 5

5 MATERIALS AND METHODS

5.1 APPROVALS AND ETHICAL CONSIDERATIONS

This research project was approved by:

- Comité Ético de Investigación Científica, CEIC; Valencia (Ethics Commettee of Scientific Research).
- Comisión Nacional de Reproducción Humana Asistida, CNRHA (National Commission of Human Assisted Reproduction).
- Comisión de Investigación (COI) IVI Valencia (Research Comission of IVI Valencia). The research project is identified by the following number: 1210-C-110-MJD

This research project respects the fundamental principles established in the Helsinki Declaration, the Convention of the Council of Europe Human Rights and Biomedicine, and the Universal Declaration on the human genome and human rights adopted by UNESCO. The research also complies with the Spanish legislation requirements in the biomedical research area, the personal data protection and bioethics.

The patients' information, related to the IVF treatment, cycle and their D+3 embryos, was obtained from the database of the IVI clinics, called SIVIS and it was extracted to excel for the following review and analysis. Database anonymity was maintained by the exclusive use of number of protocol.

5.2 AIR QUALITY CHARACTERIZATIONS

Every year, MON SOLAR (external audit firm) performs indoor air quality (IAQ) analyses, where the most common pollutants concentrations are measured, including particulate matter and biological pollution. The characterizations are done in all the IVI clinics in Spain and the IAQ is evaluated after a cleaning and disinfection protocol of the IVF laboratory.

MON SOLAR performed a broad-spectrum screening, including 54 VOCs with the purpose of evaluating the environmental concentration of most of them.

- The suction flow rate was 50 ml/min.
- Support: stationary phase film Polidimetilsiloxano (PDMS) of 100 µm thickness.
- Technique used: SPME (solid phase microextraction) technique for simultaneous identification of VOCs

- MON SOLAR obtained the compounds by an absorption pump (APEX lite), absorbing 6 litres (lt) of air.
- Sample points: inside the IVF laboratory, the semen preparation room and outside of the clinics (rooftop).

5.3 Study design

Prospective study, of cases and controls intended to investigate possible deleterious effects produced over the development of thawed human embryos since D+3 up to D+6 of development, by the presence of two common VOCs of the IVF laboratory´s ambient air, benzene and limonene, diluted in the culture media. This study is performed by two developmental toxicity tests (DTT), between a group of control human embryos and groups of exposed human embryos, analyzing in both the impact of the presence or absence of VOCs, over the morphology and their chromosome profile.

5.3.1 Embryos included in the study

The embryos collection was performed by identifying patients who had vitrified embryos on D+3 of development from the IVI clinic database (SIVIS). This stage of development was selected since it is when the embryonic genome starts its establishment.

- Inclusion criteria: Human embryos on D+3 of development meeting the following characteristics: with 6 to 10 or more cells and less than 25% of fragmentation.
- Exclusion criteria: Human embryos coming from patients undergoing IVF from advanced maternal age (=> 38) or severe male factor.

5.3.2 Study variables

- Independent variables/ exposure variables: Contaminants diluted into the culture media to different concentrations
- Dependent variables:
  - Blastocyst development rate
  - Good quality blastocyst developmental rate
  - Embryo aneuploidy rate
  - Mitochondrial DNA-copy number
- Confounding variables
  - Age
  - Body mass index

5.4 DEVELOPMENTAL TOXICITY TESTS (DTT)

All the procedures were performed inside the research laboratory of IVI Valencia that was equipped with a laminar flow hood, an incubator, a liquid nitrogen tank to storage the embryos and all the materials needed for the management of gametes and embryos.

5.4.1 Culture pollution

The culture procedure was performed inside the research laboratory, inside an incubator that is only used for research purposes.

5.4.1.1 Culture pollution materials

- Laminar flow hood
- Independent "hot plate" device
- Microscope
- Pasteur pipette (Cook® Flexipet® Adjustable Handle)
- Pipette tips
- Eppendorfs®
- Glass tubes with caps
- Tubes rack
- 2 Falcon dish 1008 (Falcon®, Becton Dickinson Labware, USA)
- Fluorodish FD5040-100. Measurements: ID: 47.5mm * OD: 49.82mm * Glass Diameter Φ: 35mm * height (inside): 7.25mm * Height (outside): 7.4mm * Access angle: 17 degrees. VOCs can be present in plastic-ware so to prevent errors of interpretation we followed chemists' advices on the use of glass. (World Precision Instruments)
- Cook® Flexipet
- Flexible capilars (Cook® Flexipet® Pipette)
- Incubator
- FERT™ culture medium
- CCM™ culture medium (Vitrolife, Göteborg, Sweden)
- Mineral oil Life®Global, Guilford, USA
- VOCs: Both chemicals were acquired from Sigma-Aldrich. For benzene it was required to fill a "Customer Declaration of Specific Use(s) of Controlled and Voluntary Monitored Substances" to confirm that the substance was only going to be used for legal purposes.
    - Benzene: Formula: C6H6
      Reagent Plus®, Thiophene free, > 99%.
      156302, Aldrich.
      N° CAS: 71-43-2.
    - Limonene: Formula: C10H16
      (R)-(+)-Limonene, 97%.

183164, Sigma.

N° CAS: 5989-27-5.

5.4.1.2 Safety measures

Because of the volatile properties and the health risks associated with the manipulation of both compounds, security measures were taken by purchasing the following:

- 3M™ Reusable Full Face Mask Respirator 6800, medium size.
- 3M™ Gas and Vapour Cartrige, A2, 6055: protection against organic vapours. They can be used together with particulate filters.
- 3M™ Particulate Filter P3 R, 5935: protection against solid and liquid particles. They can be used in together with gas and vapour filters.
- 3M™ Filter retainers 501, System Component 100 EA/ Case: to hold gas and particle filters.
- Nitrile gloves.

Table 5-1. Safety equipment to be used during culture pollution.

5.4.1.3 Culture pollution protocol

For each compound three experimental groups (EG) were created for each VOC, using three specific doses, related to previous air quality characterizations performed inside the IVF laboratories by MON SOLAR. These doses are shown in Table 5-2.

COV	Exterior (**e**)	Laboratory (**l**)	Double Laboratory (**dl**)	OLV*
Benzene (B)	268 x 10^{-5}ppm	256 x 10^{-5}ppm	512 x x 10^{-5}ppm	1 ppm
Limonene (L)	87.5 x 10^{-5}ppm	113.6 x 10^{-5}ppm	226 x 10^{-5}ppm	20 ppm

Table 5-2. Contamination levels for each dose for the three experimental groups (EG) for each VOC.

*OLV: The occupational limit values (valores límites admitidos de exposición diaria = VLA ED) are published every year by the "Instituto Nacional de Seguridad e Higiene en el Trabajo (INSHT)". They are a set of reference values for the concentrations of time-weighted averages during a normal working day of 8 hours and a working week of 40 hours. The measurements are performed in the breathing zone of air chemical agents, representing the conditions on which it is believed, based on current knowledge, that most workers can be exposed day after day, throughout their working life, without suffering adverse effects on their health.

- Control experimental group (EG): embryos cultured with manufacturer´s culture medium as received from the distributor.
- EG 1 or exterior (e): culture medium polluted with the average concentration found of the compound, outside of the clinics.
- EG 2 or laboratory (l): culture medium polluted with the average concentration found of the compound, inside of the IVF laboratories.
- EG 3 or double laboratory (dl): culture medium polluted with double value of the average concentration found of the compound, inside of the IVF laboratories.

Taking into account the solubility and density properties of the technical sheets of both compounds, we send samples of mineral water contaminated with the same doses of the study to the Mass Spectrometry Laboratory of the University of Valencia, to verify the presence of both VOCs in aqueous solutions. The presence of Benzene and Limonene in aqueous solutions was confirmed. In the case of Benzene, different concentrations were detected despite that it is described as not soluble in water.

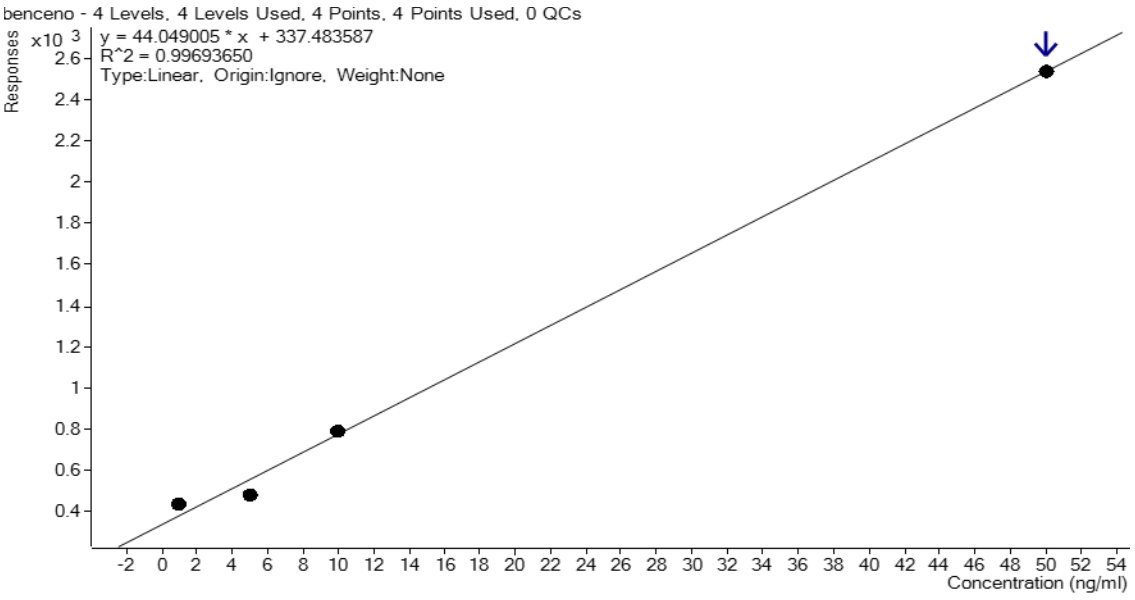


Figure 5-1. Mass spectrometry for Benzene. Values were measured in ppb or ng/ml.

Limonene is considered a very soluble compound and the mass spectrometry test confirmed the solubility in mineral water.

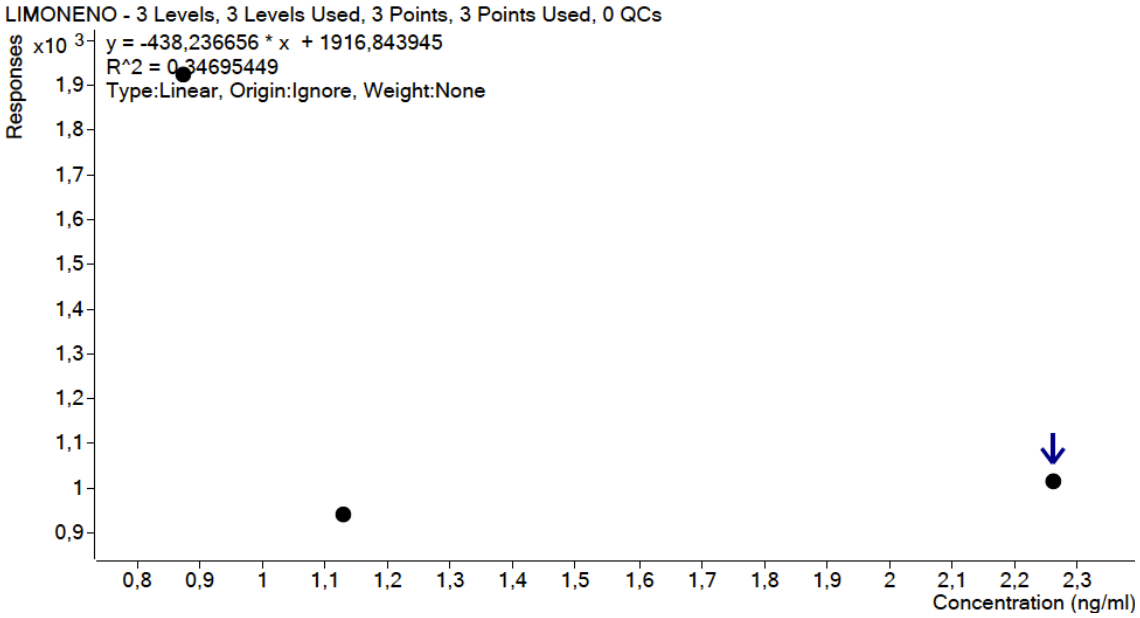


Figure 5-2. Mass spectrometry for Limonene. Values were measured in ppb or ng/ml.

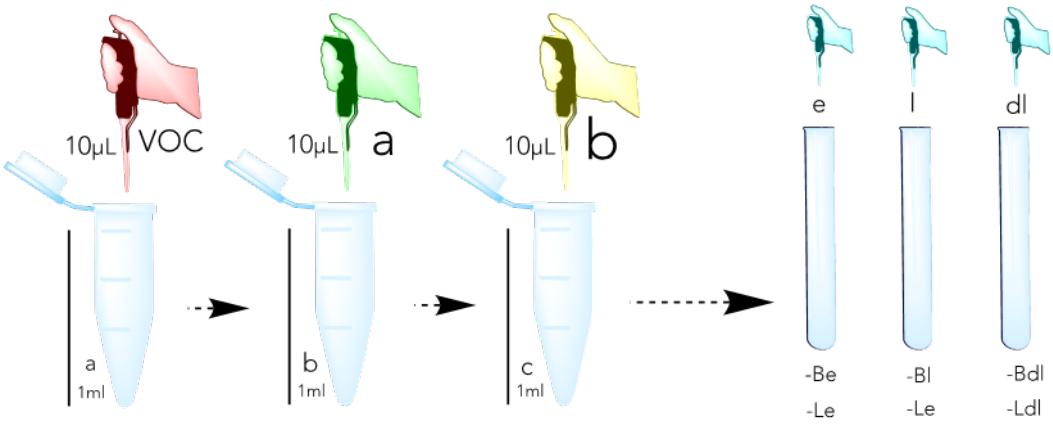
The calculations to make the dilutions were based on the density of the chemicals. Serial dilutions were prepared to contaminate the culture medium based on the solubility confirmation.

I. 1 ml of FERT™ was pipetted in 3 different eppendorf®: a, b and c.
II. 1 ml of CCM™ was pipetted in 3 different glass tubes for final step
III. 10 µl of a compound (benzene or limonene) was pipetted into the "a"-eppendorf®. It was manually agitated for 2 minutes.
IV. 10 µl of dilution "a" was pipetted into the "b"-eppendorf®. It was manually agitated for 2 minutes.
V. 10 µl of dilution "b" was pipetted into the "c"-eppendorf®. It was manually agitated for 2 minutes.
VI. The following volume was pipetted from "c"-eppendorf® and diluted in 1 ml. It was agitated for 2 minutes. Volume calculations depended on the density of each compound.

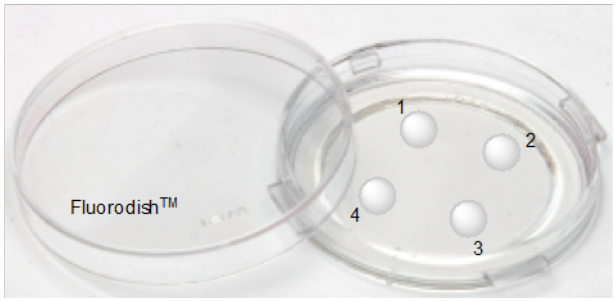
Compound	"e"	"l"	"dl"
B	5.83µl	2.92µl	3.05µl
L	2.7µl	1.35µl	1.024µl

Table 5-3. Final volumes per compound and concentration

VII. Each volume was placed into the respectively glass tubes, previously tagged with the VOC and the concentration of each dilution.



VIII. Fluorodishes were prepared with 3 or 4 drops of 50ml of the polluted CCM™ culture medium (Vitrolife®) and then 4 ml of mineral oil were put as a protective layer.



IX. Fluorodishes were put inside the incubator to gas the mediums during 24 hours. The falcon dish was also set up with CCM™ culture media without any pollutant for the cleaning step after thawing. Incubator´s environment was set with 5% de CO2 and 37°C.

5.4.2 Embryo thawing procedure

Embryos were vitrified during the clinical cycles of the patients. All embryos used were vitrified in the Cryotop system®.

5.4.2.1 Materials

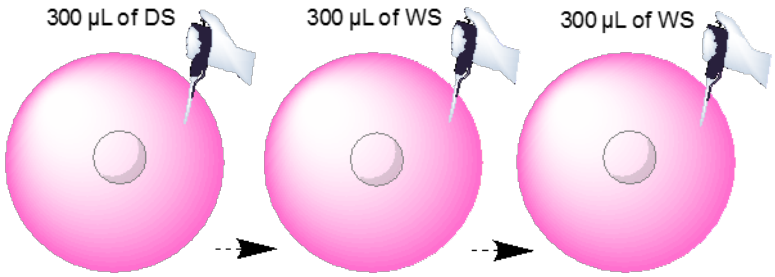
- Laminar flow hood with the heating surface off
- Independent "Hot plate" device
- Thermo-Flask® (Sigma-Aldrich, Inc., USA.)
- Pasteur pipette (Cook® Flexipet® Adjustable Handle)
- Pipette tips
- Cook® Flexipet® Pipette
- 2 Falcon dish 1008 (Falcon®, Becton Dickinson Labware, USA)
- Timer
- Tweezers
- Cooling rack: blue styrol box for liquid nitrogen
- Repro Plate® with 6 wells; KITAZATO® (BioPharma, Tokyo, Japan)
- Patients Cryotop® (KITAZATO® BioPharma, Tokyo, Japan)

- Thawing solutions:
  - Basic solution (BS): 0,1M diluted Threalose in WS
  - Dilution Solution (DS): 0,5M diluted Threalose in WS
  - Washing Solution (WS): buffer medium Hepes TCM-199 (M199) supplemented with HPC
- CCM™ culture medium for cleaning after thawing (Vitrolife, Göteborg, Sweden)

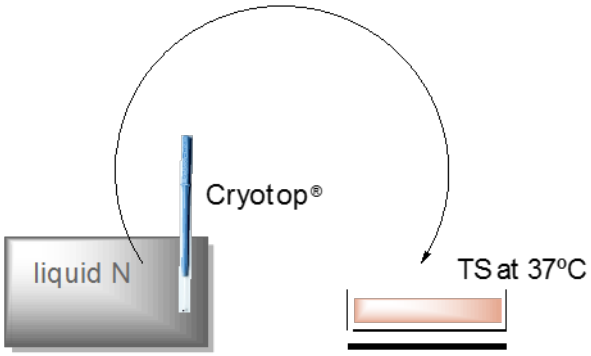
5.4.2.2 Thawing protocol

The thawing procedure was performed inside the IVI´s research laboratory at room temperature. Thawing medium and washing solutions were tempered before the procedure. The thawing solution (TS) must be used at 37 °C, so it was tempered inside the incubator. The stainless steel recipient, inside the polystyrene container, was filled completely with liquid nitrogen. Patients Cryotop® were submerged in the liquid nitrogen up to the thawing moment, avoiding changes in temperature. It is important to mention that the thawing procedure has specific steps to follow and they are the same for oocytes and embryos.

I. 300 µL of DS were pipetted in the first well of the Replo Plate and 300 µL of WS were pipetted in the second and third well.

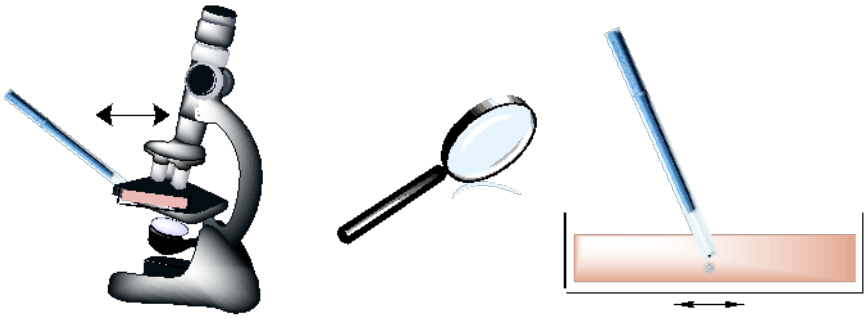


II. Each Cryotop® was taken out of the liquid nitrogen with a straight and a fast move to submerge it directly in the Petri dish with TS solution at 37 °C that allows the proper thawing speed. For this moment, we used a hot plate device.

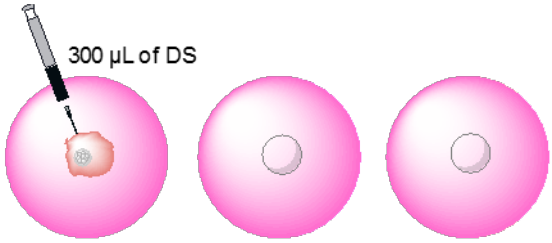


III. Embryo(s) were observed under the microscope and the 1-minute countdown started. Embryo(s) were not manipulated after 40 seconds inside the TS solution. After this time, the Cryotop® was softly moved horizontally through the bottom of the dish. When embryo(s) were still adhered to the cryotop, TS medium was

dispensed over them with the capillary but if this was not enough, movements of the capillary on the sides of the Cryotop®´s tip, were helpful to detach the embryo(s).

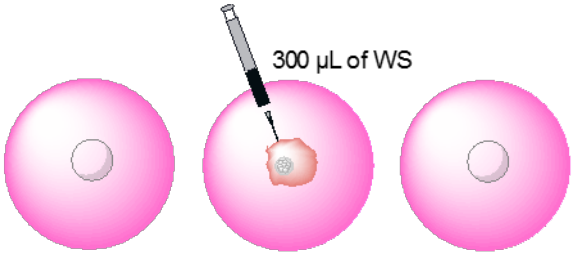


IV. After 1 minute, the embryo(s) were aspirated with the stripper and capillary and were transferred into the DS well, making sure that TS medium was blown out on the bottom of the wheel first, and then the embryo(s) were placed on the bottom of the TS layer for a gradual displacement from TS to DS. Countdown of 3 minutes.

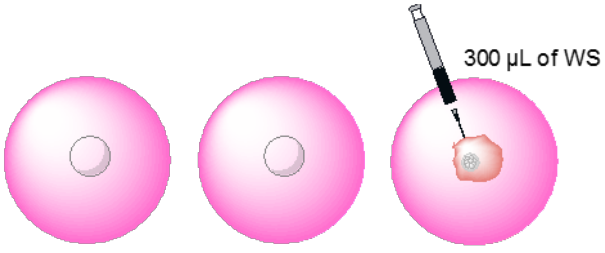


V. 3 minutes later, the capillary is immersed into DS and embryo(s) were aspirated until they reached 2 to 10 mm from the tip of the capillary.

VI. The capillary was taken to the second well, first with WS solution, releasing 1 cm of DS first in the bottom and then the embryo(s) in the bottom, for a gradual displacement from DS to WS. Countdown of 5 minutes.

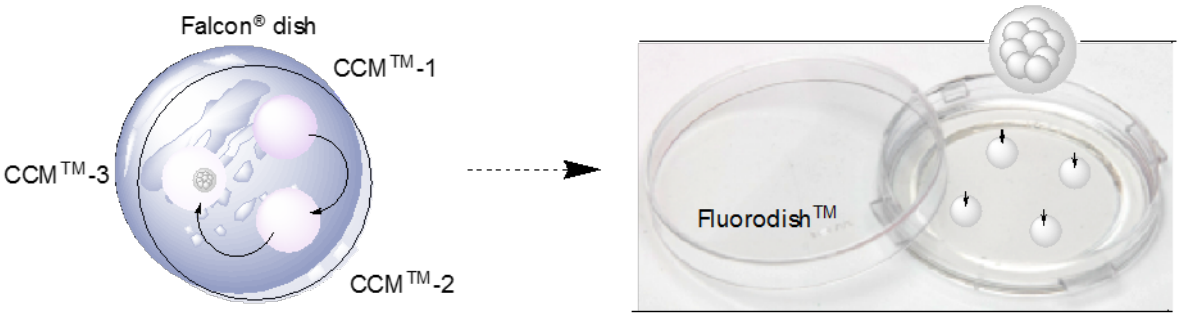


VII. Embryo(s) were aspirated with 1 cm of WS and then transferred into the third well, second of WS. Countdown of 1 minute.



VIII. Embryo(s) were then transferred to a falcon dish with CCM™ to clean the remaining thawing media from them and then they were transferred to the "Fluorodish" with

the polluted culture medium drops. Embryos(s) were then cultivated inside the incubator at 37 °C and 5% CO_2.



5.4.3 Embryo culture up to blastocyst stage through the exposition protocol

Each DTT was composed of 75 embryos, 25 embryos for each EG in both B and L. A total of 30 embryos composed the C group to be a reference of "normal" conditions inside the laboratory.

COV	e	l	dl
Control (C)	30	30	30
B	25	25	25
L	25	25	25

Embryos were selected through SIVIS, specifying that they should come from a vitrification process performed with KITAZATO® to avoid the variability between brands of systems. The embryos that survived the vitrification and posterior thawing procedure on D+3 were cultivated individually in each drop of polluted or non-polluted culture medium up to D+6 of development.

I. After the thawing procedure, the embryos were transferred from the Falcon dish with CCM™ to the "Fluorodish" (step previously described), and then they were placed inside the incubator for the next 3 days (up to D+6 of development).
II. Each embryo received an experimental name according to the pollutant, the concentration to which it was polluted and the number of the drop where it was placed:

III. On D+6, embryos morphology was assessed with the criteria of ASEBIR.

D+5	D+6			
	Expansion	ICM	TE cells	ASEBIR
"Early" or "cavitated" blastocyst (Thick zona pellucida)	From "expanded" blastocyst to "hatching"	A	A	B
			B	
			C	C
			D	D
		B	A	B
			B	
			C	C
			D	D
		C	A	B
			B	
			C	C
			D	D
		D	A,B,C o D	D
				D
Morula	Compacted Morula			Excluded

Figure 5-3. Classification scheme for D+6 embryos by ASEBIR (Asociación para el estudio de la Biología de la Reproducción, 2015).

IV. Photographs were taken of each embryo for future observations of the morphology as well.

V. Embryos were prepared on new dishes for biopsy.

5.4.4 Blastocyst biopsy and chromosomic analysis

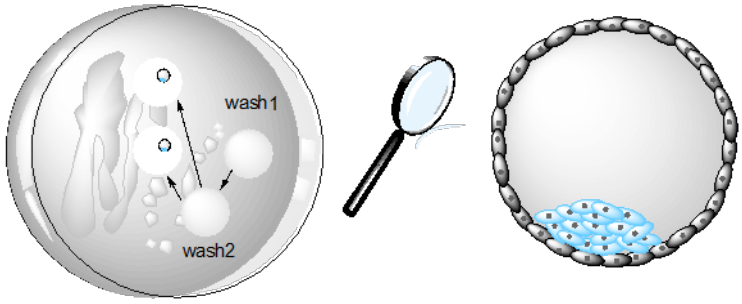
5.4.4.1 Biopsy materials

- Inverted microscope NIKON/OLYMPUS, equipped with a Hoffman objective of 40X for the embryonic biopsy and with a 10X, phase- contrast objective.
- Laminar flow Hood with UV light.
- Hot plate TOKAI-HIT (MATF-U 5530) .
- 2 hydraulic micromanipulators NARISHIGE (MO-188).
- 2 motorized micromanipulators NARISHIGE (MM-188).
- 1 microinjector Eppendorf® Cell Tram Vario.
- 1 microinjector Eppendorf® Cell Tram Oil.
- Binocular loupe.
- Holding pipette. Standard 35° Bend. Outer diameter (OD) of 120 µm and inner diameter (ID) of 25 µm (Cook® Medical, Limerick, Ireland).
- Denudation pipette (Humagen™, Charlottesville, USA).
- CCM™ culture medium for washing step and biopsy, tempered at 37°C (Vitrolife, Göteborg, Sweden).

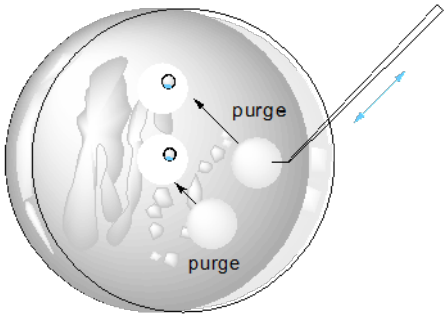
5.4.4.2 Biopsy of blastocysts protocol

The biopsy was performed on D+6 of development and it was only done on the embryos that reached any level of the blastocyst stage: from early blastocyst to hatched blastocyst.

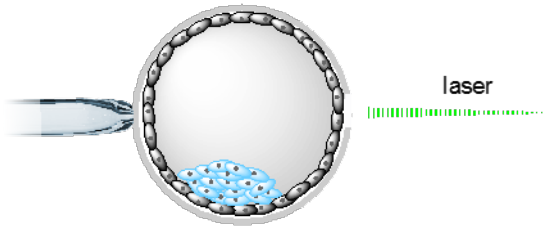
I. Blastocysts were transferred from the Fluorodish to the "biopsy-dishes". These were prepared with four drops of 50µl of CCM™: two of them are the embryos washing step before the biopsy. Drops were covered by mineral oil as well. CCM™ was tempered at 37°C 4 hours before.



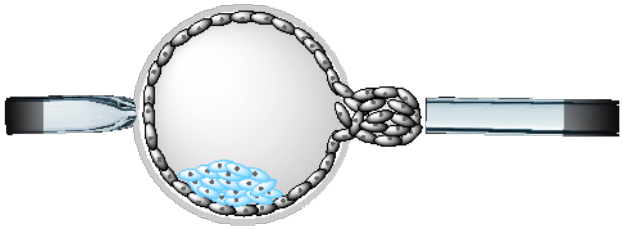
II. The holding and denudation pipettes were purged in the wash drops as well before each biopsy, to assure a good aspiration of the TE.



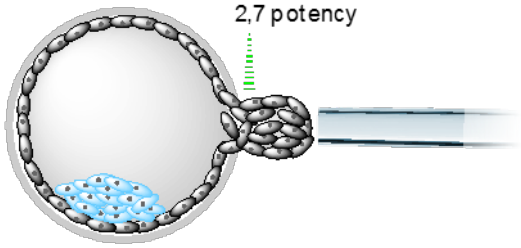
III. The zona pellucida was drilled using a laser system integrated to the microscope (Octax Laser Shot™, Fertilase®, Herbron, Germany).



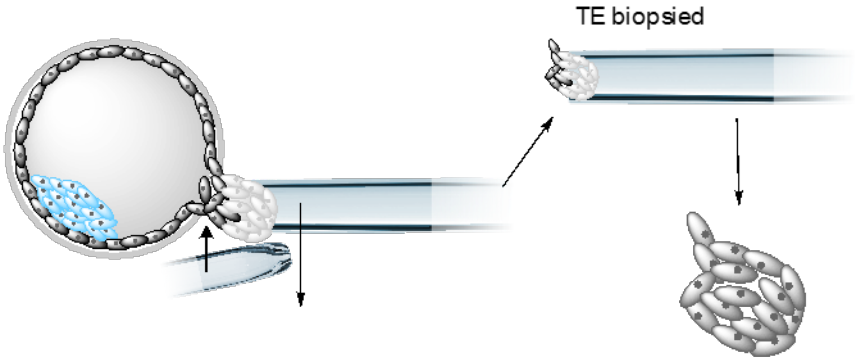
IV. Making sure that the ICM was not in the suction area, the blastomeres with visible nucleous were biopsied by a slow aspiration, through the pipette.



V. 10 to 12 cells were aspirated and then, 6 to 8 laser pulses were shot to drill between the cells junctions.



VI. Losing the opposite side with the holding pipette (making sure that it would not aspirate after) both pipettes tips were placed together to perform a cut by a rapid movement between them.



VII. The biopsied cells were then left in the culture medium, making sure that they would detach properly from the pipette to avoid their contact with the mineral oil, and closer to the remaining embryo. The mineral oil disintegrates the TE cells.

VIII. Embryos were manipulated individually by their reference ((VOC+EG+Drop number) to identify each one of them every moment and to control the time during the biopsy.

IX. When the process was finished, the remaining embryos and biopsied TE cells were taken to a laminar flow hood located in an adjacent laboratory, specially suited to prepare the cells and then send them for analysis. This process (tubing) consist on washing the TE cells in small drops of PVP medium before passing them to a 20 µL eppendorf® with 0,2µL of PBS medium. Tubes were tagged manually with each embryo reference as well.

X. TE cells were sent to Igenomix for aCGH analysis while the rest of the embryos content is being storage under refrigeration at -80°C for future analyses.

5.4.4.3 aCGH materials

- Laminar floor Hood with UV light
- Exhaust Hood
- Centrifuge for PCR Eppendorf® tubes (0.2mL and 1.5mL)
- Centrifuge for plates and slides 5430 (Eppendorf®, Hamburg, Germany)
- Centrifuge for miVac plated - DNA concentrator (GeneVac. , Ipswich, UK)
- Dual channel- array scanner (TECAN, Männedorf, Switzerland)
- Hybex® Microsample Incubator (SciGene, Sunnyvale, USA)
- Thermocycler T3000 (Biometra®, Göttingen, Germany)
- Thermal bath
- Magnetic agitator
- Thermoblock
- Vortex
- Hybridation chambers
- Glass washing bucket of 250mL y 25- slide basket
- PCR tubes of 0.2mL
- 96-well plates
- 1.5mL Safe Lock Eppendorfs®
- Crystal slide covers 22x22mm
- Positive control of amplification (genomic DNA)
- Amplification Kit Sureplex™ (BlueGnome)

- Kit Labeling System™ (BlueGnome)
- DS Hybridisation buffer (BlueGnome)
- 2xSSC/50% formamide
- Platform of array 24sure (BlueGnome)
- Washing solutions
- 20xSSC/ distilled H2O, pH: 7
- 2xSSC/ 0,05% Tween20/ distilled H2O
- 1xSSC/ distilled H2O
- 0.1xSSC/ distilled H2O

5.4.4.4 aCGH protocol

To perform arrays comparative genomic hybridization (aCGH) analysis, the trophectoderm biopsy from each embryo was amplified using the Sureplex DNA amplification system (BlueGnome).

I. To perform day-6 aCGH analysis, the TE cells from each embryo were amplified using the Sureplex DNA amplification system (BlueGnome, Cambridge, UK).
II. Amplified DNA and reference DNA were labeled and cohybridized in 24 sure arrays (BlueGnome): Whole Genome Amplification (WGA) step.
III. Amplification quality was ensured by gel electrophoresis (Lonza, Rockland, USA).
IV. Sample and control DNA were labeled with Cy3 and Cy5 fluorophores following the manufacturer's instructions. There are 2 female controls: one labeled with Cy3 and the other with Cy5. Same step with male controls.
V. Labeling mixes were combined and hybridized on 24sure arrays (V2 and V3, BlueGnome, Cambridge, UK) for 6–12 hours. Hybridization is in the control DNA and the experimental DNA
VI. Each probe is specific to a different chromosomal region and occupies a discrete spot on the slide.
VII. Chromosomal loss or gain is revealed by the color adopted by each spot after hybridization.
VIII. The technique involves the competitive hybridization of differentially labeled test and reference DNA samples.
IX. After washing, the slides were scanned and analyzed using BlueFuse Multi software.
X. Fluorescence intensity was detected using a laser scanner (Powerscanner, TECAN, M¨annedorf, Switzerland), and BlueFuse Multi software was used for data processing (BlueGnome, Cambridge, UK).
XI. The "24sure microarray product description (February 8, 2012, document version 2.3, and model number 408501- 00)" describes 10Mb effective

resolution for 24sure using BlueFuse software, being the minimum size specified by BlueGnome for segmental aneuploidies.

XII. The entire protocol can be completed in less than 24 hours.

Embryos that were able to develop up to the blastocyst stage were included in the chromosomic analysis through the CGH technology. After the reception of the eppendorf® tubes coming from the IVF laboratory, an amplification phase was initiated inside the laboratory of Genetics (Igenomix). The process was carried out under sterile conditions and in laminar flow hood that was previously cleaned with alcohol and UV light for 10- 15 minutes.

The different stages of the aCGH protocol, for the study of the 23 pair of chromosomes are described below, using the 24sure platform (BlueGnome, Cambridge, UK)

5.4.4.4.1 Complete genome amplification

I. After introducing the TE cells inside the PCR tube (tubing), 3µL of "Cell Extraction Buffer" were added to each tube. As well, 5µL of a mix of 4.8µL "dilution buffer" and 0.2µL of "Extraction Enzime" (Kit Sureplex™) were added. Tubes were introduced to the thermocycler with the amplification cycle temperatures established by the commercial house.

II. The tubes were centrifuged. Then 5µl of the compound composed by 4.8µL of the "Pre-amplification Buffer" and 0.2µL of the "Pre-amplification Enzyme" (Sureplex ™ Kit) were added to each tube. Tubes were introduced to the thermocycler with the amplification cycle temperatures established by the commercial house.

III. After centrifuging the tubes, 60µL of the mix consisting of 34.2µL of H2O (Nuclease-free water), 25µL of "Amplification Buffer" and 0.8µL of "Amplification Enzyme" (Sureplex ™ Kit) were added. The tubes were introduced to the thermocycler with the amplification cycle temperatures established by the commercial house. All reagents were from the Sureplex ™ Amplification Kit (BlueGnome, Cambridge, UK).

IV. Finally, an agarose gel electrophoresis was performed to check the presence / absence of amplicons.

5.4.4.4.2 Labeling

I. In a "96-well" plate, 5 µL of "Primer Solution" was dispensed. Then, 8 µl of the amplified DNA from each blastomere was added, setting 2 of the wells for the "SureRef male reference" (BlueGnome, Cambridge, UK) and 2 wells for the "SureRef female reference" (BlueGnome, Cambridge, UK). The tubes were introduced into the thermocycler and the DNA was denatured at 94 ° C for 5 minutes.

II. The tubes were removed from the thermocycler and immediately passed to a cooled rack (4 ° C) for 5 minutes.

III. Two eppendorfs® of 1.5mL were prepared: one for the mix with the Cy3 fluorochrome and one for the Cy5-mix, and then in both tubes, the following volumes were added:

   A. 5µL "Reaction Buffer" per sample/ control well
   B. 5µL dCTP-mix per sample / control well
   C. 1 µL of the "Klenow Labeling Enzyme" (Kit Labeling System ™) per sample/ control well.

IV. In one of the tubes, 1µL of the "Cy3 dCTP" fluorophore per sample/ control was added and in the other 1µL of the "Cy5 dCTP" fluorophore per sample/ control was added.

V. 12µL of the Cy3-mix were dispensed into the odd rows and 12µL of the Cy5-mix were dispensed into the even rows of the 96-well plate. This plate was taken to the thermocycler and, for 2 hours at 37 °C, the amplified DNA was labeled (Kit Labeling System ™, Illumina®, Cambridge, UK).

VI. 8µl of the amplified product of each TE sample were transferred to another 96-well plate and then, 16µL of the mix composed by 5µL of "Reaction Buffer", 5µL of "Primer solution", 5µL of "dCTP-mix" and 1µL of Cy3-dCTP or Cy5-dCTP fluorophore (Kit Labeling system ™), were added to each one of the samples.

5.4.4.4.3 Precipitation

I. The 96-well plate was taken out from the thermocycler and 25µL de "COT Human DNA" were dispensed.

II. Each Cy3-well was mixed with the 25 µL marked with the corresponding Cy5: the 25µL of the first row of the Cy3-marked plate were mixed with the second row of the Cy5-marked plate, the third with the forth and like this successively.

III. DNA precipitation was performed through vacuum centrifugation for 1 hour at 75 °C (centrifuge for DNA concentration: GeneVac, Ipswich, UK and Precipitation Kit, BlueGnome, Cambridge, UK).

5.4.4.4.4 Hybridation

I. Finally, the 96-well plate was transferred to the 75 °C heat-block and 21 µL of "hybridation buffer" tempered at 75 °C were added. The precipitated DNA was denatured in a Hybex® (SciGene, Sunnyvale, CA, USA) thermo-block at 75 °C for 10 minutes and then it was cooled at room temperature.

II. After cooling down at room temperature, inside of an exhaust hood, 18 µL of each sample were added to a coverslip (22x22 mm) and then it was placed over the array´s hybridation area.

III. Arrays were then placed inside a hybridation chamber that contains a SSC solution and formamide (Roche, IN, USA), and then, samples were hybridized by incubating them in a thermal bath, at 47oC during 8-16h.

5.4.4.4.5 Detection

I. The arrays were extracted from the hybridization chamber and then, inside the exhaust hood, they were submerged in the solution 2xSSC/0,05% "Tween20", slightly agitating to eliminate the coverslip by sliding it.

II. Still wet, the slides were washed for 10 minutes in 2xSSC/0,05% "Tween20" and during 10 minutes in 1xSSC, agitating at 850 rpm. Later, a 5-minute washing step was done in the 0,1xSSC tempered in Hybex® a 60oC and 1 minute in 0,1xSSC.

III. Finally, the arrays were centrifuged for 3 minutes to dry them up and were stored at room temperature.

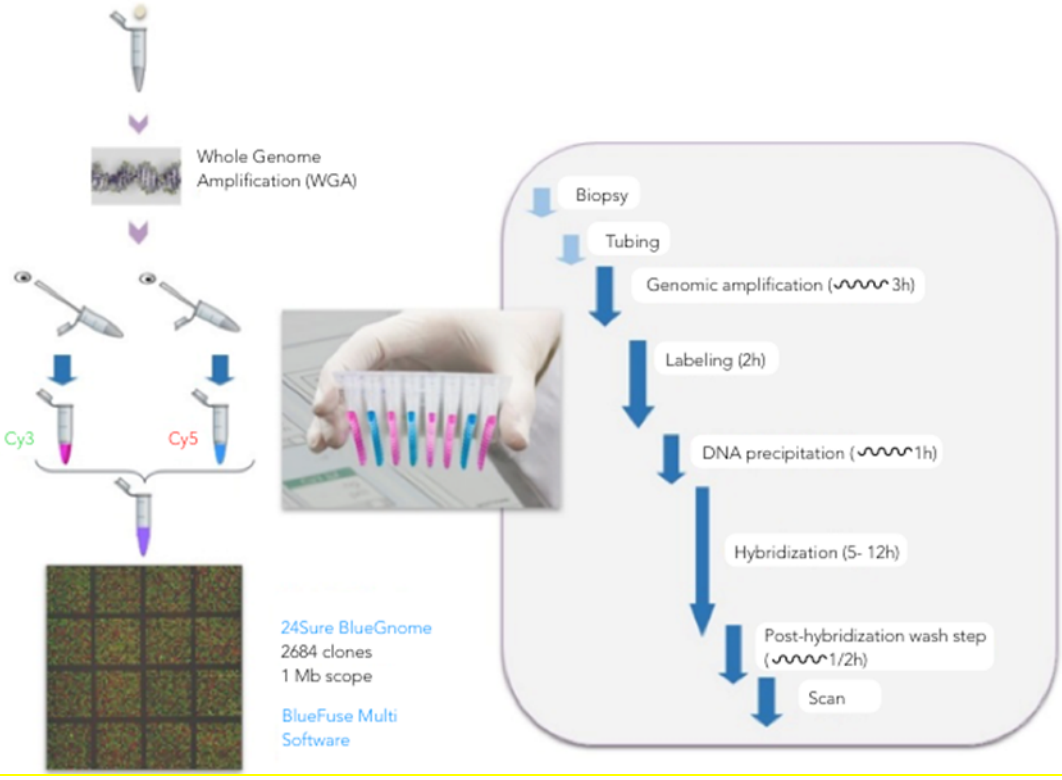


Figure 5-4. General protocol of aCGH for the study of the ploidy of 24 chromosomes over TE cells.

5.4.4.4.6 Reading and interpretation of results

The CGH array-reading was done with the Powerscanner, TECAN, Männedorf, Switzerland) equipped with a two- channel laser: a green channel (532nm) for the excitation and reading of the Cy3 signal, and a red channel (653nm) for the excitation and reading of the Cy5 signal. The images generated by the scanner were obtained in TIFF format and they were analyzed through a specific data analysis software for the arrays of CGH (BlueFuse Illumina®, Cambridge, UK), that normalizes the fluorescence

intensity of the Cy3 and Cy5 channels, and it generates a graph that helps to visually deduce the gain (trisomy) or loss (monosomy) per each of the 24 chromosomes. The gain or loss of the genetic content it's represented by dots that deviate toward the top or to the bottom of the graph, respectively.

This technique allows evaluating the gains or losses of individual chromosomes (aneuploidies), but it cannot evaluate the alterations that affect the total number of chromosomes (triploidy, tetraploid, etc.). In addition, it allows detecting the segmental chromosomic alterations that are considered clinically relevant when their size is above 20Mb.

5.4.5 Calculation of mitochondrial DNA (mDNA) copy number by NGS.

For each sample, MiSeq Reporter Software (Illumina) files in the BAM and FASTQ format were uploaded into Geneious R9 (Biomatters) to determine number of reads aligning to the mtDNA reference genome as per Genome Reference Consortium (GRC) h37. For FASTQ files, reads were aligned under maximal stringency to avoid potential multi-mapping to nuclear mitochondrial DNA segments (NUMTs) (11). The number of mtDNA mapped reads was divided by the number of nDNA mapped reads after bioinformatics' processing and filtering by MiSeq Reporter Software and displayed in Bluefuse Multi Software. Resulting values were further subjected to a mathematical correction factor described in the next section. Numbers of reads pertaining to chromosome (Chr) 1 were determined in Geneious R9 (Victor et al, 2017).

5.4.5.1 Calculation of sample size and statistical analysis

Since no previous studies were performed before in human embryos, we calculated a decrease of 35 % in the blastocyst development rate after the exposure to different toxics. So if the expected blastulation rate of human embryos with more or equal to 6 cells is 80%, a sample size of 30 embryos in each group would allowed us to detect on 35 % decrease on blastulation rate with 80% power and an a -error rate of 0.05

The statistical analysis was performed as follows. Analysis of variance (ANOVA) and Bonferroni post hoc analysis was used when need it to analyze differences among group means. Kruskall-Wallis test was used to compare medians of the experimental groups when the response variable did not follow a normal distribution. Moreover, Fisher exact test and logistic regression analysis was utilized to determine whether significant differences were observed between the expected and observed frequencies among the experimental groups. P-values < 0.05 were considered to be statistically significant.

“I gotta lot of friends, living in the ocean

you´ll never bring me down, if I have the notion

I won´t let you drown, don´t let me drown”

Chapter 6

6 RESULTS

In this chapter, the results of the study are presented. A characterization of the different pollutants found inside the IVF laboratories and their surroundings in a five-year period is presented. As it was mentioned on the previous chapter, a study to determine how these pollutants affect the embryo development was performed. The results of the developmental toxicity test (DTT) are shown as follows: first, a morphology evaluation for those groups exposed to Benzene and Limonene against a control group is made, then possible affections on the karyotype are studied, and finally, a mitochondrial DNA content analysis is performed.

6.1 CHARACTERIZATION OF POLLUTANTS

The outcomes of the air quality audits performed by MON SOLAR, to determine which were the most common VOCs inside the IVF laboratories of the IVI group in a 5-year period, were reviewed retrospectively. Three locations were evaluated per compound and year: IVF laboratories, clinics surroundings (O) and Semen Preparation Laboratory (SPL).

A total of 29 compounds (from a set of 54 VOCs evaluated) were found at least once in any of the IVI clinics in Spain, from 2010 to 2014, through the air quality characterizations performed. The Table 6-1 shows each compound and if it was present either in one, two or the three possible locations in a given year.

Compound	Number of locations per year					
	2014	2013	2012	2011	2010	Total
1,2,3-Trimetylbenzene	3	3	3	1	1	11
1,2,4-Trichlorobenzene					1	1
1,2,4-Trimetylbenzene	3	3	3	3	1	13
1,2-Dichlorobenzene	3	3	3	1	2	12
1,2-Dichloropropane	2	2	2	1		7
1,3,5-Trichlorobenzene	1	3	2	2		8
1,4-Dichlorobenzene		1		1		2
Acetic Acid	3	2	3	3	3	14
Acetone	3	3	3	3	3	15

Compound	Number of locations per year					
	2014	2013	2012	2011	2010	Total
Benzene*	3	2		1	2	8
Chlorobenzene	2	2	3	1		8
Dichloromethane			2	1		3
Diisobutylketone		3	3	3	2	11
Ethyl Benzene	3	3	3	3	3	15
Hexachloro-1,3-Butadiene				2	3	5
Hexane		2	2	3	3	10
i-Propyl Benzene (Cumene)	1	2	3	3		9
Limonene*	3	3	3	3	3	15
Methyl Acetate	1	2	2		3	8
Naphthalene		2	3		3	8
n-Decane	3	2	3	3	3	14
o-Xylene	3	3	3	3	2	14
Pentane	1	3	3	3	2	12
Pinene	3	3	3	3	3	15
m, p-Xylene	3	3	3	3	3	15
Styrene	3	3	3	3	3	15
Tetrachloroethene	1		2	3	3	9
Toluene	2		2		2	6
Vinyl Acetate			3	2		5
VOCs evaluated= 54	21	23	25	25	22	29

Table 6-1. VOCs related to the IVI clinics in Spain each year in all locations.

In general, VOCs were frequently found outside but mostly inside of the clinics, as it can be seen in *Figure 6-1* and Table 6-2, where the percentage of the total compounds found, over the total VOCs analyzed, is presented.

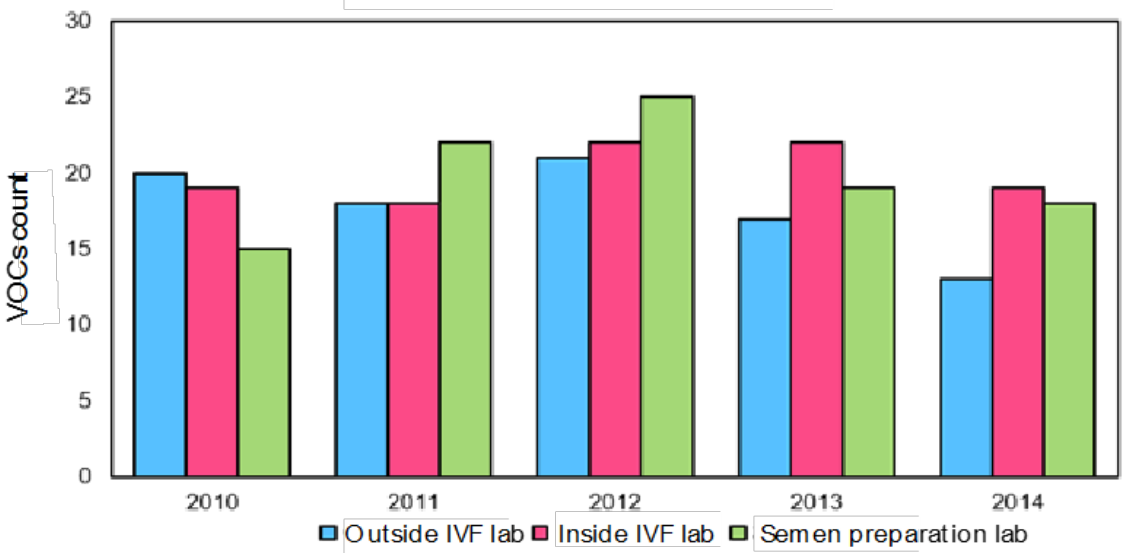


Figure 6-1. VOCs total count per location.

As it can be seen in Table 6-1, 20.7% (n=6) of the VOCs were present every year at each location: i.e., Acetone, Ethyl benzene, Limonene, Pinene, m, p- Xylene and Styrene. The details of these VOCs concentration are shown in Figure 6-2. It outstands how the concentration tends to be less or at least similar, outside the laboratory or in the semen itself. It is also worthily to mention that inside IVF and in SPL, there is a local peak of VOCs concentration in 2012; interestingly the outside concentration does not seem to follow this trend.

VOCs per year	IVF Compounds	
	N°	%
2014	19	35,2%
2013	22	40,7%
2012	22	40,7%
2011	18	33,3%
2010	19	35,2%

Table 6-2. Percentages of VOCs found inside the IVF laboratory in each year of the 5-year period evaluated.

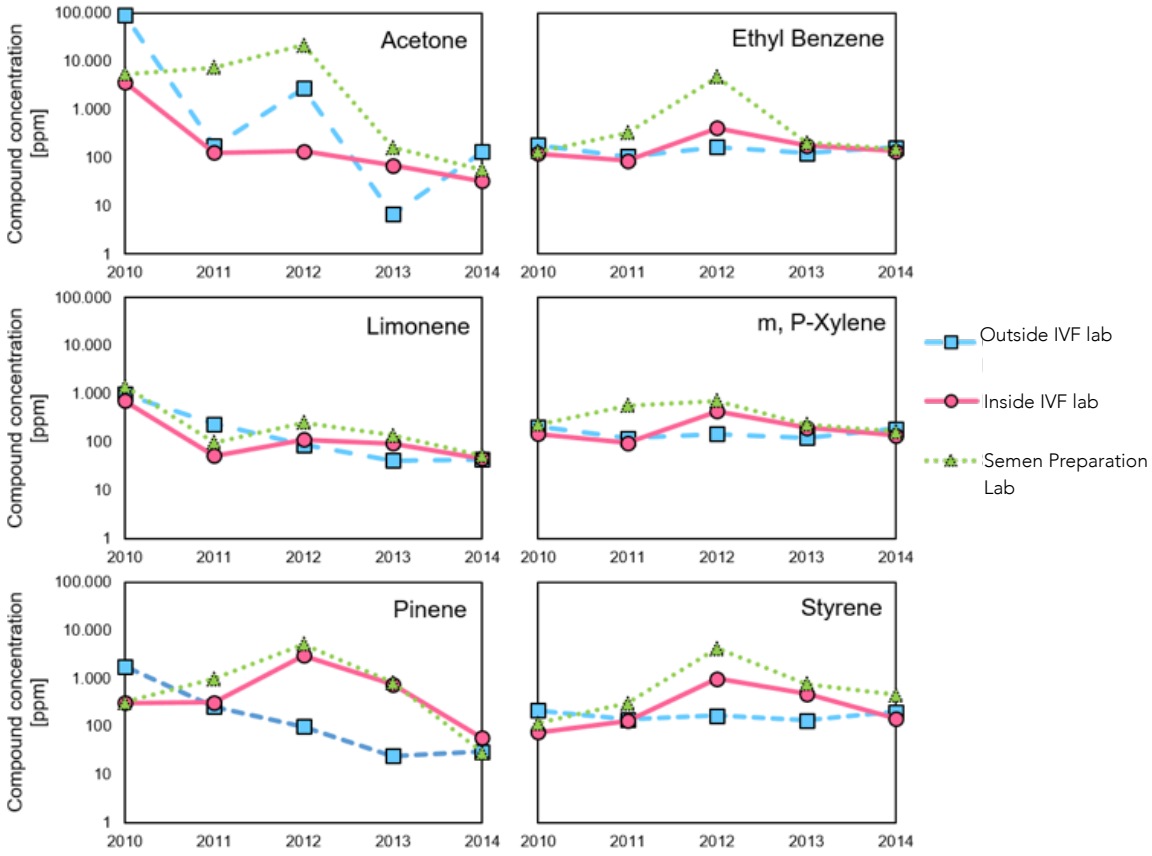


Figure 6-2.Concentrations levels of the most common pollutant in 5 years s.

In regards to frequency of appearance, from the total VOCs found, 89.7% (n=26) of them, were present inside the IVF laboratories in at least 2 years. In addition, the total concentration of VOCs found inside the IVF laboratories was 361702.2 $*10^{-5}$ ppm with an average concentration of 3617 $*10^{-5}$ ppm. In Table 6-3, it is shown how many times each VOC concentration was higher in the limits of the IVF, and the concentration values. The final column indicates the current occupational limit value for each VOC. It is also shown the occurrence of these VOCs per each location.

Compound	IVF	SPR	O	>IVF *	2014	2013	2012	2011	2010	OLV (*10^5 ppm)
1,2,3-Trimetylbenzene	3	4	4	2		160	175.1			2,000,000
1,2,4-Trimetylbenzene	4	5	4	2		105	130.8			2,000,000

Compound	IVF	SPR	O	>IVF *	2014	2013	2012	2011	2010	OLV (*10^5 ppm)
1,2-Dichlorobenzene	4	4	4	2		105	130.8			2,000,000
1,2-Dichloropropane	2	3	2	2	194	1354.9				7,500,000
1,3,5-Trichlorobenzene	3	2	3	3		132.9	157.5	2.44		500,000
Acetic Acid	5	4	5	3	672.6	159.3	64.019.9			1,000,000
Acetone	5	5	5	1		68.8				50,000,000
Benzene *	3	3	2	2		213.1			370.0	100,000
Chlorobenzene	3	2	3	1	118.4					500,000
Diisobutylketone	4	4	3	2		125.0		168.4		2,500,000
Ethyl Benzene	5	5	5	2		179.4	412.5			10,000,000
Hexachloro-1,3-Butadiene	2	1	2	1				0.47		2,000
Hexane	4	2	4	2		202.5	653.2			2,000,000
i-Propyl Benzene (Cumene)	3	2	4	3		307	346.7	55.64		2,000,000
Limonene *	5	5	5	3	45.2	95	113.6			2,000,000
Methyl Acetate	4	1	3	3	5.4	192.983	6.511.9			20,000,000
Naphthalene	3	2	3	2		88	147.4			1,000,000
n-Decane	5	4	5	2		240	6,710.2			5,000,000
o-Xylene	5	5	4	2		270	428.7			5,000,000
Pentane	5	3	4	5	5.6	19.653	19.142.0	328.7	280.7	100,000,000
Pinene	5	5	5	4	57.8	739	2,990.9	313.5		2,000,000
m, p-Xylene	5	5	5	2		198	437.8			5,000,000
Styrene	5	5	5	2		472	981.0			2,000,000
Tetrachloroethene	4	2	3	2	2.4		2.083.7		2590.2	100,000
Toluene	2	2	2	1	188.1					5,000,000
Vinyl Acetate	2	1	2	2			2.307.2	277.6		1,000,000
26	9				1	19	18	7	2	26

Table 6-3. Number of years in which each compound was found at each location. >IVF Shows the number of years in which the concentration of the compound was higher inside the IVF than in its out limits.*

In general, 2 to 21 compounds were in higher concentrations compared to the outside as it can be seen in Table 6-3 and Figure 6-3. Moreover, from the total VOCs, 31% (n=8) were not only inside of the laboratory but also in every year evaluated (Acetic acid, Acetone, Ethyl benzene, Limonene, n- Decane, o- Xylene, Pinene, m, p- Xylene and Styrene). And all of them were in higher concentration inside the IVF comparing to the values found in other locations.

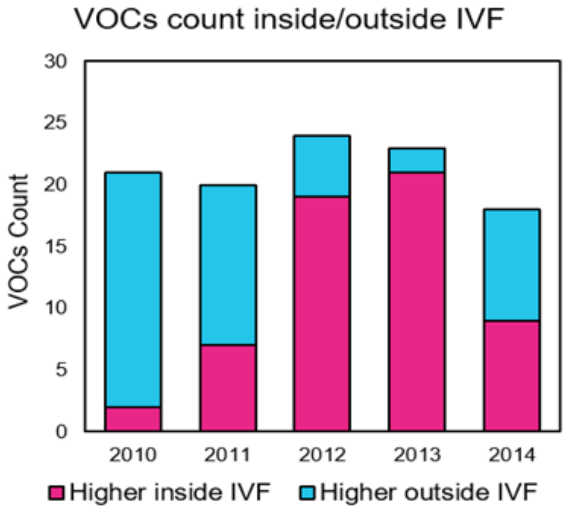


Figure 6-3. Total VOCs count inside the IVF facilities. In pink: VOCs in higher concentrations compared to the exterior. In blue: VOCs inside the facility in lower concentrations compared to the exterior.

One of the main goals of the VOCs characterization was to find which compounds, if any, exceeded the 1% of the OLV. This information is presented in Figure 6-4. In 2010, Hexachloro- 1,3 –butadiene exceeded the empirically 1%-OLV established to prevent VOCs pollution inside of the IVF laboratory (with a 2.3%), as well as Acetic acid and Tetrachloroethane in 2012 (6.4% and 2.3% respectively): only 10.3% of the VOCs found. However, in 2010 and/ or 2012, these VOCs along with Chlorobenzene, Methyl acetate, Tetrachloroethene and Vinyl acetate were also above this 1%-OLV in the SPR, which was not far from the IVF. Looking retrospectively, it was found that there were some renovations done, and because of the use of paintings and other materials that could negative influence the facilities environment.

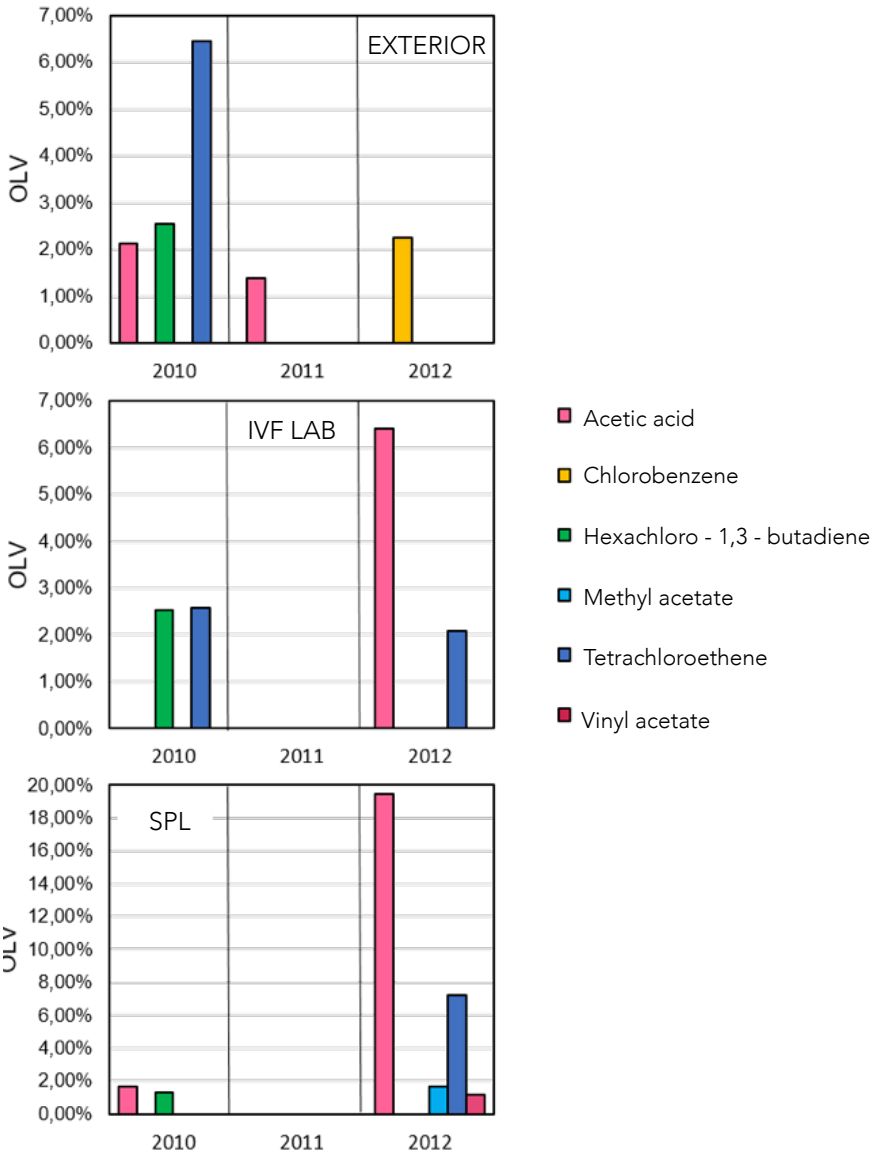


Figure 6-4. VOCs that exceeded the 1% OLV.

The distribution of each VOC through the 5-year period inside the IVF laboratory was evaluated through a Kruskal-Wallys test. Despite the increment of concentrations of some compounds, even overcoming the 1% OLV established, no significant changes within the years were obtained in the distribution per compound.

The following VOCs did not registered any concentrations in the 5-year period: 1,1,1-Trichloroethane, 1,1,2-Trichloroethane, 1,1,2,2-Tetrachloroethylene, 1,1-Dichloroethane, 1,1-Dichloroethylene, 1,2-Dichloroethane, 1,3,5-Trimetylbenzene, 1,3-Dichlorobenzene, 2-Butanone, 3-Methyl-1-Butanol, 4-Methyl-2-Pentanol, Butyl Acetate, Chloroethane, Chloroform, Chloromethane, Cyclohexane, Dibromochloromethane, Diethyl ether, Ethanol, Ethyl Acetate, Hexanol, i-Butanol, i-Propanol, Tetrahydrofurane and Vinyl Chloride.

6.2 DEVELOPMENTAL TOXICITY TESTS (DTT)

A total of 65 total patients signed the informed consent (to see the format, see annexes 10.1 and 10.2), to donate their embryos for this specific research. The average age of the patients was 31.6 years old. The mean number of embryos per patient was 2.5.

180 vitrified embryos on D+3 of development were used in this study. 150 embryos were exposed: 75 embryos to either benzene or limonene in three different doses, and 30 embryos were the control group. The embryos were obtained from different type of ART cycles: 6.5% FIV cycles, 9.2% FIV/ ICSI combined cycles and 84.6% ICSI cycles. In general, 11.7% embryos came from oocyte donation (OVODON).

6.2.1 Exposure to benzene: development and morphology results

25 embryos (100%) were exposed to each of the concentrations set up for benzene (75 embryos). When evaluating the survival of the embryos on D+6, all the embryos of the control group were able to develop up to the blastocyst stage and presented a high rate of expansion as well.

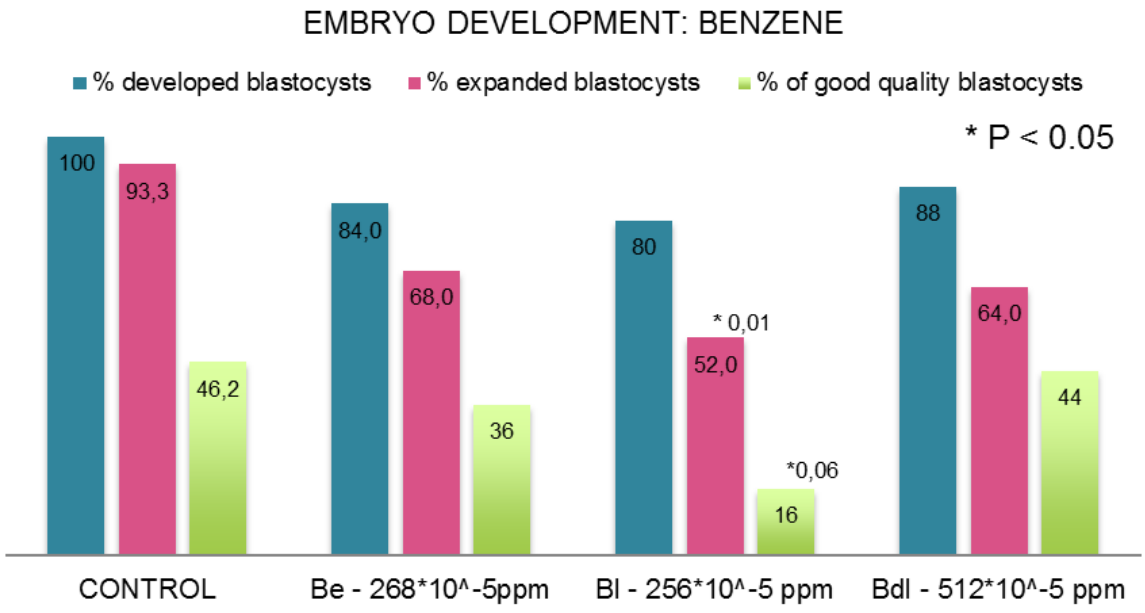


Figure 6-5. Percentages of development, expansion and good quality obtained; comparison between C-EG and each of the benzene-EG treatments.

Regarding the expansion rate, the cavitated embryos with visible ICM and TE were included in the count, being considered as "initiating expansion" embryos. When making a general comparison of development between the C-EG (100%) and the B-EG (84%), no statistically differences were obtained. When comparing against each experiment, all three doses of Benzene had lower rates compared to the control group. However, no statistically differences were obtained in development either, except for the expansion and good morphology rates, which were significantly lower in

the group exposed to the benzene-laboratory concentration only (*bl*: 256*10-5 ppm) (p-value< 0.05).

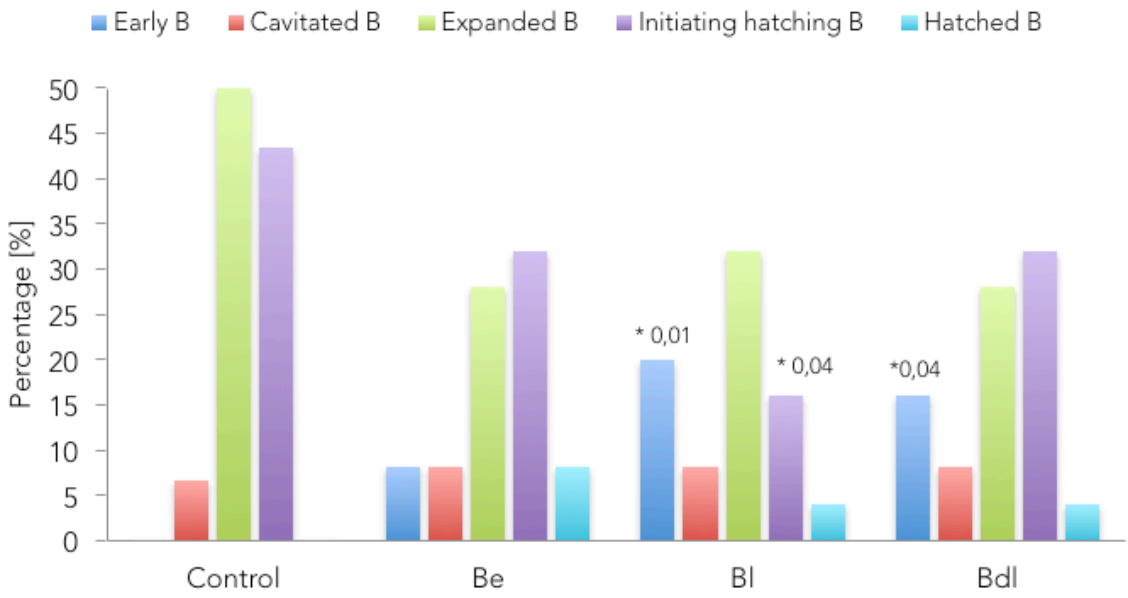


Figure 6-6. Development stages reached by B-EG embryos. Significance is per number of embryos developed in each stage.

When assessing the specific development, no embryos were found blocked either in cells, morula, and compacted morula or EB stage by D+6 in the Control-EG. In contrast, 16% (n=12) of the B-EG embryos did not develop. The embryo morphology was scored with the ASEBIR criteria.

The distribution of the embryos according to the developmental stage was also different between groups. While all embryos in the C-EG were blastocysts and were mostly in the "expansion" or "initiating hatching" stages (38.5% and 46.2% respectively), some B-EG blastocysts were seen to have had a slower development up to D+6: 14.3% and 8% of embryos were observed as early and cavitated blastocysts, respectively. On the other hand, in each of the B-treatments, there was at least 1-hatched embryo. No hatched embryos were seen in the C-EG. No statistical differences were found between the specific blastocyst stages evaluated.

6.2.2 Exposure to limonene: development and morphology results

25 embryos (100%) were exposed to each of the concentrations set up for limonene (75 embryos). The general embryo-survival rates between the C-EG (100%) and the L-EG (60%) were statistically significant (p-value= 0.0035).

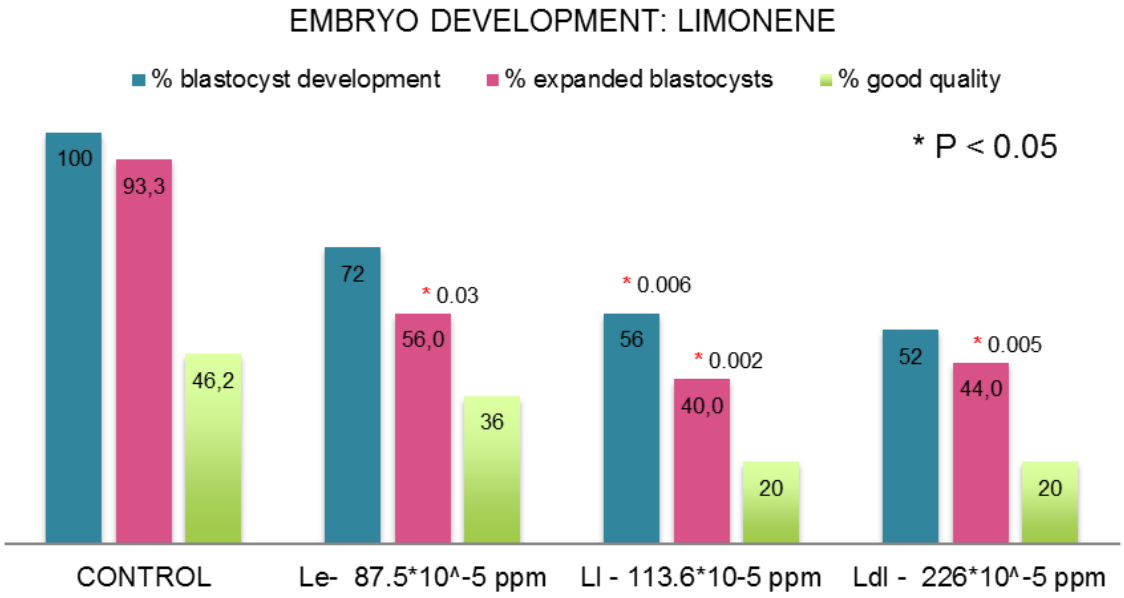


Figure 6-7. Percentages of development, expansion and good quality obtained; comparison between C-EG and each of the limonene-EG treatments.

Limonene specific doses also did influence significantly the blastocyst development and expansion parameters mostly. When comparing the C-EG against each experiment of L-EG, the development rate was significantly lower in the group of embryos exposed to "*Ll*: 113.6*10-5 ppm". The expansion rate was significantly lower in all the groups of embryos exposed to this VOC. Even though there were not statistically differences on the morphology of the blastocysts, the good quality rates were lower compared to the control group.

There is also a distribution of embryos between all stages in the L-EG as well. No significant differences were found with the control group, but fewer embryos developed further of the expanded stage in the groups exposed to "*Ll*" and "*Ldl*" treatments (Figure 6-8).

No embryos were found arrested from cells-stage to early blastocyst-stage in the control group while, in the other groups, embryos were distributed in all categories: from cells to hatched blastocysts. Because embryos were not going to be transferred, early blastocysts were included as developed embryos even though by D+6 these embryos would be considered as good quality embryos on a clinical basis.

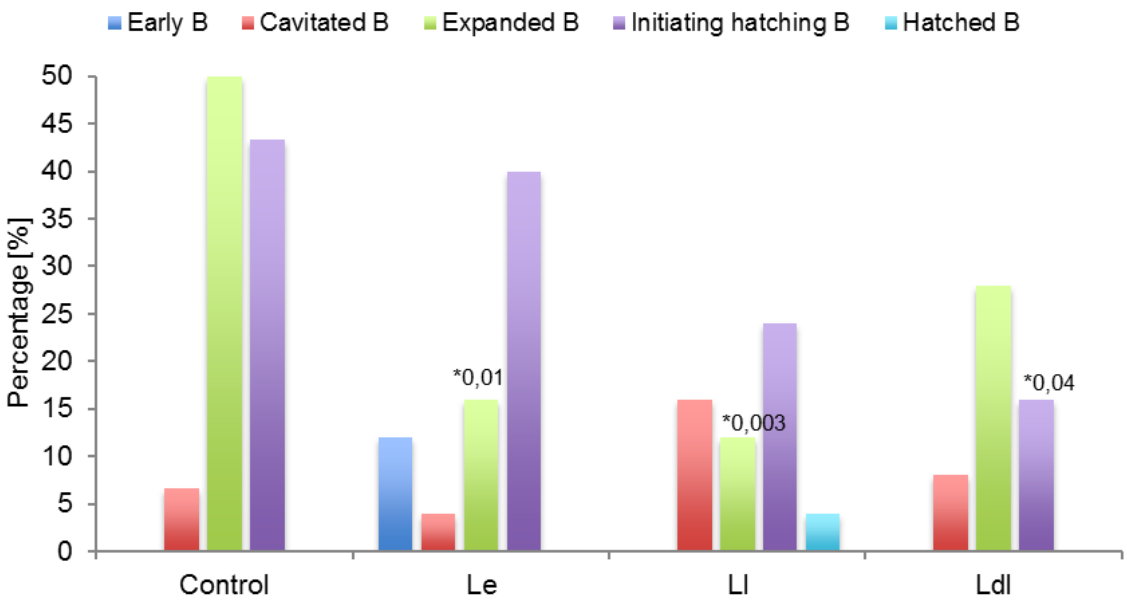


Figure 6-8. Development stages reached by B-EG embryos. Significance is per number of embryos developed in each stage.

General morphology	% General bad quality	*p-value*
C	46.7	-
B	29.3	0.1128
L	18.7	0.0064
	% ICM	
C	30	-
B	14.7	0.0980
L	13.3	0.0543
	% TE	
C	40	-
B	28	0.2516
L	16	0.0187

Table 6-4. Percentages of bad quality embryos per EG.

Bad quality rates were obtained from blastocysts with bad ICM, bad TE, or both. Limonene had a significant effect on the quality of the blastocysts evaluated.

6.2.3 Chromosomic profile

134 embryos were analyzed on D+6 of development. There were 3 genome amplification failures, so the informativity rate was 97.7%. 131 results were obtained

and aneuploidies were present in all the groups studied: 22.9% results from the C-EG (n=30), 45.8% from B-EG (n=60) and 31.3% from L-EG (n=41).

No significant differences were obtained when comparing the aneuploid embryos obtained from C-EG and B-EG; however, there was a trend toward to obtain higher aneuploidies from the embryos exposed to all three doses of Benzene:

Classification	%C	Be	Bl	Bdl
Aneuploid	23.3	38.1 (0,3514)	42.1 (0.2103)	40 (0.2275)

Table 6-5. Aneuploidy rate for the control group, and the different levels of Benzene contamination.

On the other hand, a high percentage of aneuploidies were obtained only from embryos exposed to the "*Ll*" dose but no significant differences were obtained when comparing the control group against each of the L-EG treatments either.

Classification	%C	Le	Ll	Ldl
Aneuploid	23.3	12.5 (0.4628)	42.9 (0.2878)	27.3 (1)

Table 6-6. Aneuploidy rate for the control group, and the different levels of Limonene contamination.

The comparison of percentages of aneuploidies related to each dose (exterior, laboratory and double laboratory) between both exposition groups did not result significant either. The binary logistic regression showed that the presence of Benzene in the culture medium lowers the probability of euploid embryos outcomes in a 65% ($p<0.05$; OR 0.350 CI 95%: 0.132- 0.925)

From 1 to 10 aneuploidies, between monosomies and trisomies, were detected in each embryo. Up to 2, 6 and 10 aneuploidies were detected per embryo of C, L and B experimental groups, respectively:

	N° of aneuploid embryos	N° total of chromosomes affected	N° max/ embryo
C	7	4	2
Be	8	16	6
Bl	8	16	3
Bdl	8	16	10

Le	2	3	2
Ll	6	7	2
Ldl	3	9	2

Table 6-7. Number of chromosomes affected per exposition groups.

From the set of 23 chromosomes (C#) evaluated, the C17 was not affected in any blastocyst. Based on the type of chromosome involved in the aneuploidies, Benzene (n=21) and Limonene (n=13) were significantly higher compared to the control (n=4): 0.0001 and 0.013 respectively.

	Affected chromosomes	% over 23C set	*p*-value	Most common C# (>2 times)	% Mitosis-related (C1-C14)
C	1, 2, 12, 20	17.4	-	-	75.0
Be	2, 6, 7, 9, 11, 12, 13, 14, 16, 22, XY	47.8	0.057	2, 16, 22	72.7
Bl	1, 5, 8, 11, 15, 16, 18, 19, 20, 22, XY	47.8	0.057	16, 18, 22	36.4
Bdl	1, 2, 4, 5, 6, 7, 9, 13, 14, 16, 17, 21, 22, XY	60.9	0.0058	13	64.3
Le	11, 13, 16	13.0	1	-	66.7
Ll	4, 9, 15, 16, 22, XY	26.1	0.7222	15	33.3
Ldl	2, 3, 6, 10, 21, 22, XY	30.4	0.4908	2, 21	57.1

Table 6-8. Percentage of chromosomes from the 23-chromosomes set evaluated, involved in errors in accordance of the Benzene and Limonene doses.

Benzene is significantly related to the increasing number of chromosomic errors in the set of 23 chromosomes evaluated through CGH arrays. It also affected the 85,7% of the mitosis-related chromosomes, except for C3 and C10, which are more than half of the chromosomes of the karyotype.

On the other hand, despite that Limonene had significant effects over the development of the embryos, it not significantly related to the production of chromosomic errors. However, up to 34,8% of the mitosis-related chromosomes of the karyotype were detected with errors, which is more than double than control: Benzene (n=12) and Limonene (n=8).

In the following two graphs, two comparisons are showed: percentages of monosomies (Figure 6-9) and percentages of trisomies (Figure 6-10) per type of chromosome found in each group of Benzene vs. control. Many chromosomal abnormalities, such as gains and losses of chromosomes, partial chromosome deletions and duplications were found.

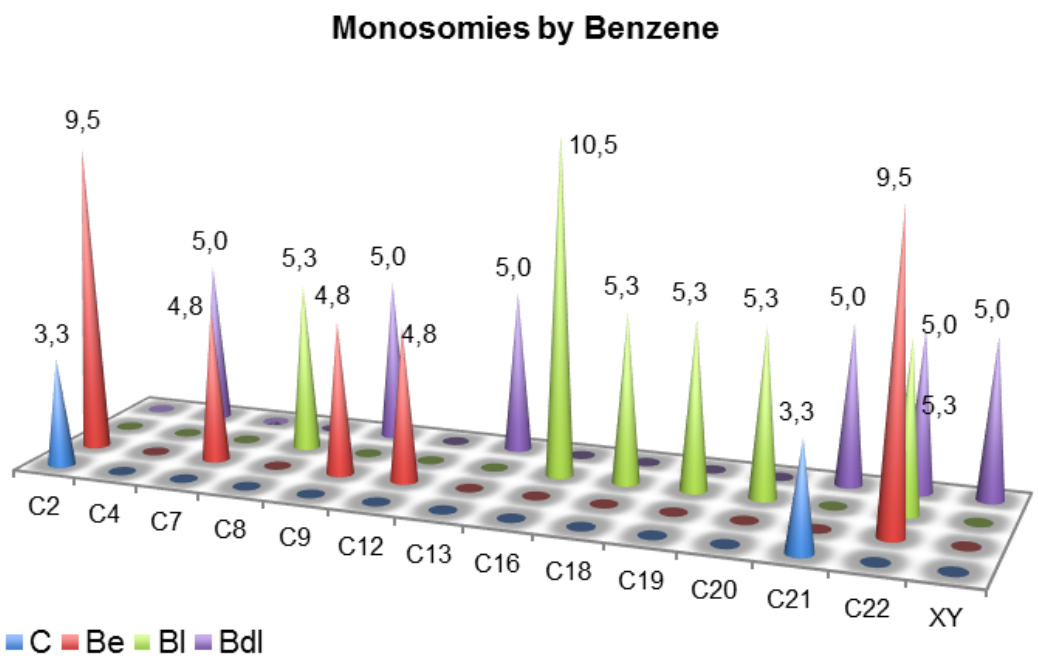


Figure 6-9. Percentage per type of chromosomes involved in monosomies related to each type of Benzene exposition group and control group.

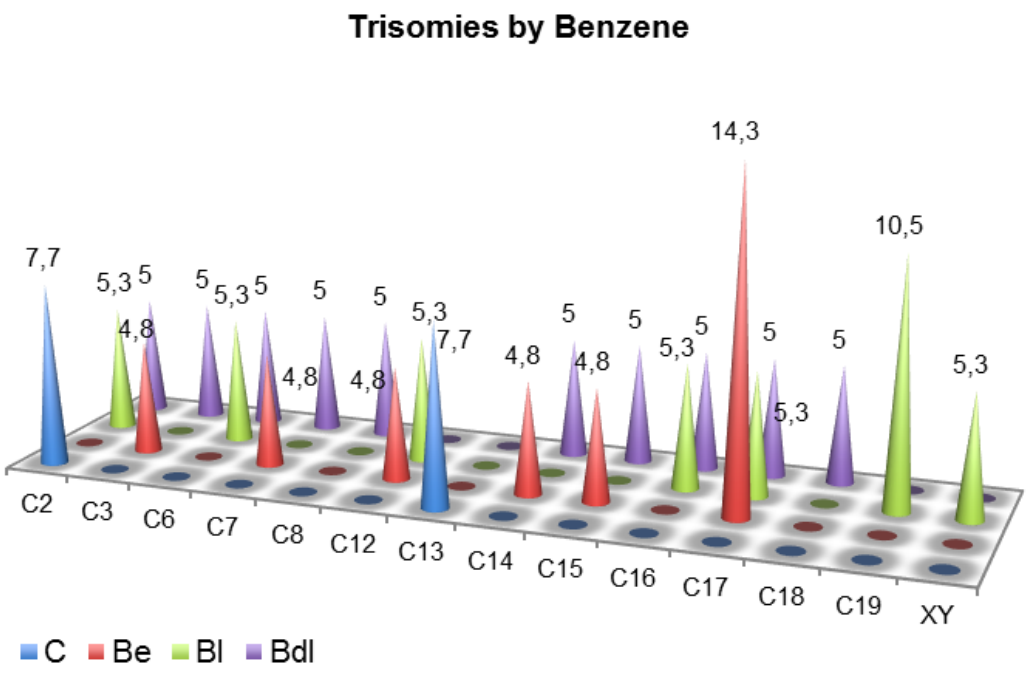


Figure 6-10.Percentage per type of chromosomes involved in trisomies related to each type of Benzene exposition group and control group.

All three doses of Benzene increased the number of errors in the embryos, specially the "*Bdl*" dose, which produced more trisomies. Limonene had higher number of trisomy errors per chromosome with the "*Ldl*" dose as well, but not that many, as the Figure 6-11 and Figure 6-12 shows

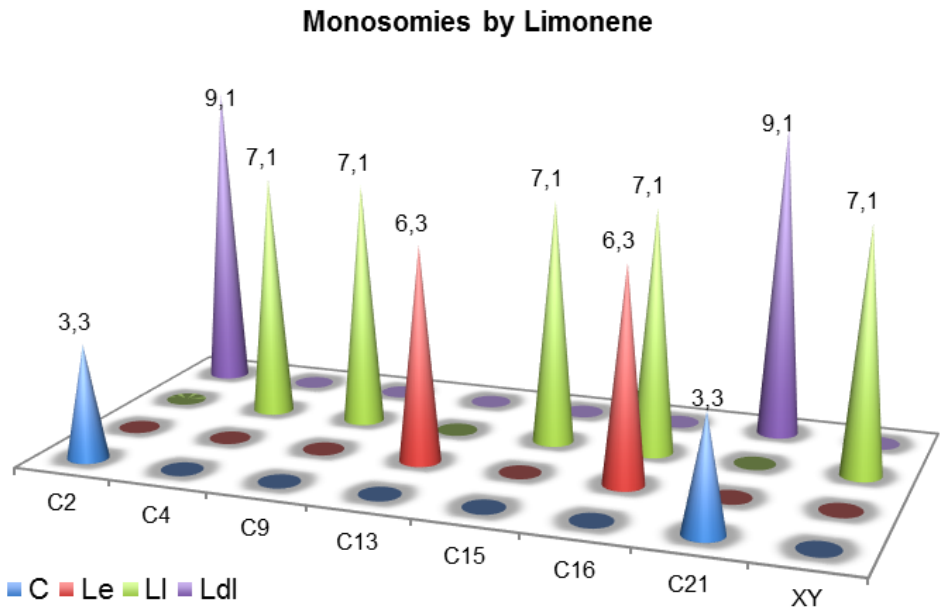


Figure 6-11. Percentage of monosomies of each chromosome affected by each concentrations of Limonene used.

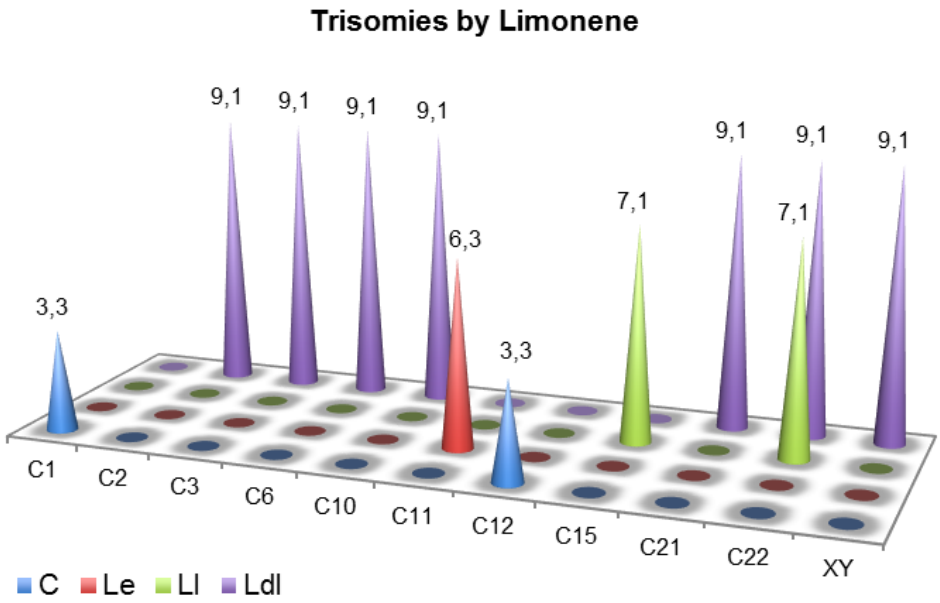


Figure 6-12. Percentage of trisomies of each chromosome affected by each concentrations of Limonene.

It seemed also that poor morphology embryos were related to a higher percentage of aneuploidies as well, although no significant differences were found between groups (Table 6-9).

Group	% Bad TE	% aneu
C	46.1	15.4
B	61.7	31.7
L	34.1	14.6

Table 6-9. Percentage of bad morphology embryos per EG with aneuploidies.

6.2.4 Mitoscore DNA content

The mitochondrial DNA content of the TE cells of 106 embryos was analyzed as well: 10.4% embryos from C-EG (n=11), 51% from B-EG (n=54) and 38.7% from L-EG (n=41) were analyzed. The summary of the lowest and highest scores obtained and the blastocysts characteristics can be seen in Table 6-10.

	Blastocysts	LMs	Blastocysts	HMs
Control	BHI (A-, A) euploid	14.7724	BC, aneuploid	37.3354
Be	BE (B, C) aneuploid	12.5448	BE (A, C), aneuploid	44.3259
Bl	BE (B, B) aneuploid	13.1023	BC, aneuploid	45.3955
Bdl	BHI (B, B) euploid	13.2999	BT, aneuploid	66.0315

Le	BHI (B, A-), euploid	14.4435	BE (B,A) aneuploid	43.3128
Ll	BHI (B, B) euploid	16.1976	BC, aneuploid	49.3170
Ldl	BE (D, D), euploid	13.8885	BC, euploid	38.3819

Table 6-10. Summary of the lowest and highest scores obtained by EG.

A comparison was performed between the mitochondrial DNA scores obtained from the embryos of the three groups. MtDNA copy values from 12.5448 to 66.0315 were obtained. The two lowest values were related to aneuploid embryos from B-EG, only one with bad TE morphology. The two highest values corresponded to two aneuploid embryos from B-EG and L-EG. The VOCs do not seem correlated with the mtDNA content as Figure 6-13 shows. No significant differences were obtained in the distribution and medians of the mtDNA copy number among the groups.

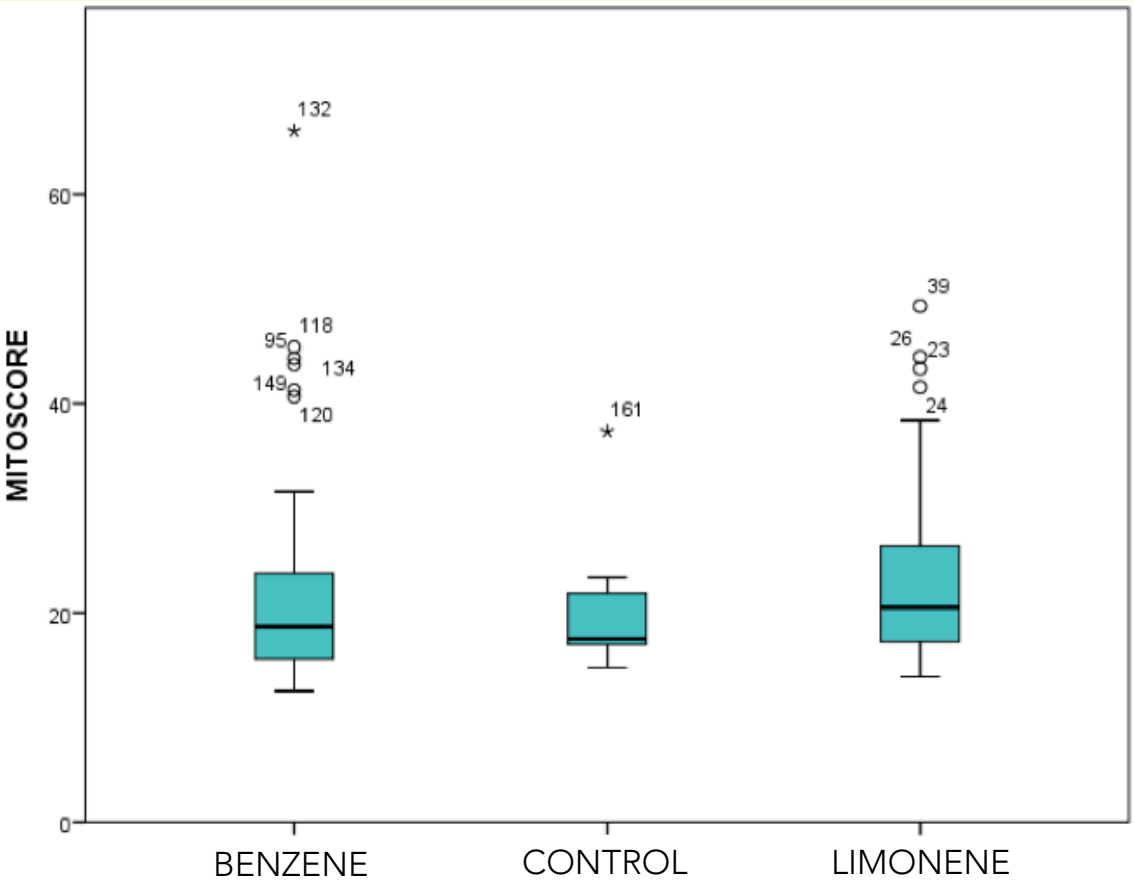


Figure 6-13. Distribution of mtDNA scores in the three EG: control, Benzene and Limonene.

Neither the distribution nor the medians of the scores of the mtDNA changed according to the increasing concentrations of neither Benzene nor Limonene. The majority of scores were similar between the three groups Figure 6-14 and Figure 6-15.

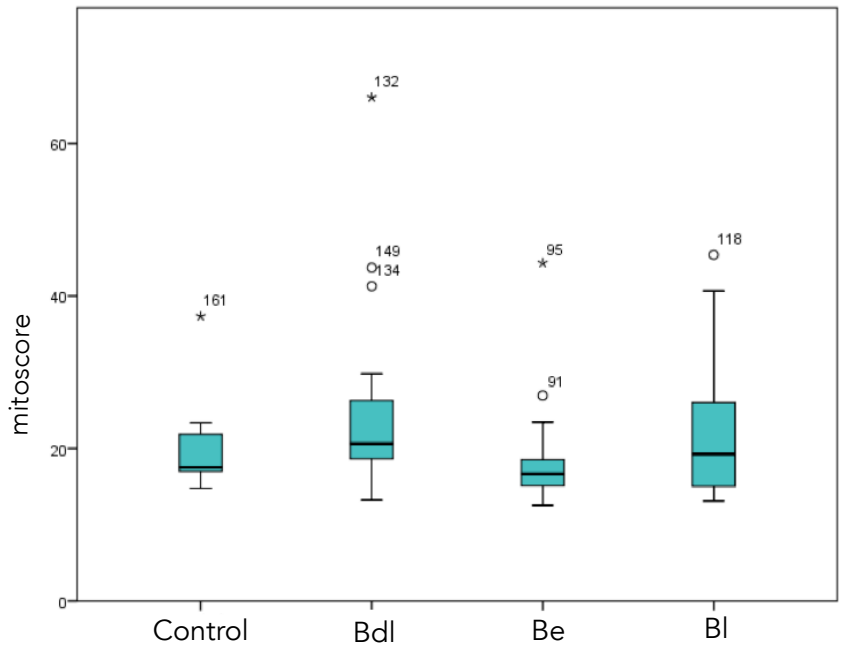


Figure 6-14. Distribution of mtDNA scores in each treatment group of Benzene. No significant differences were found.

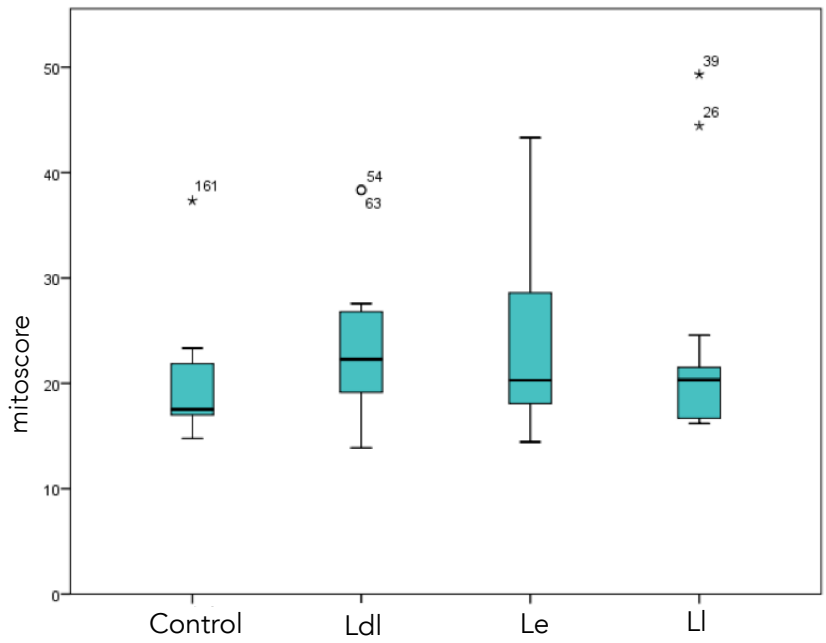


Figure 6-15. Distribution of mtDNA scores in each treatment group of Limonene. No significant differences were found.

Interestingly, aneuploid embryos with bad morphology (bad ICM and TE quality) from B-EG and L-EG presented a wide range of scores, from the lowest to the highest mtDNA scores, while the range of scores of the bad morphology control embryos was smaller (Table 6-11).

	Euploid	Aneuploid	Abnormal embryos	mtDNA content
C- Good TE	4	0		
C- Bad TE	4	3	27.3%	22,59-37.33
B- Good TE	15	5		
B- Bad TE	15	19	35.2%	12.54-66.03
L- Good TE	16	5		
L- Bad TE	14	6	14.6%	14.92-49.32

Table 6-11. Bad morphology, aneuploid embryos and their respective mtDNA rank

“Hold your breath from all those voices that are dark
look inside and hear that symphony of harps
if we tune them… well, the forest it will sing
behold this song of many queens and kings”

Chapter 7

7 DISCUSSION

7.1 IVF POLLUTION

It is common to find different types of volatile organic compounds in any type of environment, such as urban living areas. Benzene for example, is very common in areas were solvents, gasoline or gasoline-powered equipment is used, which can be related mainly to outdoor sources, while limonene is usually related to indoor areas where cleaners and deodorizers are used (Du et al., 2015). At the same time, both compounds can be produced inside of the IVF laboratories: benzene can be present due to combustion processes (pipette burning, electric equipment, etc.) but also inside the CO_2 gas cylinders of the incubators. With regards to limonene, its presence may be due as well to any kind of cleaning products of daily use that can be managed by any person that access the laboratory or any kind of staff that surrounds it, as limonene is very common in cosmetic products. At the same time, Limonene has been also known to react with the indoor ozone and with these chemical reactions they can produce submicron-sized particles and harmful by-products (Rosch et al., 2017) that have a possible association with poorer IVF outcomes (Legro et al. 2010). But so far, there are limited studies about the effects that a specific VOC can cause over the human cells while they are in the laboratory and there is still a certain level of suspicion about the specific deleterious effects over the human embryo development (Cohen et al. 1997; Khoudja et al., 2013) . ,

As mentioned in the background, the IVF laboratories are usually located in urban areas, surrounded by many types of pollutants sources (gas stations or any place that could produce combustion), and despite the fact that there is a strong line of containment for many types of compounds, it is really difficult to prevent and control the production of VOCs. The IVI laboratories use a line of pollution containment that consists in a filtration system equipped with several filtration steps comprised in the classification established by the European Committee for Standardization: EN779: 2002 and EN1822-1. The primary filtration consist on G4 filters which are coarse particles filters and that retain pollen and fog among other inhalable particles, the F7 and F9 filters that are fine dust filters for air conditioning systems which also retain inhalable particles of accumulated black carbon (F7: 85% of filtration efficiency), coarse fractions of tobacco smoke, metal oxide smoke and oil smoke (F9: >95% of efficiency), and finally the H14 filters (>99.995% of efficiency) which are HEPA filters that retain the aerosol micro particles. These filters are usually found in different types of cleanrooms (European Standard 2002, TROX® TECHNIK, 2013). The problem with the VOCs is that they can not be retained by the HEPA filters due to their very small size, so the

laboratories count with an additional filtration step with activated charcoal. However, because ketones and alcohols are not easily removed by carbon, this phase also counts with potassium permanganate that controls other compounds by oxidizing them (Esteves and Bento, 2013). The filtration phases are placed in the following order

1. Pre-filter G4
2. Filtration step 1 with F7
3. Filtration step 2 with Activated charcoal with permanganate
4. Filtration step 3 with F9
5. Absolut filters with H14

Still, in the air characterizations performed by MON SOLAR, 53,7% of the VOCs evaluated, were detected in the clinics. Limonene was one of the most common VOCs measured because it was one of the compounds present every year, although the concentrations found were below the 1% OLV; the IVF directors have established this value empirically to prevent deleterious effects of chemical and biological aggressions over very vulnerable cells, from gametes and embryos, that are routinely managed inside of the laboratories. On the other hand, although Benzene was not the most common VOC it was in higher concentrations inside of the IVF-lab in at least two of the years evaluated, which is important because its very toxic nature and the ability to sink into low-lying areas due to the fact that its vapor is heavier than air (Office of Public Health Preparedness and Response, 2013).

Other very common compounds have become the target of our attention as well, because their concentration was found to be higher inside the IVF laboratory compared to the outside were: Styrene and Ethilbenzene (which can be released from the IVF plastic ware), Tethracloroethane, Hexachloro-1,3-Butadiene, 1,2,3-Trimetilbenzene, 1,2-Dichloropropane, Acetic acid, Chlorobenzene, Hexane, Nafthalene, n-Decane, o-Xilene and Pinene. As mentioned in the background, no reports were found on the relationship between PAHs and the IVF´s environment; however, we found that Naphthalene, which is the simplest PAH, was present in at least three years evaluated. In general, the exposure to high doses of naphthalene in the environment is associated with hemolytic anemia, damage to the liver and neurological system, cataracts and retinal hemorrhage. Naphthalene is reasonably anticipated to be a human carcinogen and may be associated with an increased risk of developing laryngeal and colorectal cancer (National Center for Biotechnology Information, 2017a).

The seasonal influence described by some authors (Khoudja et al., 2013), was also confirmed in this study by the increase of the number of compounds detected per year, in our case from 21 to 25 VOCs, but also by the detection of increased doses of

some VOCs that even surpassed the empirical 1% OLV. The years 2010 and 2012 were the most representative but it was difficult to track the events that might have happen inside and outside of each IVI clinics to determine the cause of such increase. Small variations can also be produced by the degree of absorption of the small concentrations of VOCs by the mineral oil and the culture media. However, the results confirm that the filtration systems do not have the capability to eliminate these compounds and that they are being produced routinely in all the clinics.

7.2 Effects of benzene and limonene over the embryos health

Few studies have reported the relationship between IVF-air specific pollutants and the health of the human embryos that are daily manipulated inside the laboratory, which leads to many assumptions regarding the possible deleterious effects over the cells. This descriptive study, is the first known study that has addressed this topic by including parameters such as the morphology assessment and the measurement of the chromosomic and mitochondrial DNA content, to establish an objective relationship between the presence of VOCs and the effects over the development of human embryos. We divided each embryo to analyze it in two different ways: a CGH-arrays analysis and a metilation analysis that will be performed in future experiments. For this, the remaining samples are stored at -80 °C.

The conditions of the experiments were a representation of the clinic´s IVF laboratory but the study did not have a cleanroom, neither, all the recommended preventive elements to ensure that no external pollution would access the laboratory. In this case, the indoor concentration of VOCs was probably higher than an actual IVF laboratory and unfortunately we were not able to characterize our actual environment.

Preventive measures were taken during the manipulation of both VOCs, especially Benzene, although its bottle was opened once, per week of experiment, and just for a few seconds. The dilutions were prepared according to the recommendations of the Chemical Department of the University of Valencia and the MS Spectrometry evaluation of the samples of VOCs confirmed the presence of the compounds in mineral water, however, the results reported higher concentrations but allowed us to have a degree of certainty about the presence of the compounds in the culture medium. This specific test that consisted in the measurement of VOCs in aqueous solutions instead of air was set up for the first time in this laboratory, so there is a possibility of having measured the samples in an equipment that was not properly calibrated for the needs of this study. In the future more samples of other VOCs should be tested to improve this. When preparing the dilutions, it was noticed that a hard

agitation was needed until Benzene mixed properly, something that was not needed with Limonene due to its high solubility in water. Perhaps, the results could be influenced by this characteristic as well. Studies performed in bacteria have reported that hydrocarbons are hydrophobic but Benzene is still soluble in water although not completely miscible (Sikkema et al., 1994; Sikkema et al., 1995).

In any case, the presence of VOCs inside the culture medium, for four consecutive days, did influenced the development giving clear evidence of the deleterious effects that can be produced while human embryos developed *in vitro*. When assessing the survival status of the embryos on D+6 of development, it was determined that the developmental rates were lower compared to control group in all the experimental groups, mostly in the "*Ll*" treatment ($113.6*10^{-5}$ *ppm*) where the rate was significantly lower. At the same time, all experiments lowered the possibility of the surviving blastocysts to grow properly; the expansion rates where also lower in all experimental groups, with significant effects associated to "*Bl*" ($256*10^{-5}$ *ppm*) and all Limonene treatments. For the expansion parameter, the "initiating expansion" (IE) blastocysts, which are usually graded as "cavitated", were included because it was viable to see the ICM and the TE in them. Around half of the embryos were able to expand: 52%-68% embryos after benzene exposure and 40%- 56% after Limonene exposure while in the control group 92,3% developed.

Good morphology was also higher in the controls compared to all the experimental groups. Blastocysts exposed to "Bl" had a statistically worst quality of their morphology. There were no aneuploid embryos in any of the cases of excellent morphology (ICM=A and TE=A), which has been seen in other studies (Irani et al., 2017). Despite these outcomes, it was observed only one blastocyst with excellent morphology in the control group, while more excellent quality embryos were obtained in the polluted groups (See annex 10.3).

In vivo development is done in an environment with a variety of detoxification mechanisms, as well as excretory routes. This is a deficiency of the IVF facilities where each embryo culture becomes an experiment of embryo toxicology. Any pollutant exposure happens at the same time that the embryo is initiating the development, differentiation through gene activation and transcription (Gilligan, 2010). Benzene, which is an aromatic hydrocarbon, is known to be a very hazardous compound, which has been related to the production of reactive oxygen species (ROS) and in consequence, genotoxicity and even carcinogenesis. Inside the IVF laboratory, the early embryo has to be protected against the generated damage that happens through *in vitro* manipulations, because it also generates ROS as a byproduct of the embryo´s own metabolism. So the presence of Benzene, Limonene or other aggressive chemicals in the environment will increase the probability of deleterious consequences

for the embryos developing *in vitro*, surpassing any intrinsic or environmental defense mechanisms such as an enriched culture medium.

Benzene produces cellular oxidative stress, which its known to alter the integrity of proteins, lipids and DNA, because ROS can pass through cell membranes and alter cellular molecules that can also produce other oxidation products. All of these can alter sensitive signaling pathways and produce more toxicity as well (Guerin et al., 2001; Menezo et al., 2010; Tsutsui et al. 1997b, Winn 2003). Benzene is a lipophilic, hydrophobic and non-polar compound and it can pass the cellular membranes, because the center of the lipid bilayer (the fatty acid tails) is non-polar. Also, when the surface area of the membrane increases so does the fluidity, so it can also be in the intracellular space. Benzene can be metabolized in a body through the liver, by oxidation (catalyzed by the hepatocyte´s CYP2E1) and then through enzymatic and non-enzymatic routes of bio-activation, producing highly toxic metabolites and causing DNA damage (Barreto et al., 2009). However, it seems that the mechanisms through which Benzene metabolizes inside the human embryo have not been elucidated. For example, the biotransformation and bio-activation of benzene is necessary in terms of embryotoxic and dysmorphogenic effects, especially for its metabolites (Chapman et al. 1994) but it has been demonstrated that the CYP2E1 are poorly expressed in fetal liver; its expression rapidly increases within hours after birth (Vieira et al., 1996). There could be another CYP or other type of enzymes with higher activity in early stages of development (Stoilov et al., 2001) because embryos are capable of bioactivating xenobiotics leading to the production of reactive metabolites and ROS. Through the evaluation of the epigenetic regulation of CYP genes (Cytochrome-P450 enzymes), Park *et al.*, confirmed that there are some genes that have major transcript levels in human pluripotent stem cell-derived hepatocytes (hESC-Hep) and others in human primary hepatocytes. However, so far the hESC-Hep cannot fully replicate a drug metabolism (Park et al. 2015). Benzene itself does not seem to affect as much as its metabolites. Phenol, catechol and hydroquinone can be further metabolized into reactive intermediates such as benzoquinone that is considered the most harmful metabolite (Winn, 2003) . All the metabolites enhance ROS generation and can roduce lipid peroxidation as well. Another hypothesis is that something during the culture period could produce reactions over Benzene and then without needing a bio-activating system, its metabolites could produce the embryotoxic effects. During the biopsy procedure, specifically during the aspiration of TE cells, it was observed that embryos easily released many fragments. Fragmentation could be related to the ability of the embryos to produce apoptosis as a mechanism of defense against oxidative stress.

On the other hand, Limonene, which is a very common IVF laboratory´s compound, has been tested in liposome model systems and it has been demonstrated that it

accumulates in the membrane, which causes a loss of the membrane integrity and dissipation of the proton motive force as well as other terpenes (Sikkema et al., 1995). Limonene can metabolize through different pathways as well (Ekelman and Benz, 2015). Some of the most common metabolites are the perillic acid and diydroperillic acid.

However, no reports of Limonene´s embryo metabolism were found for this discussion. The only reports associated to deleterious effects in reproduction and development are in animal models, where skeletal anomalies, delayed ossification and growth retardation in the offspring was seen after exposing to high doses of Limonene (Kim et al. 2013b). Limonene is not considered as harmful as Benzene because is a non-genotoxic compound and is one of the less polar monoterpenes; however, even though is less harmful compared to other terpenes, it increases the cellular membrane permeability and produces some cytotoxicity in fibroblast cells (Mendanha et al., 2013) as well as some morphological transformations and a minimal affection of the GJIC in Syrian hamster embryo cells (Rivedal et al., 2000). More studies determining possible ways of metabolism and effects should be perform and it is necessary to investigate if the human embryonic cells or their membrane and organelles could be specific targets for this compound. In this study, we confirmed that not only Limonene is one of the most common VOCs in the environment of the IVF laboratory but also it is a associated with low rates of development of cultured human embryos, even under values obtained for Benzene.

For the proper culture of human cleavage embryos up to blastocyst stage the culture medium needs to be renewed every 48 hours, to ensure minimal accumulation of embryotoxic substances, such ammonium. However, the embryos of this study were exposed to the polluted medium for a longer time than recommended before the morphology assessment (72 hours), which is another limitation of this study.

7.3 Chromosomic content of embryos exposed to VOCs

The PGS results obtained through the arrays technology had an informativity rate of 97,7%, which is considered as a successful amplification (Mir et al., 2013). NGS is a comparative technique for detecting not only aneuploidies but also for analyzing samples with mosaic patterns; it can properly detect whole-chromosome and segmental aneuploidies as well (Vera-Rodriguez et al., 2016).

Morphological evaluation of embryos is a very important task to perform, because it allows the follow up after the fertilization and through the development of embryos *in vitro;* however, it is a fact that the pre-implantation embryos showing normal morphology are not entirely correlated with euploidy (Irani et al., 2017). A high rate of human embryos produced *in vitro* present chromosomal aneuploidies due to age-

dependent chromosome segregations during the meiosis, especially in cases with patients of advanced maternal age (Munne et al., 1995), with recurrent pregnancy loss (Rubio et al., 2009) or patients that carry chromosomal aberrations for other reasons.

The chromosomal results of the embryos evaluated in this study confirm that the culture environment is one of these reasons that has influenced the chromosomic content of the exposed embryos, and that the obtained aneuploidies sometimes seem to be related to mitotic errors due to the low rate of development up to blastocyst stage. As expected, not all the aneuploid embryos had bad morphology. The analysis performed on the three groups revealed different effects over the chromosome content of the embryos. As expected, the control group had a low percentage of aneuploid blastocysts (23.3%) as well as the Limonene group (26.8%) in general, but despite that there were not significant differences, Benzene showed a higher rate of damage reaching 40% of aneuploidies. The differences between exposition groups were not significantly different either, however, the "Ll" group reached an aneuploidy percentage similar to that obtained in each of the Benzene groups.

The presence of Benzene increased the probability in blastocysts to have more aneuploidies compared to control and the presence of Limonene in the culture medium. This was seen in the number of chromosomal errors, which in Benzene were up to 10 errors per embryo, and also in the type of chromosome affected, which was also related to an increased frequency of detection of specific chromosomes. Taking the B-EG as a whole, there were up to 16 chromosomes types and some were detected up to three times (per exposition group). C2, C13, C16, C22 and XY were the most common chromosomes in type and frequency (half of them sub or metacentric and the other half acrocentric ones). At the same time and as described previously, because Limonene is not a mutagenic compound, less aneuploidies were detected but they were still more than the control group. C2, C15, C16 and C21 (of them sub or metacentric and the other half acrocentric ones were found up two times in Limonene group as a whole. No error results were found in the chromosome 17 in either of the groups. Monosomies and trisomies were also found in all the groups and specific doses. Benzene and Limonene showed a dose-related increase of chromosomes with trisomies. It is possible that monosomies were present in arrested embryos at early stages, so no chromosomal analysis would have been performed but, while Limonene´s effect was produced by the "Ldl"-dose, the three doses of Benzene targeted different chromosomes errors; mostly whole chromosome abnormalities. Finally, the mitotic-related chromosomes rates were not significantly different among groups but there were more mitotic-related chromosomes (C1- C14) affected in the groups exposed to both VOCs, especially to Benzene.

Transition to blastocyst stage should be associated with a decrease in the aneuploidy

rate and therefore a good rate of viable embryos. The arrested embryos rate should be high as well while aneuploid embryos are not able to develop. After the activation of the embryonic genome at the cleavage stage, the cell-cycle regulatory mechanisms become active, reducing the risk of further errors occurring and beginning a process of clearing abnormal cells, especially those affected by multiple aneuploidies (Fragouli et al., 2013). However, this study hypothesized that the presence of either Benzene or Limonene can alter not only the development rates but also increase the aneuploidy errors in cleavage embryos developing to blastocyst.

The high toxicity produced by Benzene and its metabolites has been linked to many chromosomal aberrations such as gains and losses of chromosomes, polyploidy, structural changes, translocations, deletions, DNA mutilation, etc., all related to altered cell differentiation, because these compounds target genes and very important pathways, specially through chronic exposure (Badham and Winn, 2010; McHale et al., 2012; Zhang et al., 2002). According to McCoy *et al*, mitosis-errors rates increase in patients with genetic issues and they could be influenced by environmental factors as well. Mitotic errors are very common during the initial post-zygotic cell divisions and produce mosaic embryos containing multiple types of karyotypes and they seem to be more related to larger chromosomes and whole chromosome abnormalities as well. These errors can vary widely from the initial embryonic divisions up to the blastocyst stage when these should have been reestablished (McCoy et al., 2015). This is one of the reasons why around 50% of the embryos cohort is able develop up to blastocyst stage. Normal post-zygotic mitotic errors possible arise from different mechanisms such as anaphase lag, mitotic non-disjunction and endoreplication. However, at the same time, *in vitro* procedures can produce mitotic aneuploidies as well. External factors such as the ART techniques (ovarian stimulation, ICSI, freeze and thaw procedure, etc.) or the environment (oxygen concentration, visible light, temperature fluctuation, culture media, and anything considered pollution) can influence different factors of cells divisions. All these factors are closely related to the presence of oxidative stress as mentioned in the last section. ROS can not only accelerate apoptosis in the cell by direct DNA damage, but also affect the DNA repair mechanisms along with alterations in important checkpoints in cell cycle (Gupta et al., 2006; Mantikou et al., 2012). Lipid peroxidation initiated by ROS may also react with DNA to cause oxidative DNA damage (Shen et al., 1996).

7.4 Mitochondrial DNA content of embryos exposed to VOCs

Because mitochondria can be target organelles of pollutants, this study evaluated how the presence of VOCs inside the culture medium could affect the content of

mitochondrial DNA (mtDNA) in the embryos. This analysis is based on the belief that a high amount of mtDNA in the oocytes can be used as a biomarker correlated with proper fertilization and future embryo viability.

The mitochondrial DNA (mtDNA) does not have additional replications between the fertilization and the early post-implantation stages (Hashimoto et al., 2017). As a result, the total amount of mtDNA must be split between cells during the embryo divisions. Thus, by D+6 of development, each embryonic cell should contain very few copies of mtDNA, because the embryo´s energy comes from the energy accumulation in the oocyte. However, the mitochondrial proliferation is considered a pathogenic consequence of mitochondrial distress. In viable embryos, the total amount of mtDNA equally split during division and then moderate energy consumption will be needed to accommodate in the environment. In embryos experiencing energetic stress, mtDNA biogenesis increases during early development compensating the mtDNA reduction caused by cell division. Thus, increased mtDNA in euploid embryos is an indication of reduced amount of energetic reserve during oocyte maturation that is reflected in reduced ability to implant (Diez-Juan et al., 2015).

Also, following the hypothesis that the VOCs studied altered the membrane permeability and that could have induced oxidative stress in the embryos, leading to growth impairment, we could also hypothesize that this may have lead to mitochondrial alterations in some of the embryos, as an indirect target of lipid peroxidation as well. Oxidative stress can induce mitochondrial damage because mitochondrial DNA is especially susceptible to mutation because its lack of histones (Meyer et al., 2013). During oxidative stress mtDNA mutations can also be more frequent than nuclear DNA mutations and there could be a dysfunction on the embryos metabolism that will disturb their development in consequence (arrest). It could be also related to apoptosis. At the same time that there is an inability of the mtDNA to auto-repair, it will lead to a decline in the mitochondrial function (Guerin et al., 2001). Euploid embryos under suboptimal intrinsic or environmental factors could elicit higher levels of mtDNA (Victor et al. 2017).

However, this study did not find a clear relationship between the mtDNA values obtained. The comparison between the whole control, Benzene and Limonene groups did not have any significance between mtDNA levels, neither between each specific treatment (exterior, laboratory and double laboratory doses) and the control group. The distribution of the medians of the scores of the mtDNA was similar between the three groups.

MtDNA values should only be considered after taking into account the aneuploidy screening result and embryo morphology. This study did not find a correlation between the presence of any type of VOC and "abnormal" mtDNA levels either.

Anyhow, a wide spectrum of mtDNA levels was found (from 12,5448 to 66,0315).

Specific morphologic parameters of the gametes, zygotes, and embryos should be assessed systematically when evaluating air quality due to evidence that pollutants can affect development at the earliest stages (the four cell stage and compaction up to the differentiation of the ICM and TE), which have fundamental roles in embryo survival, implantation, and fetal viability. However, since embryos often develop even in the presence of contaminants, other molecular parameters such as chromosomal abnormalities or epigenetic modifications should be considered, because the epigenetic bio-monitoring is necessary and it needs an international methodological accordance (Pacchierotti and Spano, 2015). It would be remarkable to ascertain the role of embryonic self-defense and repair mechanisms against pollutants, such as the mechanisms of fragmentation and implantation or pregnancy failure, as well as long-term effects on children conceived through IVF techniques.

Finally, with the results obtained with this study there has been demonstrated that very low concentrations of both compounds are harmful for the human embryo health in many parameters as the development and the karyotype. With these outcomes it is suggested that IVI clinics use as an *in vitro* environment limit the lowest values used for this research until further studies in both compounds can be performed. Therefore, it is also recommended to study values below $256x10^{-5}$ppm and 87.5×10^{-5}ppm for both Benzene and Limonene, respectively, to start establishing an official "*in vitro* environment limit values" database.

Chapter 8

8 CONCLUSIONS AND FUTURE WORKS

8.1 CONCLUSIONS

- Despite all the preventive and control measures applied in the clinics, many VOCs are present in the ambient air of the IVI clinics, but mostly, inside the IVF laboratory, sometimes at doses above 1% of the official OLVs.
- The presence of specific VOCs, such as Limonene, seems to be constant through the years inside the IVF laboratory and the presence of certain VOCs, such as Benzene, can be due to seasonal incidences.
- Benzene IVF-related concentrations do affect the progression of development and expansion from cleavage and beyond the expanded blastocyst state.
- Limonene IVF-related concentrations also affect the progression of development and expansion from cleavage and beyond the expanded blastocyst state.
- The probability of obtaining aneuploid embryos is higher when they are exposed to Benzene compared to control and Limonene-exposed embryos.
- Benzene and Limonene do not seem to be involved in the scores obtained of the mtDNA.
- The results presented could be used as basis to set reference values to establish an *in vitro* environment limit values for human embryos.

8.2 Future works

This study is part of the first phase of a research about the potential effects of different types of VOCs over the human embryo development in vitro. The other compounds that will be studied in the future are: Styrene, Tetrachloroethylene and Hexachloro- 1,3 – butadiene.

Because it is unknown how VOCs may affect the epigenetic profile or which mechanisms could be implicated on the embryos, that apparently had overcome the chemical aggression, further studies should be perform. In the near future, it will be analyzed the possible mutations of the remaining stored samples of embryos exposed to Benzene and Limonene.

"and let all your mistakes

become your greatest gifts

in disguise"

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"and no one said it was gonna be easy, and I´m not afraid to try

and with the odds stacked up against me, I will have to fight

one life, one chance, gotta do it right"

Annexes

10 ANNEXES

10.1 RESEARCH INFORMATION DOCUMENT FOR PATIENTS

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HOJA DE INFORMACIÓN AL PACIENTE

TÍTULO DEL ESTUDIO: Evaluación de contaminantes ambientales en los laboratorios de Embriología Clínica y su efecto sobre la salud del pre-embrión humano

CÓDIGO DEL PROMOTOR:

PROMOTOR:

INVESTIGADOR PRINCIPAL *Mª José de los Santos Molina*

CENTRO: IVI Valencia Tel: 963050991

INTRODUCCIÓN

Nos dirigimos a usted para informarle sobre un estudio de investigación en el que se le invita a participar. El estudio ha sido aprobado por el Comité Ético de Investigación Clínica de IVI Valencia y la Comisión Nacional de Reproducción Asistida.

Nuestra intención es tan solo que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación. Además, puede consultar con las personas que considere oportuno.

PARTICIPACIÓN VOLUNTARIA

Debe saber que su participación en este estudio es voluntaria y que puede decidir no participar o cambiar su decisión y retirar el consentimiento en cualquier momento, sin

que por ello se altere la relación con su médico ni se produzca perjuicio alguno en su tratamiento.

Su participación no implica visitas ni analíticas ni pruebas adicionales

DESCRIPCIÓN GENERAL DEL ESTUDIO:

La presencia de contaminantes ambientales es cada vez más frecuente en nuestros días y desconocemos muchos de los efectos nocivos que su presencia puede crear sobre los preembriones humanos durante su cultivo in vitro. El objetivo del estudio, es recopilar información sobre la presencia de ciertos contaminantes ambientales en los laboratorios de Fecundación In Vitro diversas clínicas IVI, ver el impacto que la presencia de sobre la salud embrionaria.

Para ello solicitamos el permiso para la utilización de los preembriones que tiene usted criopreservados en la clínica IVI Valencia y que previamente decidió darles fines de investigación con el objetivo de realizar los estudios de toxicidad sobre ellos.

BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO

Este estudio permitiría encontrar en un futuro en las condiciones de cultivo más óptimas para los embriones humanos y mejorar así las tasas de éxito.

CONFIDENCIALIDAD Y TRATAMIENTO DE LOS DATOS

El tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los sujetos participantes se ajustará a lo dispuesto en la Ley Orgánica 15/1999, de 13 de diciembre de protección de datos de carácter personal. De acuerdo a lo que establece la legislación mencionada, usted puede ejercer los derechos de acceso, modificación, oposición y cancelación de datos, para lo cual deberá dirigirse a su médico del estudio.

Los datos recogidos para el estudio estarán identificados mediante un código y solo su médico del estudio/colaboradores podrán relacionar dichos datos con usted y con

su historia clínica. Por lo tanto, su identidad no será revelada a persona alguna salvo excepciones en caso de urgencia médica o requerimiento legal.

Sólo se transmitirán a terceros y a otros países los datos recogidos para el estudio que en ningún caso contendrán información que le pueda identificar directamente, como nombre y apellidos, iniciales, dirección, nº de la seguridad social, etc. En el caso de que se produzca esta cesión, será para los mismos fines del estudio descrito y garantizando la confidencialidad como mínimo con el nivel de protección de la legislación vigente en nuestro país.

El acceso a su información personal quedará restringido al médico del estudio/colaboradores, autoridades sanitarias (Agencia Española del Medicamento y Productos Sanitarios), al Comité Ético de Investigación Clínica y personal autorizado por el promotor, cuando lo precisen para comprobar los datos y procedimientos del estudio, pero siempre manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente.

COMPENSACIÓN ECONÓMICA

El promotor del estudio es el responsable de gestionar la financiación del mismo. Para la realización del estudio el promotor del mismo ha firmado un contrato con el centro donde se va a realizar y con el médico del estudio.

Su participación en el estudio no le supondrá ningún gasto y le serán reintegrados los gastos extraordinarios (p. ejem. comidas y traslados)

INFORMACION RELEVANTE

Al firmar el contrato sobre donación de embriones adjunto, se compromete a cumplir con los procedimientos de estudio que se le han expuesto.

10.2 INFORMED CONSENT FOR PATIENTS

CONSENTIMIENTO INFORMADO

CÓDIGO/NOMBRE DEL ESTUDIO:

Yo (nombre y apellidos) ……..

Yo (nombre y apellidos) cónyugue

……..

N° Historia: .....

Hemos leído la hoja de información que se me ha entregado.

Hemos podido hacer preguntas sobre el estudio.

Hemos recibido suficiente información sobre el estudio.

Hemos hablado con: Mª José de los Santos

Comprendemos que nuestra participación es voluntaria.

Comprendemos que podemos retirarnos del estudio:

1° Cuando queramos

2° Sin tener que dar explicaciones.

3° Sin que esto repercuta en nuestros cuidados médicos.

- Prestamos libremente nuestra conformidad para participar en el estudio y damos nuestro

consentimiento para el acceso y utilización de nuestros datos en las condiciones detalladas en la hoja de información.

- Accedemos a que las muestras de sangre o tejidos obtenidas para el estudio puedan ser

utilizadas en el futuro para nuevos análisis relacionados con la enfermedad o fármacos

del estudio no previstos en el protocolo actual (quedando excluidos los análisis

genéticos, siempre y cuando no formen parte de los objetivos del estudio):

SI NO

Firma de la paciente: Firma del paciente

Nombre: Nombre:

Fecha: Fecha:

Firma del médico/investigador:

Nombre:

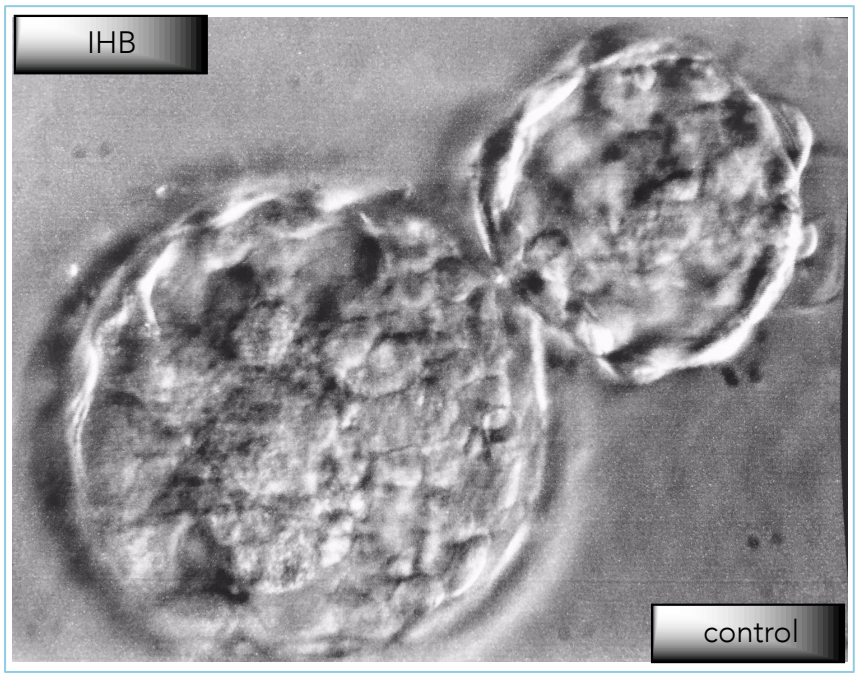
Fecha:

Este documento se firmará por duplicado quedándose una copia el investigador y otra el paciente

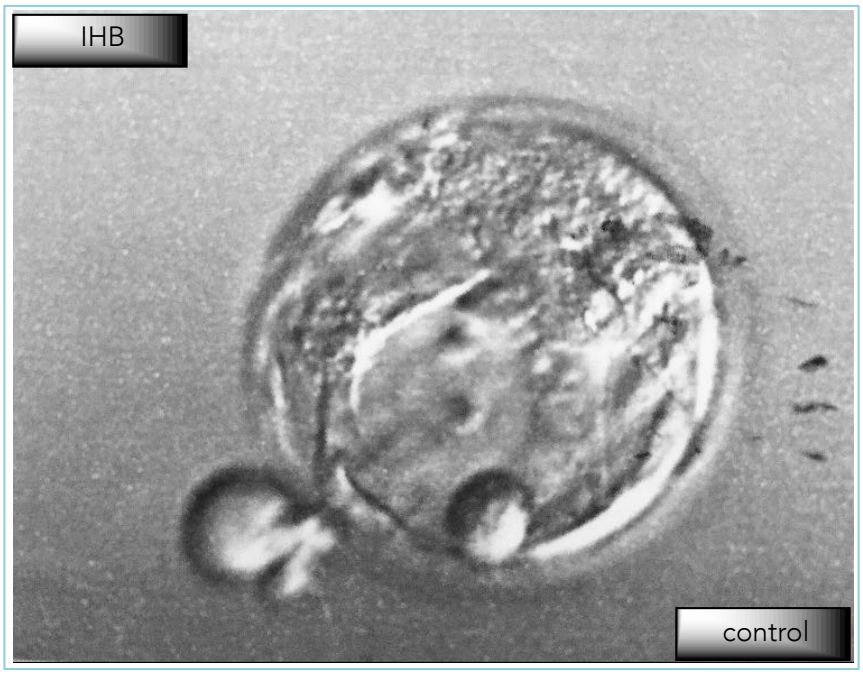
Versión: Fecha:

10.3 MORPHOLOGY

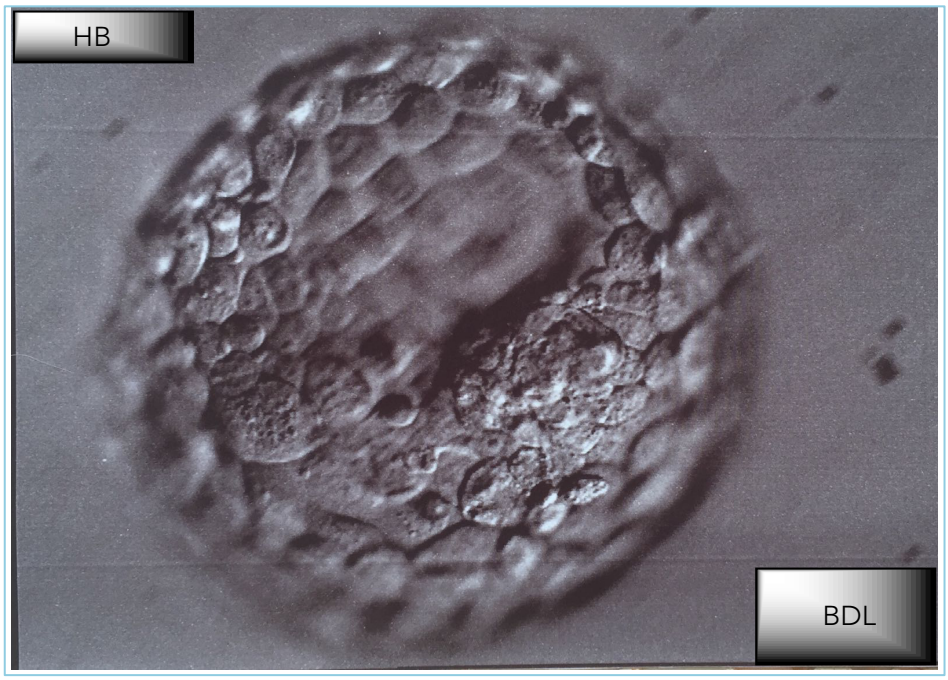
- CONTROL IHB (A-A), EUPLOID – MS 14.7724



- CONTROL IHB (C-D), ANEUPLOID – MS 23.3483



- BENZENE double laboratory HB (A-A), EUPLOID - MS20.2727



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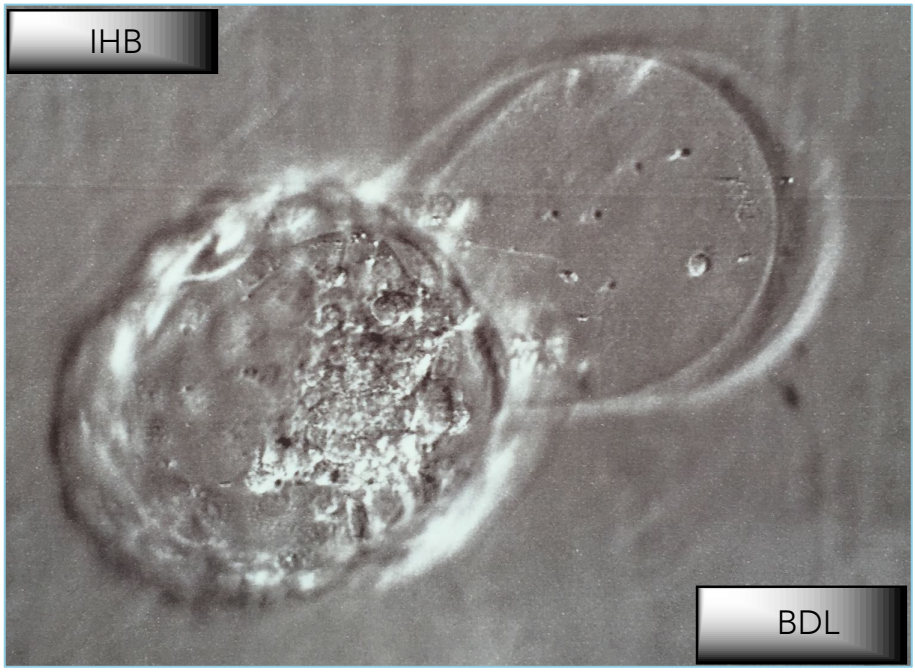
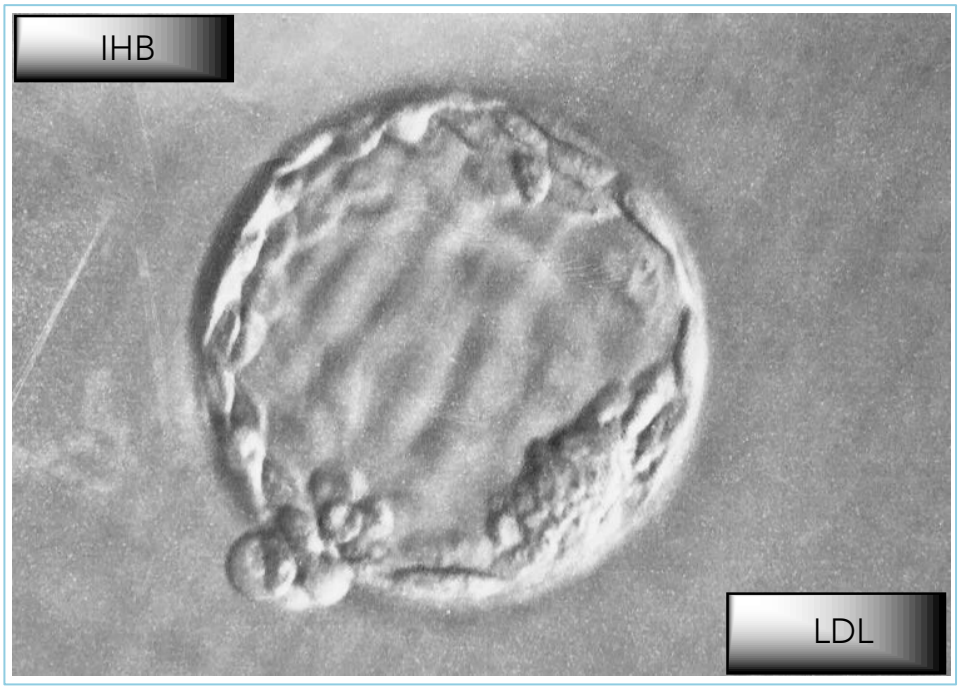
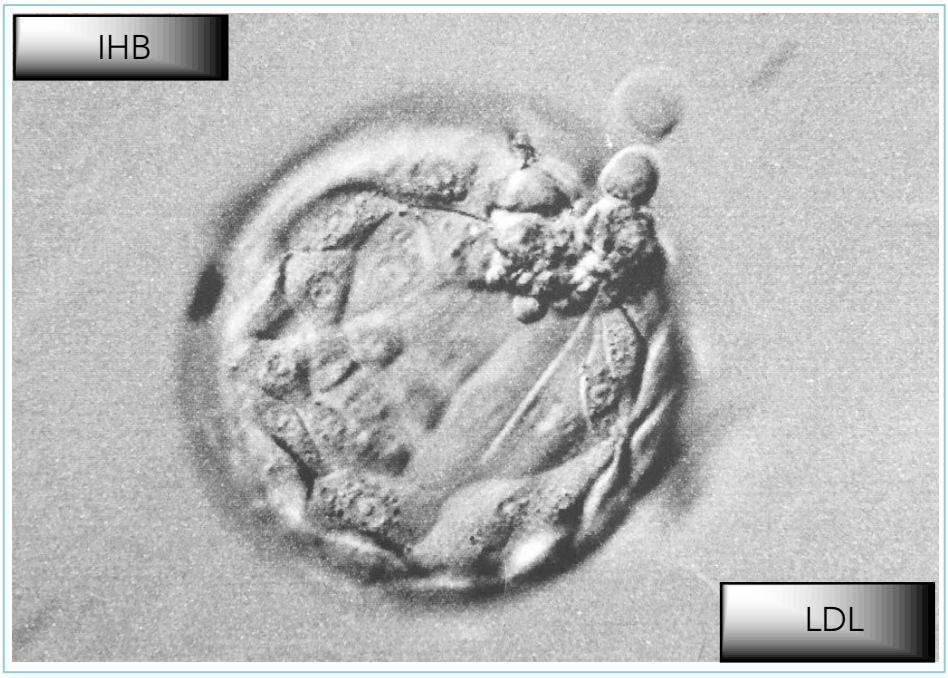


- LIMONENE double laboratory IHB (A-A), EUPLOID – MS 18.5224



- LIMONENE double laboratory IHB (C-B), ANEUPLOID – MS 38.3148



“When life gives you lemonade, make lemons
Life will be all like: “What?”

Thank you

No seré formal, no lo deseo; prefiero contribuir con otro tipo de verdad a éste documento. Quiero dar las gracias como vale la pena hacerlo.

Mi MariaJo, podré llamarte así con cariño, pero ante todo, te tengo un enorme respeto. Nos encontramos en un pasillo y con una maravillosa actitud, como siempre, decidiste creer en ésta desconocida que tenía todas las ganas del mundo de investigar y aprender. Desde ese momento has confiado en mí y así mismo, cuando no he sabido continuar y te he buscado, has salido a mi rescate con una gran sonrisa y mucha sabiduría. Muchas gracias por permitirme ser parte de todo esto… sigo con muchas ganas de seguir aprendiendo de tí y de todos los que te rodean.

A Marcos, porque sin tu guía ni esa entrevista informal y tan positiva jamás hubiera conocido a MariaJo ni a mi bonito proyecto. Así mismo, gracias por animarme a realizar este trabajo a pesar de tener todos los "odds" en mi contra.

Quiero agradecer a todo el equipo del laboratorio de FIV de IVI Valencia, porque sin su desinteresada colaboración, cada vez que aparecía por allí, nunca hubiera podido hacer mis tardes y hasta mis noches tan efectivas.

Muchas gracias al super equipo "PiGiDi": a Amparo ☺, a Lauri, a Arancha y a mi Pili, porque sin vuestro respaldo, conocimiento, sonrisas y abrazos no hubieran sido tan fáciles mis sesiones de trabajo. Sois maravillosas! A Pili, tienes un enorme corazón. Muchas gracias por toda tu ayuda y asesoría con tanto cariño. Muchas gracias sobretodo a mi Diani, mi divertida compañera hace unos años y ahora mi loca maestra de biopsias, porque podamos seguir creando ciencia, pero en ingles ^·^

A mi Glor, mi gran amiga de un muy pequeño laboratorio. Gracias por recibirme con los brazos abiertos, por enseñarme con tanta nobleza y por quedarte hasta tarde para poder planear como conquistar nuestro mundo.

A Ro, mi gran amiga de otro pequeño laboratorio, compañera de un inolvidable viaje, de magdalenas en taza, de risas y locuras. Para mí eres la más estupéndida!

A Julie, mi científica loca. Sin un cerebro tan brillante y tan demente como el tuyo el mundo se caería a pedazos.

A todos mis amigos de la tierrita, por ser capaces de ver de lo que soy capaz aún cuando yo misma no lo he visto. Sin ustedes no estaría viviendo éste gran momento. Muchas gracias por darme fortaleza desde lejos y a través de tantos cambios, de cada nuevo reto y por siempre estar presentes.

A todos mis amigos de la terreta por todo lo que han hecho por mí, por todo lo que hemos vivido y todo lo que me siguen enseñado. Gracias especialmente a Cris, Oscar, Manu, Alberto, Ilse, Leslie, Mariale, Nayeli, Rosa, Elsa, etc. A Marta, porque eres demasiado importante para mí! y amo tener un pedacito de ti aquí.

A mi mundo paralelo cerca del océano… gracias por permitirme, aprender y enseñar mientras admiraba a los seres más maravillosos del mundo. Gracias también por todo el apoyo para yo poder sacar adelante éste proyecto. Una parte de mi corazón se quedará siempre nadando con vosotros.

A Danilo, mi papá. Porque no buscas que el trabajo te apasione, tú le apasionas y así te diviertes y ese ejemplo me ha servido a comprender y llevar mejor todos mis múltiples trabajos. Gracias por todo tu apoyo!

A Nubia, mi mama. Porque a pesar de todo por lo que hemos pasado siempre estás ahí para preocuparte y recordarme que debo ser positiva. Por enseñarme a no dejar de emocionarme como una niña pequeña cuando sucede hasta la cosa más simple.

A mis bebés: Liv, porque mis problemas desaparecen a tu lado y porque me diste una nueva oportunidad de proteger, aprender y enseñar a través de la paciencia, el conocimiento y la inocencia. A Riot, porque me haces reír con cada travesura y por permitirme darte amor cuando lo necesito. A Rogue, porque eres el equilibrio, el detalle y la dulzura. Gracias por sentarte a mi lado durante tantas noches de escritura.

A tí, porque la venganza es dulce ^^… y te amo · Gracias por llenar a mi corazón de vida y por llenar mi mente de orden, o por lo menos intentarlo. Gracias por tu ternura (hasta cuando no sabía recibirla). Gracias por ayudarme pero sobretodo por creer en mí. Amo nuestra pequeñita familia.

Y finalmente, al gran amor de mi vida… a Nalú. Mi gran inspiración de nobleza y fortaleza, de inteligencia y coraje, de amor y lealtad. Tu corazón siempre laterá en mí.