

# Blastocyst culture: Co-culture needs to be restored. Available culture media are still far from being optimal

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## Abstract

Since the introduction of human IVF a number of efforts have been made to optimize embryogenesis in-vitro. The implementation of co-culture was successful in supporting embryo development to blastocyst stage. The environment provided by feeder cell lines was shown to increase cell numbers, the embryo constitution and implantation rates. The improved embryo development with co-culture can be attributed to several mechanisms involved in the secretion of cytokines, growth factors and antioxidants by the feeder cells. However, concerns about the use of non-human cell lines in co-culture put a sudden halt to this practice. The industrially prepared culture media has gained popularity in the use of IVF. It has been reported that the insufficient supplementation of the culture media has a direct relationship with oxidative stress causing errors in methylation and imprinting. The capacity of feeder cell line to mimic the in-vivo environment makes it more effective for the development of the human embryo and it's a concept that should not be abandoned. Hence, more studies are required to optimize the quality of the media used for IVF.

## Introduction

Human birth as a result of the implementation of in-vitro fertilization (IVF) with embryo transfer has revolutionized the concept of assisted reproductive technology (ART). In the beginning, one of the main limitations in IVF was the incapability of the simple culture media to support the embryo growth to more than a 2 to 8 cell stage forcing its early transfer into the uterus. In normal physiological conditions, embryos at this stage are not found in the uterine cavity rather, they are in a different environment in the fallopian tubes [1]. Therefore, the incapability of reproducing the in-vivo conditions for the proper development did not yield high success rates in IVF. For over five decades, a number of efforts have been made to optimize in-vitro culture ability to support the embryologic development and to achieve increased pregnancy rates.

In 1965 Cole and Paul conducted a study that concluded with an increased blastulation rate in mouse embryo cultured with an immortalized feeder cell line [2]. The outcome of this study presented an opportunity for more early research with animal embryos. The evident success of various studies suggested that the cross-contact with the feeder cells was responsible for the enhancement of in-vitro embryologic development. Therefore, the use of co-culture with feeder cell lines was implemented in human IVF during 1989 as an attempt to optimize culture conditions for embryonic growth. Randomized clinical trials in co-culture technology in human IVF noted the improvement in the quality of the cultured embryos and an increase in clinical pregnancy as compared to the conventional media used at that time. In the early 90's the practice gained popularity using human and non-human origin feeder cells. Some examples of non-human cells utilized for co-culture are: bovine uterine fibroblast, bovine oviductal epithelial cells, buffalo rat liver cells [3] and Vero cells from African green monkey (kidney epithelial cells) [4]. It was noted that these cells were capable of supplying the essential requirements for blastocyst growth,

development, and implantation. In turn, this efficient environment promoted an improved pregnancy rate that was documented in further studies.

During the year 2002 the U.S Food and Drug Administration (FDA), formally transmitted some apprehension regarding disease transmission from non-human feeder cells to human embryos. They classified this practice as xenotransplantation with a possibility of transmission of known and unknown infective agents. After this concern was addressed, they discouraged the use of nonhuman cell line as feeder cell for IVF. In addition to the FDA recommendation, the complex sequential media introduced in 1990 started gaining popularity because it showed an improved blastulation rate compared to the standard media [5]. The sum of the recommendations made by the FDA, along with the increased use of the complex sequential media put an abrupt stop in the use of co-culture technology. The objective of this paper is to evaluate the need of scientific studies comparing the outcomes of embryo development using co-culture versus the use of standard media in order to understand how we can improve the culture techniques used in IVF.

## Human embryo development and the proposed mechanism in co-culture

Maternal protein and mRNA that are stored in the oocyte during maturation initially drive the development of human embryo. These reserves provide the environment for early metabolic mechanism such as, cell division, energy metabolism and protection against different insults [6]. Although the maternal oocyte reserves are important for

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the quality of the embryo and implantation capacity, they are finite and decreasing rapidly with maternal age. The environment provided during and after this early phase is also critical for pregnancy continuation. In the later phases of embryonic preimplantation development, in-vivo environment offers the factors that are necessary for DNA repair, energy metabolism, protein synthesis, removal of reactive oxygen species (ROS), protection against metal ions and more. But, post genomic activation (MZT; maternal to zygotic transition), the embryo acquires gradually its autonomy: In the in-vivo environment, the embryo has the proper regulation of these different mechanisms, which often results in normal development and pregnancy. The objective of IVF is to mimic the physiological environment to reach normal development of blastocyst and then allows ongoing clinical pregnancies.

Several studies concluded that feeder cells used in IVF are able to provide the paracrine stimulations that the embryo needs to get to the blastocyst stage. This is particularly obvious as demonstrated by Schmalz-Panneau *et al.* [7,8]. The presence of the embryos on a feeder layer can modify the mRNA expression of these cells; this indicates exchanges of information at the molecular level. The embryo emits signals that are recognized and captured allowing a modulation of the "milieu". The feeder cells are able to secrete a variety of embryotrophic factors that are needed to nurture the human embryo. Some factors like Leukocyte Inhibitory Factor (LIF), Insulin Growth Factor (IGF), Interleukin-1 and 6 (IL1, IL6) had been shown to promote the development of the embryo and increase the implantation [1]. Furthermore, Granulocyte-Macrophage Colony Stimulating factor (GM-CSF) and Transforming Growth Factor  $\alpha$  (TGF $\alpha$ ) promotes the blastocyst formation and the increase in the cell number [9] which in turn, increases the availability of blastomere cell numbers for biopsy and genotypic evaluation [10]. The synchrony of the interaction using co-culture techniques gives a better opportunity for transfers of embryos and it favors the blastocyst constitution.

Another proposed mechanism of action in co-culture is the detoxification of the media by the removal of ROS and metabolic insults aiding the embryo to overcome development block [5,11]. This concept is very important because it has been observed that there is an increased production of ROS in embryos developed in-vitro and this can be partially responsible for the developmental arrest and cell apoptosis in the embryo. Helper cells used in co-culture produce protective sulfur-derived antioxidants such as Hypotaurine and Glutathione, free radical scavengers that are also found in the natural environment [12]. This is of particular importance if we consider that the classical IVF culture media generate spontaneously ROS during incubation [13]. Oxidative stress impairs epigenetic/DNA methylation regulation as a consequence; the imprinting process can be defective. Furthermore, co-culture allows blastocyst to be more resistant to the cryo-preservation methods by decreasing ROS induced damage, lipid peroxidation and decreasing fracture zones in the freezing process. The use of co-culture can increase the frozen embryo pregnancy rate by ten times as compared to early-stage embryo implantation [6].

### Comparison of the use of co-culture of human embryos with standard culture media in IVF

After 2002 the industrially prepared culture media were systematically used in ART laboratories for embryo culture. Vitoulikas *et al.* [14] conducted a study in order to analyze the concentration of growth factor released in co-culture using autologous cells and compare them to the concentration of standard culture media. The supernatants of 19 cases were assayed with the use of immunofluorescence

cytometry with a factor-specific magnetic bead. The investigations found statistically significant elevated levels of Vascular Endothelial Factor A and C (VEGF-A and VEGF-C) in cases that used co-culture. However, VEGF-A and VEGF-C were not detectable with standard culture media. The study reveals similar findings with other growth factors such as Fibroblast Growth Factor-2 and Insulin Growth Factor-1 (FGF-2 and IGF-1). The conclusions suggest standard culture media cannot meet the requirements of the embryos.

The understanding of several embryologic requirements is necessary for the proper supplementation in prepared medium with key growth factors that will result in an increase in the quality of blastocyst. More recently, molecular studies have been able to detect receptors on the surface of early embryos. The encounter of these reports indicates the importance of the cross-communication for embryo development [14].

Culture media are not optimal for the early embryo development. The inferior conditions and supplementations of the media along with the lack of regulation observed in a physiologic environment can contribute to developmental abnormalities. One example of this is the observed decrease in cell number in the embryos cultured in standard media in comparison to co-culture. This effect reduces the possibility of biopsy for genetic testing. This difference in cell numbers can be attributed to the better cross communication with the use of feeder cells and the protection conferred to the developing embryo.

Culture media for human embryo are currently designed using mouse embryo as a model (MEA: mouse embryo assay). Although human and mouse embryos appear to have some similarities, there is some questionable difference in the timing of DNA methylation and the rate of cellular cleavage [12]. Evidence indicates that some embryos grown on standard media can show reduced growth rates, disruption of genomic imprinting errors caused by altered DNA methylation leading to increased risk of genetic imprinting diseases [15]. Moreover, in the mouse embryo culture in all commercial media systems results in imprinted methylation losses [16]. In a study conducted by Hiura *et al.* [17] there is a noted increment of imprinting disorders related to the use of ART. The current IVF culture media do not allow the correct embryo epigenetic/DNA methylation maintenance during the time spent in vitro. The MEA is definitively not an accurate model to design Human IVF culture media.

In 2008 a meta-analysis study was conducted with 17 prospective randomized trials comparing the use of co-culture with the conventional media used in IVF. After eliminating confounding bias, such as maternal age, the study concluded that there is a statistically significant increase in implantation by 3%, an increase in clinical pregnancy of 8.1% and a difference in ongoing pregnancies by 8.7% in the use of co-culture versus conventional media [5]. Once again, the conclusion of the study indicates a superior quality in the embryos developed in co-culture media.

### Discussion

In summary, there is sufficient evidence that supports the benefit of using helper cells in co-culture to obtain a better quality of human embryos and an increase in clinical pregnancy rate. This optimized environment is related to the growth factors, cytokines, and antioxidants provided by the layer of feeder cells.

Co-culture with cumulus of donor endometrial cells is not a concept that should be abandoned and is a favorable alternative to the use of non-human cell lines and to the use of autologous endometrial cells. Even though the autologous endometrial cells are physically related to the

embryo [14] the exposure to the treatment of ovarian hyper stimulation affects the natural environment. This makes the use of donor epithelial endometrial in co-culture a better option. Epithelial endometrial cells can be obtained from biopsies and are cultured without difficulty. Co-culture with endometrial cells can resemble the natural environment and provide the metabolic requirements that are able to support the embryo growth, if we consider that tubal and endometrial cells have the same embryotrophic potential. Scientific reports record improved embryo morphologies and implantation rates when co-cultured with donor epithelial endometrial cells. [18,19]. More studies should be directed in the comparison of the complex media and the outcomes in co-culture with endometrial cells.

The complexity of the bi-directional communications between embryos and feeder cells for proper regulation of metabolic pathways is difficult to catch since the current technology is not able to detect the small messages. The protein content of a human preimplantation embryo can be estimated at 45-50 ng. Some of the important aspects of interactions between ROS and methylation/epigenetic errors have not been studied thoroughly [12]. The consequence the anomalies observed for these metabolic pathways in the development of the human embryo are not fully understood. Hence, further randomized control trials for the analysis of the interaction with co-culture are necessary to comprehend the complexity of the complete requirements for embryogenesis in-vitro.

## Conclusion

The knowledge of biochemical process in the normal physiological state involved in embryogenesis and pregnancy is rapidly growing and is making more obvious the insufficiency of the current media utilized for IVF to support and nurture human embryo. The recurrent problem is that in human, the in vivo model is inaccessible, contrarily to animal models. However, the animal models used to re-create the physiological environment have differences in the genetic regulation process. It is important to remember that the objective of IVF technology is to culture better quality embryos and increasing pregnancy rates. It should be taken into consideration that more efforts have to be directed to improve the quality of the media that is currently used to develop human embryos in order to decrease the metabolic/ physiological errors and better outcomes of clinical pregnancies.

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