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REVIEW

Extended embryo culture up to 14 days

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KEYWORDS
human embryo development;
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Abstract
Introduction: The early stages of human embryonic development prior to implantation have been extensively studied to improve reproductive outcomes. However, subsequent events are unknown. *In vitro* advances have allowed extended culture of embryos up to day 14, considered the legal limit. We aim to review aspects related culture conditions, morphogenesis events, potential applications of extended culture of human embryos as well as some ethical issues surrounding this topic.
Materials and methods: This literature review was carried out using different databases and websites by screening articles.
Results and discussion: Extended culture consists of culturing embryos up to day 14 of development. This requires specific culture conditions of temperature and gas concentration, as well as a medium composition that is different from that used in clinical practice in IVF laboratories. In addition to having made it possible to document *in vitro* the morphological events and structures of the embryo after implantation, the study of mosaic embryos at these hitherto hidden stages could provide valuable information for clinical practice. All this has been achieved within the 14-day limit, but due to scientific progress this can be considered restrictive and different reasons have been put forward why it should be extended.
Conclusions: Although prolonged culture has provided a better understanding of the events occurring between implantation and gastrulation, culture conditions must be improved to optimise the technique, and once this is achieved, consideration should be given to extending this limit. Furthermore, alternatives to the use of embryos, such as the development of embryos

Abbreviations: 2D, 2 dimensions; 3D, 3 dimensions; ART, Assisted Reproductive Technologies; ATP, adenosine triphosphate; BSA, Bovine Serum Albumin; CO_2, carbon dioxide; DMEM/F12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; DNA, Deoxyribonucleic acid; ESC, embryonic stem cells; GATA6, GATA Binding Protein 6; HFE, Human Fertilization and Embryology; HSA, Human Serum Albumin; ICM, Inner Cell Mass; ISSCR, International Society for Stem Cells Research; ITS-X, Insulin-Transferrin-Selenium-Ethanolamine; IVC1, *In vitro* Culture 1; IVC2, *In vitro* Culture 2; IVF, *In vitro* Fecundation; Nanog, Nanog homeobox protein; NCBI, National Center for Biotechnology Information; NGS, Next-Generation Sequencing; O_2, oxygen; OCT4, octamer-binding transcription factor 4; PKC, protein kinase C; SBF, Bovine Serum Fetal; Sox17, SRY-Box Transcription Factor 17; TSC, trophectoderm stem cells; XENC, extraembryonic endoderm cells

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from stem cells, should be further explored. All of this could have a future clinical application in terms of choosing the best embryos or finding out about diseases that affect pregnancy and lead to miscarriage.

PALABRAS CLAVE
desarrollo embrionario humano;
desarrollo embrionario *in vitro*;
pre-implantación;
cultivo embrionario *in vitro*;
regla de los 14 días

Cultivo embrionario extendido hasta 14 días

Resumen
Introducción: Las primeras etapas del desarrollo embrionario humano antes de la implantación han sido ampliamente estudiadas para mejorar los resultados reproductivos. Sin embargo, se desconocen los eventos posteriores. Los avances *in vitro* han permitido cultivar embriones hasta día 14, considerado el límite legal. Nuestro objetivo es hacer una revisión sobre las condiciones de cultivo, los eventos de morfogénesis, las posibles aplicaciones del cultivo extendido de embriones humanos, así como algunas cuestiones éticas que rodean este tema.
Materiales y métodos: Esta revisión bibliográfica se ha realizado empleando diferentes bases de datos y páginas web mediante el cribado de artículos.
Resultados y discusión: El cultivo extendido consiste en cultivar los embriones hasta día 14. Para ello se requieren unas condiciones de cultivo determinadas de temperatura y concentración de gases, así como una composición del medio que es diferente a la empleada en la práctica clínica de los laboratorios de FIV. Además de haber permitido documentar *in vitro* los eventos y estructuras morfológicas del embrión tras su implantación, el estudio de embriones mosaicos en estas etapas hasta ahora ocultas podría dar información valiosa para la práctica clínica. Todo esto se ha logrado dentro del límite de 14 días, pero debido al avance científico puede considerarse restrictivo, y se han expuesto diferentes motivos por los que debería ampliarse.
Conclusiones: Aunque el cultivo prolongado ha permitido comprender mejor los acontecimientos que ocurren entre la implantación y la gastrulación, se deben mejorar las condiciones de cultivo para optimizar la técnica, y una vez conseguido, plantear la ampliación de este límite. Además, se deben seguir estudiando alternativas al uso de embriones como el desarrollo de embriones con células madre. Todo esto podría tener una aplicación clínica futura a la hora de elegir los mejores embriones o conocer enfermedades que afecten al embarazo y acaben en aborto.

Introduction

Human early embryonic development from long before pre-implantation to gastrulation has always been a very active but difficult field of developmental biology.

It begins with a series of morphogenetic divisions and rearrangements leading to the formation of the blastocyst, constituted by the inner cell mass (ICM) and the trophectoderm, which floats freely between days 5 or 6 of development. Around the seventh day, this human embryo, now consisting of epiblast and primitive endoderm derived from the ICM and trophectoderm, must implant in the maternal uterus to survive (Zhai et al., 2021). Implantation of the blastocyst is a milestone in early mammalian embryonic development, as it is the moment at which a coordinated program of lineage diversification, cell fate specification and morphogenetic movements establishes the generation of extraembryonic tissues and the embryo proper, and determines the conditions for successful gestation and gastrulation (Deglincerti et al., 2016).

Errors in this process can lead to adverse pregnancy outcomes, including miscarriages and fetal defects, so understanding the mechanisms underlying this early development is of great importance not only for basic developmental biology but also for regenerative medicine (Zhai et al., 2021) and for resolving many cases of infertility and early pregnancy loss (Lusine, 2020).

The first days of pre-implantation development have been extensively studied using methods pioneered in *in vitro* fertilization (IVF) (Edwards et al., 1969; Edwards et al., 1970). Most of our knowledge about human embryogenesis from pre-implantation to gastrulation derives from studies of existing anatomical and histological collections of human embryos (Zhai et al., 2021) and the majority of observations and experiments have been carried out using other mammals as models. For example, monkey embryos (Enders et al., 1986) as well as *in vitro* implantation systems in mice (Bedzhov et al., 2014) have been used as models for human embryo development.

Recent advances in the *in vitro* embryogenesis, including the development of extended *in vitro* culture systems for mouse and primate embryos and the construction of stem cell-based embryo models, with the help of multi-omics

studies and single cell-based imaging techniques, open new doors to study human embryonic development and have significantly improved our understanding of the characteristics and mechanisms of human embryos from pre-implantation to gastrulation (Zhai et al., 2021). However, although embryology presents a similar general program in all mammals, it should be noted that the extrapolation of data to humans is limited, because mammalian embryos show species-specific differences in post-implantation morphology (Rossant, 2015).

Recently, in order to explain what happens in the early stages after human embryo implantation, the groups of Deglincerti et al. (2016) and Shahbazi et al. (2016) have adapted a protocol for *in vitro* culture of mouse embryos, allowing human embryos to develop up to the maximum number of days legally allowed.

In the present work we aim to review aspects related culture conditions, morphogenesis events, potential applications of long-term *in vitro* culture of human embryos as well as some ethical issues surrounding this topic.

Materials and methods

In the following study, an electronic bibliographic review of several papers and publications obtained from different databases, including Pubmed and the National Center for Biotechnology Information (NCBI), has been carried out. In addition, the academic search engine "Google Scholar" and different websites, such as ThermoFisher Scientific, have been used for some components of the culture medium. The research of information took place between the end of 2021 and the beginning of 2022, and the main materials used were papers in English or Spanish from the last five years. The keywords used for the research of information were: human embryo development, *in vitro* embryo development, pre-implantation, *in vitro* embryo culture, and 14-day rule.

Results and discussion

What is extended embryo culture up to 14 days and what are its conditions?

This extended culture consists of culturing fresh or thawed embryos that are at day 5 or 6 of development up to the maximum of 14 days allowed by law. But how has it been possible to culture human embryos *in vitro* up to this limit?

Before answering this question, it should be noted that, although long-term *in vitro* culture of mouse and primate embryos is promising, the current conditions need to be optimised in terms of reproducibility and efficiency. High-quality embryos and optimised cell culture environments (culture medium, supplements, and gas regulation mechanism) are essential for stable and efficient protocols for long-term mammalian embryo culture. Furthermore, in current long-term cultures, extra-embryonic tissues tend to grow vigorously with embryo development, hindering the development of the inner embryo by absorbing nutrients from the culture medium. In this sense, strategies to equilibrate extra-embryonic and embryonic growth may also be a good starting point to achieve long-term embryo culture, as human embryo development is highly dependent on the simultaneous growth of extra-embryonic tissues that provide essential signals to help extend embryo development to certain stages (Zhai et al., 2021).

Meanwhile, the group of Zernicka-Goetz et al. (2016) established a culture system that allowed human embryos to develop *in vitro* through post-implantation stages. This was based on a system for culturing mouse embryos through implantation (Bedzhov et al., 2014).

This procedure basically consists of thawing the embryos (if vitrified), removing the zona pellucida with tyrodes acid, and placing them in culture until day 14 (figure 1). Interestingly, as in the sequential approach used in IVF to culture embryos from day 3 to blastocyst culture to provide the embryos with a specific nutrient environment, long-term culture requires a gradual replacement of culture media containing decreasing amounts of serum. The first 48 hours of culture that is from day 5-6 to day 7-8, embryos are cultured using first culture medium (IVC1). On day 9-10, half of the culture medium is replaced by another medium (IVC2) and renewed daily until day 14 by removing half of the medium and adding the other half. More detailed information can be found in Shahbazi et al. (2016) and in Zernicka-Goetz et al. (2016).

The conditions and components of the extended culture medium are explained below

Like conventional embryo culture, long-term culture has, with some particularities, similar culture conditions requirements to conventional embryo culture in IVF.

Temperature

With regard to the optimal culture temperature, it depends on the body temperature of the animal from which the cells are collected and the regional temperature variation in that animal. Some studies have shown that the temperature within the female reproductive tract can be lower than 37°C, indicating that a lower temperature may be more beneficial for embryo development (Baak et al., 2019). Indeed successful fertilization, blastocyst formation rates and pregnancies can be achieved at 36°C (Hong et al., 2014).

Currently, the temperature used in the long-term culture is also 37°C (Bedzhov et al., 2014; Deglincerti et al., 2016; Popovic et al., 2019; Shahbazi et al., 2016; Shahbazi et al., 2020; Weimar et al., 2013; Zernicka-Goetz et al., 2016).

Gas environment

Carbon dioxide (CO_2) and oxygen (O_2) levels inside the culture incubator must also be controlled. CO_2 is essential to maintain the right pH in the culture medium, which ranges between 7.2-7.4, and allow proper embryo development (Reimundo et al., 2021; Rendón Abad, 2018). As in IVF, 5% CO_2 concentration is commonly used for embryo culture up to day 14 (Bedzhov et al., 2014; Deglincerti et al., 2016; Popovic et al., 2019; Shahbazi et al., 2016; Shahbazi et al., 2020; Weimar et al., 2013; Zernicka-Goetz et al., 2016).

Regarding O_2 concentration, the trend in IVF clinics over the past years has been to culture pre-implantation human embryos in hypoxic conditions (5% O_2) (Christianson et al.,

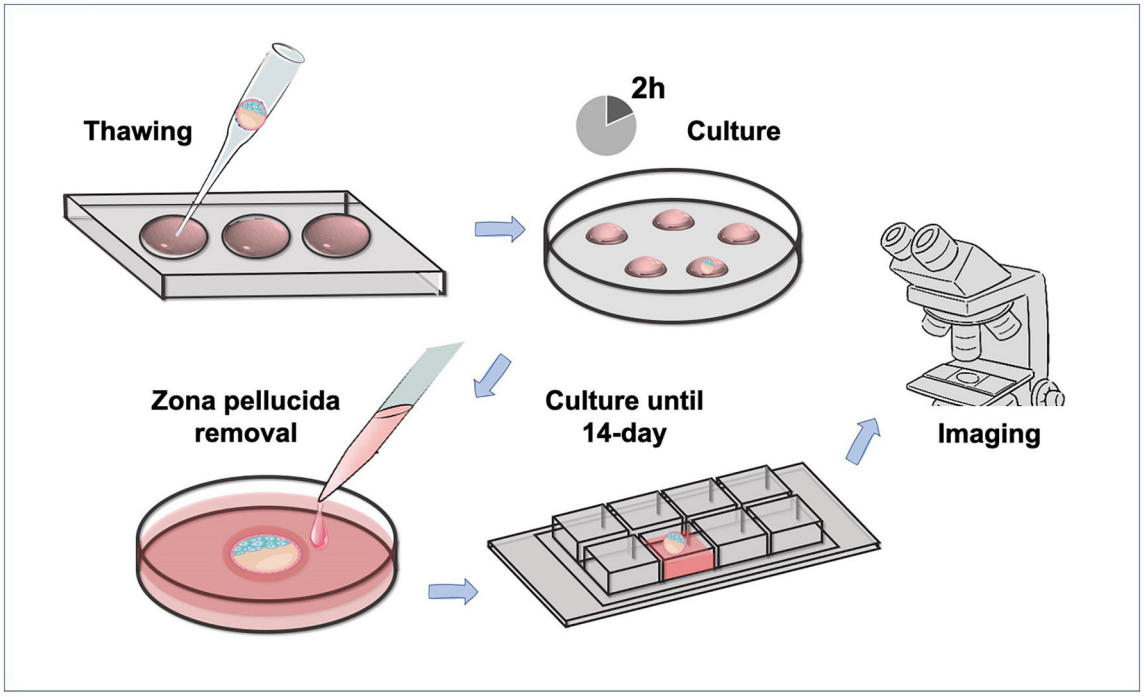

Figure 1 Extended culture procedure up to day 14. The embryos are first thawed and then placed in culture medium for two hours for reexpansion. The next step is to remove the zona pellucida with tyrodes acid and then transfer them to the culture plate for culture until day 14 of development. Each day they can be observed under the microscope and their evolution can be evaluated.

2014). Low O_2 tension improves both embryo development and IVF outcomes (De los Santos et al., 2013; van Montfoort et al., 2020). When data from the meta-analyses were stratified for duration of culture under low O_2, it was demonstrated that even though embryo quality was lower at cleavage stage (De los Santos et al., 2013), pregnancy or live birth rates were only higher when the blastocyst stage (Nastri et al., 2016).

As the embryo is more vulnerable to oxygen in the pre-compaction stage as compared to the post-compaction stage, low O_2 is recommended from the initial stages of development (Wale and Gardner, 2010; Kirkegaard et al., 2013). However, it has been observed that beyond day 7 of development, embryos may benefit from higher O_2 concentrations (21%), possibly due to the increased size of the embryo, which may decrease the O_2 pressure in the embryonic nucleus. Hypoxia clearly has a complex influence on embryonic development and its effects need to be better understood in the intrauterine environment to determine exactly how it affects embryogenesis (Shahbazi et al., 2016). With regard to the concentration of this gas, although some authors have used hypoxia conditions (Popovic et al., 2019), most of them have done the extended culture until day 14 with an O_2 concentration of 21% (Deglincerti et al., 2016; Shahbazi et al., 2016; Shahbazi et al., 2020; Zernicka-Goetz et al., 2016).

Culture media composition

The composition of the long-term culture medium is slightly modified compared to the embryo culture media widely used in IVF laboratories such as G-2™ (Vitrolife, Sweden) or Sydney IVF Blastocyst Medium (Cook Medical, United States) as they do not contain hormones, nor serum from animal sources.

Indeed, both IVC1 and IVC2, are made up of the same components such as advanced DMEM/F12, ITS-X, L-glutamine, β-oestradiol, progesterone, penicillin/streptomycin, and N-acetyl-L-cysteine. Whereas IVC1 is supplemented with 20% heat-inactivated FBS, IVC2 by 30% Knockout Serum Replacement.

Advanced DMEM/F12 (ThermoFisher Scientific, United States) which used as basal medium, is composed, like the conventional IVF media mentioned above, of amino acids, inorganic salts, vitamins, proteins, and glucose. However, it has certain peculiarities. It does not contain human serum albumin (HSA), but instead includes other proteins such as AlbuMAX® II (Gibco, United States), human transferrin and recombinant full-chain insulin. Furthermore, it contains additional components such as fatty acids like linoleic acid and linolenic acid, reducing agents like glutathione, monosodium, and other components like putrescine or thymidine (all components and concentrations are specified in Table 1) (ThermoFisher Scientific, 2020a). Despite this, the *in vitro* function of some of these components on the embryo has not been completely described.

On the other hand **ITS-X**, which is composed of: (1) insulin, which promotes glucose and amino acid uptake, lipogenesis, intracellular transport, and protein and nucleic acid synthesis; (2) transferrin, which acts as an iron carrier and can also help reduce toxic levels of oxygen radicals and peroxide; (3) selenium, as sodium selenite, which acts as a cofactor for glutathione peroxidase and other proteins that is used as an antioxidant in the medium; and (4)

Table 1 Composition and concentrations of basal medium Advanced DMEM/F12 and ITS-X. This table shows the different components that make up the basal medium Advanced DMEM/F12, as well as ITS-X, all with their respective concentrations in mg/L. Data obtained from ThermoFisher Scientific (2014a, 2014b).

Advanced DMEM/F12	Concentration (mg/L)		Concentration (mg/L)
Non-essential amino acids:		***Essential amino acids:***	
Glycine	18.75	L-Arginine hydrochloride	147.5
L-Alanine	4.45	L-Histidine hydrochloride-H_2O	31.48
L-Asparagine-H_2O	7.5	L-Isoleucine	54.47
L-Aspartic acid	6.65	L-Leucine	59.05
L-Cysteine hydrochloride-H_2O	17.56	L-Lysine hydrochloride	91.25
L-Cysteine 2HCl	31.29	L-Methionine	17.24
L-Glutamic acid	7.35	L-Phenylalanine	35.48
L-Proline	17.25	L-Threonine	53.45
L-Serine	26.25	L-Tryptophan	9.02
L-Tyrosine disodium salt dihydrate	55.79	L-Valine	52.85
Inorganic salts:		***Vitamins:***	
Calcium chloride	116.6	Ascorbic acid phosphate	2.5
Cupric sulphate	0.0013	Biotin	0.0035
Ferric nitrate	0.05	Choline chloride	8.98
Ferric sulphate	0.417	D-Calcium pantothenate	2.24
Magnesium chloride	28.64	Folic acid	2.65
Magnesium sulphate	48.84	Niacinamide	2.02
Potassium chloride	311.8	Pyridoxine hydrochloride	2.0
Sodium bicarbonate	2438.0	Riboflavin	0.219
Sodium chloride	6995.5	Thiamine hydrochloride	2.17
Sodium phosphate dibasic	71.02	Vitamin B12	0.68
Sodium phosphate monobasic	62.5	i-Inositol	132.6
Zinc sulphate	0.864	***Other components:***	
Ammonium metavanadate	3.0E-4	D-Glucose (Dextrose)	3151.0
Manganous chloride	5.0E-5	Ethanolamine	1.9
Sodium selenite	0.005	Hypoxanthine Na	2.39
Proteins:		Linoleic acid	0.042
AlbuMAX® II	400.0	Lipoic acid	0.105
Human Transferrin	7.5	Phenol red	8.1
Insulin Recombinant Full Chain	10.0	Putrescine 2HCl	0.081
Reducing agents:		Sodium pyruvate	110.0
Glutathione, monosodium	1.0	Thymidine	0.365
ITS-X			
Proteins:			
Insulin	1000.0		
Transferrin	550.0		
Trace elements:			
Sodium selenite	0.67		
Other components:			
Ethanolamine	200.0		

ethanolamine, a precursor of phosphoglycerides that are essential for the structure of the plasma membrane and cellular organelles (Concentrations are specified in Table 1). All these components have been shown to reduce the need for serum supplementation of conventional cell culture media and to promote optimal performance of serum-free media (ThermoFisher Scientific, 2014b). This is the case for the culture media up to day 14, IVC1 and IVC2 respectively, where IVC1 contains only 20% inactivated FBS and IVC2 contains Knockout Serum Replacement, which replaces FBS. Possible reasons for using reduced serum reduced serum are unknown exact composition, batch-to-batch variability, unintended interaction with culture substances, potentially leading to unexpected or undesired results, experimental variability, and limited reproducibility (van der Valk et al., 2018). In addition, with Knockout Serum Replacement, many of the disadvantages of using FBS in culture can be mitigated (ThermoFisher Scientific, 2020a).

As discussed, both conventional and long-term culture media contain **amino acids** in their composition. These molecules have numerous functions, and their importance is attributed to acting as precursors for protein and nucleotide biosynthesis, substrates for energy metabolism, osmolytes, antioxidant, regulators, buffers, and chelators. From studies

in mice, non-essential amino acids stimulate the rate of cleavage, blastocyst formation and hatching. Essential amino acids stimulate cleavage rates after the eight-cell stage and increase the development of the inner cell mass in the blastocyst (Sunde et al., 2016).

As in IVF culture media, L-glutamine is not present, instead **GlutaMAX™** (ThermoFisher Scientific, United Stated) is added as an alternative. This supplement consists of the dipeptide L-alanyl-L-glutamine, which is more stable in aqueous solutions, making it suitable for suspension and adherent cultures of mammalian cells, such as embryos cultured up to day 14. Moreover, compared to L-glutamine, GlutaMAX minimises toxic ammonia accumulation, improves cell viability and growth by having lower ammonia levels, and remains stable over a wide temperature range because it does not spontaneously degrade (ThermoFisher Scientific, 2020b). It should be noted that, although this supplement as such is not added to conventional culture media used in IVF laboratories, they contain stabilised glutamine in the form of L-alanine-L-glutamine.

Another peculiarity of the basal medium of the long-term culture is that it contains a compound called **AlbuMAX® II**. Conventional culture medium contains HSA with associated lipids. However, this supplement consists of lipid-rich bovine serum albumin (BSA), and like ITS-X, is effective in reducing or replacing the requirement for serum supplementation in culture media. In addition, the chromatographic separation process contributes to the preservation of the natural lipids associated with the purified albumin, resulting in excellent growth stimulation characteristics (ThermoFisher Scientific, 2014c).

Additionally, to these protein-associated lipids, the long-term culture medium contains other **lipids**, specifically fatty acids, which are not added normally in conventional IVF media such as those mentioned above. It is known that embryos need lipids at very early stages. Most of the ATP for pre-implantation development comes from fatty acids. The proportion of different types of lipids plays an important role. An increase in unsaturated fatty acids can compensate for the negative effects of saturated fatty acids. Polyunsaturated fatty acids (linoleic acid and linolenic acid) have a beneficial role in embryonic cells signalling and may influence gene expression in lipid metabolism, oxidation and fatty acid synthesis as seen in animal models (Pawar and Jump, 2003). The impact of trans-unsaturated fatty acids on embryonic development is unknown. However, it is thought that their effect on polyunsaturated fatty acid metabolism could affect embryonic development (Fürstova et al., 2008). This means that lipid balance may be important for pre-implantation embryo development and implantation (Zander-Fox et al., 2021).

The basal medium also contains, although at low concentration, polyamines such as **putrescine**. These play a key role in cell proliferation, growth, and differentiation. They are involved in the regulation of DNA and protein synthesis, proliferation and differentiation (Igarashi and Kashiwagi, 2000). They act as antioxidants protecting DNA, proteins and lipids from oxidative damage (Chattopadhyay et al., 2003). There are evidences that these molecules regulate early embryogenesis, placental trophoblastic growth, implantation and embryonic development (Halloran et al., 2021; Wu and Morris Jr, 1998). In addition, supplementation of polyamines such as putrescine has been shown to be involved in embryo survival, ultimately resulting in improved fertility (Hussain et al., 2017).

Another component present in the culture media is **vitamins**. In contrast to conventional media, the culture up to day 14 contains an abundance of vitamins B and C. Although the role of some of them in embryonic development is known, the role of others is unknown. In hamsters, vitamins B have been shown to promote embryonic development *in vitro*; however, they may be inhibitory in early mouse embryos. Vitamins B2 (riboflavin), 6 (pyridoxine), 9 (folic acid), 12 (cobalamin) are known to be necessary for homocysteine recycling (Ménézo et al., 2013). Vitamin C (ascorbic acid) acts as an antioxidant by reducing oxidative stress-induced embryo toxicity, and in mice it also improves blastocyst development rates (Wang et al., 2002).

In addition to the mentioned molecules that act as antioxidants (amino acids, proteins, vitamins), the basal culture medium of the extended culture contains **N-acetyl-**

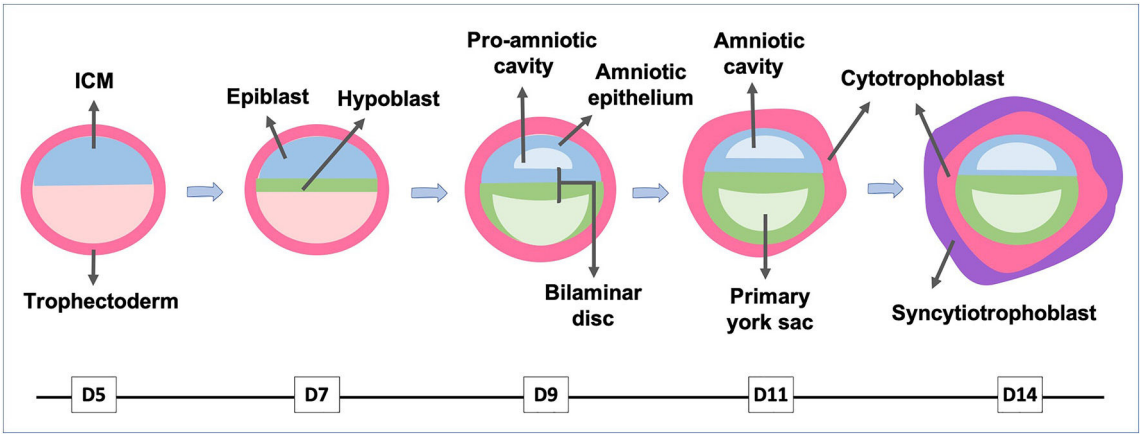

Figure 2 Events of human implantation morphogenesis. The embryo formed by the ICM (inner cell mass) and the trophectoderm is shown first. On the one hand, from the ICM, the epiblast and hypoblast segregate, forming the bilaminar disc. In the epiblast, a cavity called the pro-amniotic cavity begins to appear, which will later give rise to the amniotic cavity, and the hypoblast extends to form the yolk sac. On the other hand, the trophectoderm differentiates into cytotrophoblast and syncytiotrophoblast.

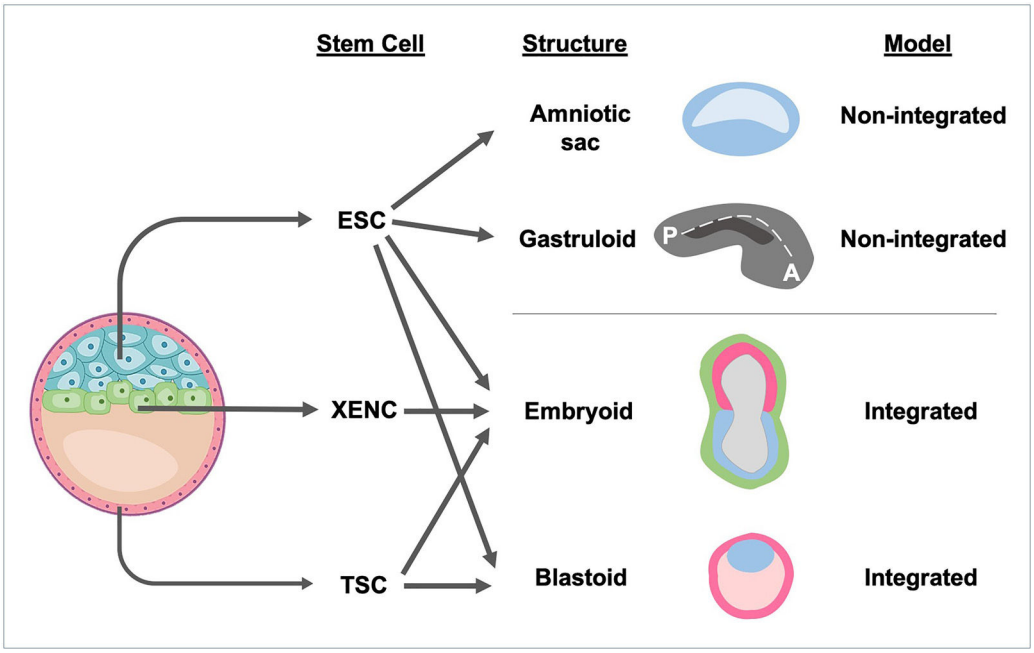

Figure 3 Non-integrated and integrated Stem cell-based embryo models. The upper figure of the schematic corresponds to an amniotic sac-like structure obtained from ESC. The second represents the elongated gastruloid with an anterior-posterior axis, also obtained from ESC. The third structure represents the embryoid, which has been obtained from ESC, XENC and TSC. And the last structure is known as the blastoid, made up of cells from ESC and TSC. (ESC: embryonic stem cells; XENC: extraembryonic endoderm cells; TSC: trophectoderm stem cells) (adapted from Rossant and Tam, 2021).

L-cysteine and **reducing agents** such as glutathione, monosodium which are not present in conventional IVF media.

As mentioned before, the culture medium up to day 14 also contains **hormones** which are not present in the routine culture media used in IVF laboratories. There is a wealth of evidence that growth factors and hormones in the reproductive tract and in the pre-embryo may be potential regulators of embryonic development. Their benefits are thought to include decreased apoptosis, increased cell proliferation, and consequently improved progression to blastocyst. The need for their presence and usefulness in culture media is unclear due to the unknown long-term effects they may have on pre-embryos. However, culture media containing different growth factors and hormones can be found on the market (Rendón Abad, 2018). Long-term culture medium, in its initial protocol and unlike conventional IVF media, contains progesterone and β-oestradiol.

Extended culture medium will also include other supplements such as **antibiotics** (e.g. penicillin and streptomycin). These are added to the medium to prevent or reduce likelihood of contamination. Although gentamicin has been found to be the antibiotic used par excellence in culture media as a prophylactic measure, it should be noted that the absence of antibiotics is beneficial for the development of pre-embryos as they develop faster (Rendón Abad, 2018).

What structures can we see with the culture up to day 14?

In vitro culture systems up to day 14 of embryo development have made it possible to document the key events of normal human embryogenesis from pre-implantation to gastrulation. By comparing embryos developing *in vitro* with the Carnegie series of human embryos *in vivo* (Hertig et al., 1956), the main morphogenetic events of human implantation morphogenesis were discovered (figure 2). These include:

1. Segregation of the epiblast and hypoblast from the inner cell mass of the blastocyst and morphogenetic movements to form the bilaminar disc. While the epiblast is represented by increased levels of OCT4 and Nanog (pluripotency transcription factors), the hypoblast is determined by increased levels of GATA6 and Sox17 (hypoblast-specific transcription factors).
2. Pro-amniotic cavity formation within pluripotent epiblast cells of the embryonic lineage (via apically located atypical PKC, leading to apical polarity in epiblast cells and lumen formation) and amniotic epithelium.
3. Formation of the embryonic disc from the epiblast (embryo proper).
4. Formation of the primary yolk sac within the hypoblast.
5. Differentiation of the trophoblast to produce its two characteristic cell types: the mononucleated cytotrophoblast and the multinucleated polyploid syncytiotrophoblast.

Despite not all embryos will be able to undergo the former morphogenic event, the system brings *in vitro* cultured embryos to the point of gastrulation (Shahbazi and Zernicka-Goetz, 2018). Interestingly, all of them can take place *in vitro* in the absence of maternal tissues, indicating that human embryos have a previously underestimated potential for self-

organization (Shahbazi et al., 2016; Zernicka-Goetz et al., 2016).

Although this system may not be able to fully recapitulate all aspects of human embryogenesis *in vivo*, it has revealed that even in the absence of endometrial cells, human blastocysts have remarkable capacity for self-organization that was previously unknown (Shahbazi et al., 2016).

Stem cells and culture

In recent years, the cultivation of stem cell-based embryonic models to unravel the mysteries of early embryonic development in humans has gained ground.

The stem cell-based embryo model is a simplified model that attempts to mimic the characteristics of a natural embryo. The knowledge gained from their experimentation opens a new way to model embryogenesis, cell lineage differentiation, tissue morphogenesis, and organogenesis in mammalian development (Rossant and Tam, 2021). Moreover, it can improve the understanding of the mechanisms of embryo development from blastocyst stage to gastrulation, as it is more scalable, versatile, accessible than natural embryos, and exhibits similar self-organization as natural embryos during *in vitro* culture (Zhai et al., 2021).

There are two types of stem cell-based models (figure 3). On the one hand, *non-integrated models* use a single stem cell type (ESC) and are limited to the formation of specific parts of the embryo such as amniotic sac structures and shed light on selected morphogenetic events such as gastrulation or developmental processes such as germ layer differentiation. This type of model includes amniotic sac models (Zheng et al., 2019) and the elongated gastruloid resembling the posterior elongation body axis (Beccari et al., 2018). On the other hand, *integrated models* are formed from several stem cell types (ESC, XENC, TSC) and contain all parts of the conceptus, that is, embryonic and extra-embryonic tissues, giving rise to the blastoid, a blastocyst-like structure formed by the aggregate of ESC and TSC, where TSC encloses ESC in a cavity (Rivron et al., 2018) and the embryoid, an embryo-like structure made up of various types of stem cells but not capable of developing into a functional human embryo (Sozen et al., 2018). Both types of models can be achieved by means of 2D micropatterning techniques, microfluidic devices, or 3D aggregation methods, exhibiting a self-organization similar to that of natural embryos during *in vitro* culture. It should be noted that most of this type of work has now been carried out in animal models such as the mouse (Rossant and Tam, 2021).

Limited knowledge about the mechanisms regulating cell fate determination during early human embryogenesis hampers the creation of stem cell-based embryo models. This could only be remedied by further research with human embryos per se, non-human primate embryos *in vitro* and embryo models (Zhai et al., 2021). And, although these culture systems could reproduce the events of early mammalian embryogenesis *in vivo*, a critical prerequisite of experimental models is that they must accurately reconstruct the events that occur *in vivo*, particularly in the early stages when the basic body plan is established and when the *in vivo* embryo is most inaccessible for experimentation (Rossant and Tam, 2021). By collecting more data on this, extended *ex vivo* phenotype, new markers of embryo competence and sustained implantation could be identified to enhance IVF outcomes.

Potential applications in embryology

The contributions of post-implantation embryo research, apart from allowing to observe *in vitro* the events and structures mentioned above, could include advancing the understanding of gene function during embryogenesis; a scientific basis for the prevention of early pregnancy loss, birth defects and teratogenesis; and an understanding of how the widespread epigenetic programming that occurs during this stage of development might affect disease progression in later life (Pera, 2017).

Advances in *in vitro* culture systems have allowed the modelling of chromosomal instability and the investigation of how mosaicism and chromosomal abnormalities can affect development in the pre-implantation period.

Before post-implantation *in vitro* culture, the capacity of mosaic blastocysts had only been studied in mice (Bolton et al., 2016) or through clinical outcome data (Greco et al., 2015) with no blinded studies evaluating the IVF outcome of the reported mosaicism. Without knowing the implications of mosaic embryos in IVF transfers, it is difficult to determine whether to discard them and waste a potentially viable embryo or transfer a potentially harmful one. Popovic et al. (2019), using NGS and extended culture to day 12 of development, are believed to be the first to investigate the fate of chromosomal abnormalities and mosaicism. On the one hand, they showed that many mosaics in the day 5 or 6 blastocyst biopsy were no longer mosaic at 8 or 12 days, others were still mosaic, and others had become uniformly aneuploid. In general, when mosaic blastocysts had more abnormal cells, they were more likely to be non-viable at 12 days. On the other hand, they also showed that NGS can have an 18% misdiagnosis rate for mosaicism, so improvements and further studies are needed before clinical application. However, it shows the possible developments of these advances in extended culture (Williams and Johnson, 2020).

Consensus on the clinical management of embryonic mosaicism is still under debate. *In vitro* performance of specific mosaics are needed to understand their developmental behaviour and improve diagnosis. Furthermore, assessing chromosomal instability during these hitherto hidden developmental stages may provide valuable information on the predictive value of reporting mosaicism in clinical practice, ultimately improving the embryo selection process and clinical outcomes.

14-day limit

The 14-day limit for *in vitro* human embryo culture dates back to the 1970s, more specifically to 1978, when the ability to develop embryos *in vitro* for research and assisted reproduction was demonstrated (Steptoe and Edwards, 1978). But it was not until 1984 that it was first established in the Warnock Report in the UK (Warnock, 1984), and subsequently enshrined in the Human Fertilization and Embryology (HFE) Acts of, 1990, 2008. It is now one of the most internationally accepted standards in reproductive medicine (Appleby and Bredenoord, 2018).

The Warnock Report (1984) elicited different responses from the public and committee members as there was a wide range of opinions on the moral status of human embryos.

However, most of the committee agreed that such research should be allowed under licence. To allay public fears it was necessary to set a clear limit, which was 14 days, that could be justified on the basis of this moral status of the embryo (Blackshaw and Rodger, 2021).

One of the reasons for setting the maximum at 14 days was that this point in development was prior to the formation of the primitive line, which appears on day 15 of human embryonic development. This occurs at the onset of gastrulation, a period when the three germ layers are formed and the axes and body form are established, marking the point of individualisation (Williams and Johnson, 2020). The committee's view was that from this point onwards the embryo could be considered as a defined individual and a potential person, an entity with rights, such as the right to life, since from this point onwards twins cannot be formed. Furthermore, he pointed out that this boundary was prior to the formation of the central nervous system, around day 22 of development, which would exclude the possibility of the embryo experiencing pain (Blackshaw and Rodger, 2021). Other reasons were the substantial loss of embryos from fertilization to this point and that until the implantation process is not complete, the embryo has no potential for further development (Pera, 2017).

However, given that scientific progress has brought us to a point where viable pregnancies can be obtained from day 7 blastocyst transfer, could be reasonable to question whether 14 days is too restrictive from a research point of view (Williams and Johnson, 2020). Furthermore, as discussed before, recent studies have demonstrated the successful growth of human embryos up to developmental stages close to this limit (Deglincerti et al., 2016; Shahbazi et al., 2016). Other research has reported the generation of embryo-like structures from mouse and human pluripotent stem cells that mimic the gastrulating embryo in form and cellular content (Etoc et al., 2016; Harrison et al., 2017; Shao et al., 2017; van den Brink et al., 2014). And recently, successful *in vitro* culture of *Cynomolgus* macaque embryos beyond the gastrula stage and up to day 20 of development has been reported (Niu et al., 2019). This may fill the knowledge gap on human embryogenesis beyond day 14, and it is even suggested that such a culture period should also be feasible for human embryos (Williams and Johnson, 2020).

All these advances have led to a re-examination of the current restrictions on human embryo culture (Hyun et al., 2016), with various positions or sides emerging. There are those in favour of such an extension (Hyun et al., 2021; McCully, 2021); others who think that it should not be extended and should remain as it currently stands (Blackshaw and Rodger, 2021; Green et al., 2021; McLaren, 1984); and even the most extreme who argue that human embryos should not be experimented on. Making changes to existing legislation is a complex process and there is a risk of creating new restrictions that could have unintended consequences for future research (Pera, 2017).

So, should the 14-day limit be extended?

The period between days 14 and 28 of human embryonic development is called the "black box" (Hurlbut et al., 2017). Until now, embryonic development in this period has been difficult to study and understand from a scientific and regulatory point of view. However, the Nuffield Council on Bioethics (2017) argues that any attempt to extend research beyond day 14 would have to result in significant advances in science. Furthermore, it should examine the committee's reasoning and justify the extension on this basis. The two key considerations are maintaining public confidence and providing a moral justification for embryo research. The committee provided two reasons for morally justifying early embryo research: the utilitarian argument, based on the benefits of such research, and its argument based on the moral status of early embryos (Blackshaw and Rodger, 2021).

Due to the current benefits of embryo research and the potential future benefits, supporters of extending the 14-day rule are calling for its revision. Extending this period to 28 days would allow to study the developmental processes of gastrulation, when the first primitive tissues are formed. In addition, it would allow learning about the developing nervous system without the risk of neural connections and a better understanding of early organ development (Hurlbut et al., 2017). It would also be possible to advance our understanding of cell fate decisions during early embryonic development (Shahbazi et al., 2016). The safety and success of current IVF procedures could be further improved by understanding the nature of some birth defects and predicting which embryos are most likely to result in a successful pregnancy (Hurlbut et al., 2017). Finally, more could be learned about the physiology of pregnancy beyond day 14, including the processes surrounding implantation, a greater understanding of the causes of embryonic defects and why events such as miscarriages occur (Hurlbut et al., 2017). Castelyn (2020) offers several reasons why a re-evaluation of the 14-day rule may be justified.

Sophia McCully takes this side, listing several potential advantages to extending the limit to day 28 of development (McCully, 2021). She focuses mainly on the utilitarian argument to justify her idea, suggesting that it will help reduce miscarriage rates, improve IVF outcomes, and test the safety of new techniques such as gene editing. And, while acknowledging that the current limit has generated public confidence, she argues that concerns about a slippery slope are unjustified because the regulations are strict, making it illegal to slide down such a slope. Other authors advocate a more conservative extension of about 2-3 days, and that only after broad public consultation (Hyun et al., 2021).

However, although it has important potential scientific and medical benefits, it is unclear whether extending the 14-day limit in human embryo culture is the best way to achieve them or not, as the possibility of prolonged normal development of human embryos *in vitro* during and beyond the primitive line stage is unknown. In mice, pre-implantation embryo culture has so far not generated structures that resemble post-implantation embryos, in contrast to recent results with stem cell cultures. In humans, degenerative changes have occurred as they approach the 14-day limit with the current methodology. In mice, embryos have been grown *in vitro* up to day 10.5 of development, the equivalent of about 4 weeks in humans, but this is obviously unethical in our species. So, the question is whether it is feasible to extend *in vitro* development of high-quality human embryos to this stage. However, even if it were, the extremely limited supply of such embryos means that any line of research requiring large numbers of cells would be

extremely difficult to conduct and replicate in multiple laboratories (Pera, 2017).

Green et al. (2021), despite supporting human embryo research, are not very receptive to the recommendations of the International Society for Stem Cell Research (ISSCR) to extend the 14-day limit, as they consider that there are compelling reasons based on important biological events to maintain it, and believe that this institution should offer more convincing arguments for abandoning the current limit.

Another group that is opposed to this extension is Blackshaw and Rodger (2021). In response to McCully, they argue that the Warnock committee's reasons for the 14-day limit still apply despite advances in culturing human embryos *in vitro*. These authors believe that McCully seems unaware that such an extension is precisely the kind of slippery slope that opponents of the original law warned about. She also fails to take into account the argument based on the moral status of embryos: that only after the primitive line appears is the embryo a distinct individual and potential person. Moreover, research on embryos between 7 and 14 days old has only just begun to be exploited and it seems rash to extend it to 28 days. It is also not known how precisely post-implantation embryo culture would replicate the *in utero* environment, which may limit its applicability beyond 14 days.

Therefore, several practical obstacles must first be overcome to facilitate this extended study. For example, conditions must be devised and improved to keep an embryo sustained and alive in a suitable environment (Aach et al., 2017). Although this challenge is difficult, it could be overcome with the help of 3D bioprinting and organoids (Aach et al., 2017; Bredenoord et al., 2017). To culture embryos at later developmental stages, such as beyond day 10.5 in mice or day 30 in macaques, it is essential to establish a functional placenta and a functional vascularised umbilical cord. This has been achieved artificially to help extremely premature lambs develop to near maturity (Partridge et al., 2017). Although this has not yet been achieved in humans, in the future, culture systems incorporated with a 3D printed vascularised umbilical cord and a functional artificial placenta *in vitro* could help extend *in vitro* embryo culture to later stages of development (Zhai et al., 2021).

Ultimately, any decision to revise the 14-day rule must depend on whether the proposed changes can maintain the two main objectives of the rule: to support research and to accommodate various moral concerns (Hyun et al., 2016). It requires a careful assessment of the scientific feasibility and effective benefits of embryo research; it needs extensive research on public opinion regarding embryos, and a deliberative process that takes these elements into account. It does not need positions that only consider the beneficence of the research and its technical feasibility. This would be undemocratic and potentially a measure unsupported by a rigorous assessment of the science behind embryo research (Cavaliere, 2017).

Conclusions

The extended culture of embryos up to day 14 of development is leading to a better understanding of the events that take place between implantation and gastrulation. Despite this, it is necessary to improve the current culture conditions in order to optimise the technique and reach the maximum number of days allowed by law, as in most studies of this type, the embryos are blocked before day 14. Furthermore, it should be borne in mind that access to and availability of human embryos is limited, so other alternatives such as embryo development from stem cells should also be further explored.

Once the culture conditions up to day 14 have been optimised, the idea of prolonging extended culture beyond day 14 is under debate, since with the current knowledge and with the help of existing technology, events such as gastrulation, hitherto unknown in humans, could be studied, allowing a better understanding of human embryonic development at later stages. In addition, this could be applied to areas such as assisted reproduction when making decisions on the choice of the best embryos or on the knowledge of diseases that may affect pregnancy and lead to miscarriage, for example. All of this with clear moral and ethical boundaries.

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Conflict of interest

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