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## 9.1 Introduction

In the last decade, several efforts have pointed to a better awareness of the embryo physiology and biochemistry, leading to significant advances in systems for embryo culture.

It is well known that a short *in vitro* culture does not allow for a reliable embryo evaluation, requiring the transfer of more than one embryo and thus increasing the risk for multiple pregnancies. However, only up to half of human embryos conceived *in vitro* develop to the blastocyst stage and ~18 % of them arrest at or prior to the 4-cell stage [1, 2]. Beyond the substantial genetic defects that are intrinsic to the embryos, suboptimal culture media composition or physical culture parameters (or a combination of them) may be responsible for these observed rates of development arrest.

The preimplantation embryo is a free-living organism that can regulate its own cell division and differentiation using transcripts accumulated during oogenesis [3] and produced after the activation of the embryonic genome [4]. Additionally, this autonomous organism produces embryotrophic factors such as platelet-activating factor and interleukin-1 regulating the early events of

embryo development [5]. The first crucial steps of mammalian development such as first cleavage, activation of maternal genome, compaction, and differentiation are the result of precisely programmed and orchestrated events. The embryo is also endowed with the ability to adapt to changing environmental conditions that maintains cellular homeostasis and preserves viability. Despite this embryonic plasticity, the exposure to suboptimal environmental conditions that can exceed its adaptive capacity may cause change in epigenetics, transcription, metabolism, and cell allocation with potential long-term consequences [6, 7].

In the recent years, many researches pointed to improve embryo culture conditions and to introduce novel devices and platforms to provide a more appropriate microenvironment for the embryos. The majority of acquired knowledge has led to enrich media formulation, refining them by introducing salts, amino acids, energy substrates, growth factors, and other supplements. Overall, these advances have made feasible to extend embryo culture to the blastocyst stage, allowing single embryo transfer while accomplishing consistent pregnancy and live birth rates, thus increasing significantly the efficiency of human-assisted reproduction procedures.

However, not only the chemical supplies of the developing embryo need to be considered but also potential physical requirements (mechanical and surface interactions, cell movement) may influence embryo development and may be important factors in the continuing pursuit of

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improved *in vitro* conditions. To this end, very recently novel culture and surface platforms have been developed, allowing dynamic culture through the employment of media flows.

Despite several aspects remain to be analyzed, these new approaches and emerging technologies may optimize the efficiency of embryo production, creating a more appropriate microenvironment for gamete function and support embryo developmental competence.

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## 9.2 Embryo Culture Platforms

### 9.2.1 Static Culture Platforms

Until now, human embryos have been commonly cultured on inert plastic supports that create a “static microenvironment” as these platforms do not produce any active movements and limited cell surface contact [8]. During routine IVF procedure, culture media are conventionally placed in disposable polystyrene multiwell or Petri dishes, in 10–80  $\mu\text{l}$  drops of media covered with oil and equilibrated overnight in the proper gas mixture at 37 °C to stabilize the pH, temperature, and achieve proper gas saturation. Generally, the embryos are cultured individually or in small groups and incubated for days, in either single or sequential medium [7, 9, 10]. However, *in vivo*, embryos are exposed to a more dynamic environment, developing in the virtual space of oviduct. As previously underlined [9], considerable differences exist between the conventional culture system and the natural environment of the oviduct. The female reproductive tract is surrounded by ciliated epithelia that sustain embryo movement; moreover, during this progression embryos are exposed to several unknown constituents of oviductal fluids that fulfill the metabolic needs of the embryo.

This is in sharp contrast with the *in vitro* environment, where embryos are cultured on artificial surface and no dynamic movements are ensured and where autocrine factors are often diluted and diffused into the oil layer.

### 9.2.2 Enhanced Static Platforms

Recently, novel devices and new culture approaches are being developed in order to handle physical parameters and to improve the *in vitro* microenvironment, exploiting different potential beneficial aspects of embryo culture such as increased embryo density, decreased media volume, and retention of autocrine/paracrine factors.

Embryo density, expressed as the embryo-to-volume ratio, is the number of embryos in a defined volume of culture medium. The same density can be achieved by manipulating either the number of embryos in a given volume of medium, or manipulating the volume of the medium for a given number of embryos. In different animal models, it has been observed that increased embryo density may improve developmental competence, probably through the production and secretion of various factors able to affect embryo homeostasis [11–14]. Recently it has been shown that group culture improves rates of human blastocyst development, when compared to individual culture [15, 16].

Embryo culture may be successfully performed in small volume to effectively benefit of retention of autocrine factors. In fact, the mixture of compounds embryo-secreted is challenging to be replaced by exogenous biomolecules.

Moreover, utilization of exogenous growth factors may be inadvisable since an appropriate spatial or temporal exposure may lead to developmental abnormalities such as large offspring syndrome [17, 18].

In order to confine embryos to a small area, microdrop systems have long been used. Generally, these drops varied from 10 to 50  $\mu\text{l}$  of volume and can be used with group or individual embryo culture, although most embryologists prefer individual culture for easily identification and follow up. A limitation of this approach is related to the potential drop flattening or coalescing, entailing a variation in the amount of media where embryos are cultured and hampering the embryo tracking during handling and evaluation. Specialized dishes are now available, specifically

designed for embryo culture and employing small round bottom wells inside a traditional Petri dish that allows for retention of putative embryotrophic factors while preserving the individuality of each single embryo.

Another variation of this approach utilizes ultralow volumes of media, the “ultramicrodrop system”. The volume of these drops ranges from 1.5 to 2  $\mu\text{l}$  and allows to culture and confine groups of embryos in a small area and to concentrate autocrine/paracrine factors. This approach has resulted in improved embryo development, although tested only with very few embryos. However, further and detailed analysis including pregnancy and implantation rates are necessary to investigate the potential risk of using very small volumes of media, where rapid evaporation with dangerous increase in osmolality can occur [8].

New culture platforms have been developed utilizing extremely low volume of media with a limited surface area. The submicroliters platforms are composed of a culture chip of polydimethylsiloxane (PDMS) containing a small vertical channel. During the culture, 2-cell embryos in the vertical channel are surrounded by submicroliters volume (100 nl) [19]. Rates of blastocyst development obtained using this culture system were comparable with 20- $\mu\text{l}$  culture systems, but significantly greater than 5- $\mu\text{l}$  microdrop cultures. Thus, this novel device allows embryos to take advantages from reduced culture volume and spacing while avoiding issues correlated with small microdrop volume; however, it is limited by a complicate embryo recovery [8].

A novel solution is represented by the Well-of-the-Well (WOW) system, a culture device where embryos are confined in small area while sharing a larger reservoir of media. Basically, it consists of small microwells of conical shape created inside of a well of a 4-well dish or in a Petri dish. First described by Vajta [14], this approach has been successfully used with embryos from several species such as mouse, pig, and human. The advantage of this system is that embryos can be cultured individually in each microwell while sharing the same overlying medium; this creates a

microenvironment around the embryos, increasing the point of contact between them. According to an initial human trial, higher blastocyst rates were observed when embryos were cultured in WOW devices compared to microdrop system (56 vs. 37 %) [20]. Although this system appears very promising, data regarding pregnancy and birth rates are still preliminary and further investigations are required.

The “glass oviduct (GO) system” was proposed by Thouas et al. in 2003 [12] as alternative solution. This culture system is composed of 2- $\mu\text{l}$  sterile open-ended capillary with 200- $\mu\text{m}$  inner diameter. Embryos are loaded by immersing one end of the capillary in a standard microdrop system. Initially, a small oil column enters into the glass capillary, followed by the medium with the embryos, finally upon retraction, oil enters again into the column and closes the solution. Then the capillary is cultured in vertical position in a carbon dioxide incubator and the medium surrounding embryos is approximately 1  $\mu\text{l}$ ; this allows creating concentration gradients for several factors selected or discarded by the embryos. Although blastocyst rates obtained in mouse model were similar to those achieved by traditional culture methods, culturing embryos in the GO system has allowed to improve others parameters such as blastocyst total cell number and hatching rates [21].

The GO system can be considered as an extremely simplified and static version of the microchannel system. More sophisticated and purpose-designed versions of microchannels have been regarded as the greatest promise to establish a multipurpose automated system for in vitro production of preimplantation embryos.

### 9.2.2.1 Specialized Surface Coating

Enhancing culture conditions entails also the revision of the surface of the devices where embryos are cultured.

Several synthetic polymers have been tested on mouse embryos to investigate the potential toxicity due to contaminants or different additives. Generally, conventional devices are made by

polystyrene and glass, materials that are heat-stable and tolerate the temperature and humidity of the incubator without interfering with media [8]. The use of PMDS as IVF device is particularly critical, since it could modify media composition or cause detrimental osmolality shifts [22, 23]. However, the static inert devices used for embryo culture are extremely different from the dynamic interactive surfaces to which embryos are exposed in the uterine cavity. *In vivo*, embryos are surrounded by several macromolecules and components of extracellular matrix that are thought to support embryo cellular homeostatic mechanisms, imparting responsiveness or plasticity to the embryo [24–26]. These macromolecules are supposed to act in a physical sense to stabilize the chemical environments along the oviduct, interacting with biological fluids and inducing significant modifications of the fluid surrounding the embryos. The inclusion of constituents, such as glycosaminoglycans, could improve embryo culture, altering surface properties such as hydrophilicity and aiming to reproduce more closely the female reproductive tract. Equally, glycoproteins are believed to act as carrier molecules to present cations and metabolic substrates at appropriate concentration to the embryo [24].

Figueiredo and collaborators found that laminin added to culture media was detrimental to embryo development decreasing cell number in mouse blastocyst, whereas fibronectin was compatible with mouse embryo development, even if no positive effect was observed compared to controls. Other investigations found that fibronectin and laminin could improve human blastocyst hatching rates if used at 50 µg/ml [27], underlying the different species-specific actions and the importance of concentration. Also, Heparin, hyaluronic acid, and chondroitin sulfate have been added to culture media improving blastocyst development in bovine embryos [28]. These macromolecules can act as anchor for different growth factors, thus their proper orientation is important to influence embryo development. It has been demonstrated that the employment of matrigel (a solubilized basement membrane preparation, rich in Ecm protein) as plate coating increased rate of mouse blastocyst hatching at 96 and 120 h,

even if other authors have shown a detrimental effect of the same coating on mouse blastocyst development, probably due to a different mouse strain used [29, 30].

Conflicting data exist regarding the use of hyaluronic acid, since after preliminary encouraging results in mouse and bovine models, the use of hyaluronic-coated culture surface has significantly reduced mouse blastocyst cell number [31]. A different approach utilizing agarose-made microwells did not display any benefits during embryo culture.

In 1965, Cole and Paule [32], in the attempt to more closely mimic the *in vivo* microenvironment, provided the proof of concept that mouse embryos could benefit from coculture with somatic cells. The use of feeder cell lines was then investigated in human *in vitro* culture, with conflicting clinical results. Initial studies in human IVF, using bovine uterine epithelial cells and human oviductal cells [33], showed promising results and led to a great deal of optimism that coculture may improve embryo development. The observed benefits in terms of improved embryo quality were due both to the secretion of embryotrophic factors and the detoxification of the culture medium [34–37]. Limited studies in this field have been performed; a systematic review of randomized controlled trials was performed by Kattal et al. [38] in order to objectively determine the potential benefits of coculture in human IVF, revealing a statistically significant improvement in embryo morphology and clinical outcome when coculture is performed.

However, the use of biological materials has been complicated by the potential risk of contamination or transmission of disease from feeder layers to the developing embryos. As a consequence of the limitations introduced by the U.S. Food and Drug Administration in 2002 (limiting the use of nonhuman coculture cell lines for human IVF), autologous endometrial cells have been introduced for coculture.

Currently, there is still a lack of information regarding these novel culture platforms and human embryos. Although several proteoglycans and oviductal-specific proteins have been identified, the comprehension of the real impact

of these biomolecules on human embryo development requires more exhaustive studies. Because of these controversial results, the use of coated platforms is not as widespread as expected, yet.

### 9.2.3 Dynamic Culture Platforms

As discussed above, static embryo culture has been the mainly employed method so far. Although these platforms are not completely static, because of convection currents and movement of dishes that can shake media during the observations, they are not fully proper to satisfy the ever-changing needs of preimplantation embryos. *In vivo*, embryos are exposed to a dynamic and gradually changing microenvironment sustained by peristaltic contraction of the smooth muscle of the fallopian tube and kinetic friction forces with ciliated epithelia. During its journey alongside the reproductive tract, the embryo is exposed to constant vibrations of around 6 Hz with the periodically repeating increase to 20 Hz that stimulate embryonic mechanoreceptors and induce the cell-to-cell communication [39].

Conversely, conventional static embryo culture systems require several washing and changing of media during the preimplantation period and expose embryos to suboptimal atmosphere and sudden changes in microenvironment conditions. Furthermore, the accumulation of toxic substances, such as oxygen-derived radicals [40] and ammonia [41], may have a detrimental effect on embryo development. Moreover, studies monitoring the mouse embryo physiology have measured gradients of potassium, calcium, and oxygen around unperturbed embryos [42], due to the secretion or depletion of media components by the developing embryo.

Therefore, new dynamic platforms, specifically assembled in order to produce flow of media, have been proposed to disrupt these gradients and to create a more homogenous environment around the embryo, thus mimicking closely the *in vivo* conditions.

Although providing mechanical stimuli may improve embryo developmental ability, several limitations characterize these promising novel

culture systems; first of all their complexity and lab compatibility with respect to static culture platforms. Besides biocompatibility, that is of utmost importance, other factors such as friction and flow rate have to be carefully considered. Excess mechanical stimuli or overhandling of embryos can induce transient changes in embryo homeostasis and significantly impair embryo viability [43, 44]. Moreover, a continuous rough refreshment of medium may lead also to the elimination of beneficial auto- and paracrine factors [45].

To sum up, there are different hypotheses that explain the potential benefits of dynamic culture systems: the gentle agitation of media that remove waste products around the embryos with replenishment of fresh substrates, the disruption of environmental gradient and the physical stimulation able to activate mechanoreceptors, or signaling pathways involved in embryo development. Unfortunately, not all dynamic culture platforms can have all the characteristics mentioned above; thus, several approaches to generate dynamic culture have been examined.

One of the first approaches to perform a dynamic embryo culture is the use of an orbital shaker placed inside the incubator [46]. Using this culture system, embryos were agitated at 60 rev/min, cultured in a volume of 0.5 ml overlaid with oil. The first promising results came from mouse embryos and ovarian tissue culture [47]. Higher rates of blastocyst development (98.5 %) have been obtained using orbital rotation on flat surface with respect to static culture platform (86.3 %) [31].

While different volumes of media and times of agitation do not have a significant effect on embryo viability, instead the rate of rotation seems to have an impact on embryo development, having detrimental effect when rates of orbital movement arrived at 60 rev/min [8].

Another easy-to-implement alternative to conventional static platforms is represented by the tilting embryo culture system (TECS). A motorized tilting platform is composed of a control unit to set the speed, the angle, and the period of tilting and of a motor unit to drive stage tilting and to place conventional culture dish. While embryos

are tilted, the rolling and media agitation try to mimic the movement through the reproductive tract. Mouse and human embryos have been successfully cultured in TECS, showing an enhanced cell division and blastocyst quality compared to controls [48]. A benefit of this system relies on its lab compatibility that may allow a widespread utilization; however, additional clinical investigations are required to analyze the potential benefits and the limitation of TECS.

To induce dynamic culture conditions, a simple vibration may be sufficient. Initially, pulsatile mechanical microvibration has been successfully used to mature porcine oocytes improving developmental competence and subsequently embryo growth [49]. Also, human zygotes were cultured using gentle vibration of 20 Hz for 5 s [50]. Although the introduction of microvibration did not influence fertilization rates compared to static controls, a significantly higher percentage of high-quality cleavage stage embryos was observed compared with static culture system (90.1 vs. 77.9 %,  $p < 0.05$ ). Moreover, the percentage of embryos that reached the blastocyst stage was 10 % higher than that recorded for the static culture system. This enhanced in vitro embryo development in vitro resulted in a significantly higher pregnancy rate regardless of the day of embryo transfer, highlighting the benefits of gentle vibration during embryo culture.

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### 9.3 Microchannel Microfluidic System

The replenishment of culture media and removal of harmful factors produced by embryos is not accomplished with novel culture devices mentioned above. Moreover, embryos are exposed to suboptimal conditions during handling and the great amount of media to which are exposed may temper the presence of embryotrophic factor.

The great advantage of microfluidic system is that all the requisites to obtain an optimized embryo culture can be accomplished at once. This system allowed performing culture of embryos in precisely defined, submicroliter volumes minimizing the risk of evaporation and

to maintain the surface area-to-volume (SA/V) ratios in a physiological range. The spacing theory is already supported by several investigations demonstrating that improved embryo development can be achieved using ultramicrodrops [51], glass capillary tubes [12, 13], and WOW technologies [14].

Others benefits may arise from the gradual replenishment of media around the embryo and from the mechanical induction of cellular pathways involved in embryo development.

The microchannel microfluidic system is not a recent technique since it has been developed during the 1980s of the last century with multidisciplinary purpose and applications in different fields from physics and chemistry to micro- and biotechnology. The approach of dynamic media flow obtained with microfluidic platforms varied greatly in design and are used for various aspect of ART such as in vitro oocyte maturation [52, 53] and sperm selection [54, 55] and recently also as platform for embryo culture [56, 57].

The microchannel system is essentially composed of the following parts: a glass microscopic slide base and plastic layer with the channels and valves connected with automatic or mechanical pumps.

A critical aspect of microfluidic system is the flow rate that must be finely regulated to determine the range for beneficial effects, since shear stress can influence negatively embryo development, causing damage to blastomeres and embryo degeneration [44].

The early devices used in ART employed passive flow driven by gravity; others used manually applied pressure created by syringes connected externally or programmable syringe infusion pump [55, 58–60]. However, these approaches require constant and difficult regulation of the flow; thus, they are not of easy employment [61]. Very recently, a new Braille pumping system using electric piezo actuators has been successfully introduced, aiming to create a peristaltic movement of media along microchannels. This system assures computerized regulation of the flow without constant supervision and allows gradual variation of media flowing toward the embryos [61].



Glasgow and coauthors are first to demonstrate that embryo manipulation and movement in a microfluidic system is feasible at low flow rates without injuring the embryos [62]. Once the safety of microfluidic system was proved, some authors [56] have shown that 2-cell mouse embryos could be cultured using microchannel system with sub-microliter culture volume, with significantly higher blastocyst rate at 48 h and at 72 h (17.6 vs. 2.4 % and 72.9 vs. 42.9 %, respectively) and hatching blastocyst rate at 72 h and 96 h (4.1 vs. 0 % and 26.5 vs. 8.8 %, respectively). Although the effective volume surrounding the embryos was 250  $\mu\text{l}$ , the employment of very tiny channels (from 100 nm to several hundred micrometers) avoids the occurrence of turbulence and maintains a laminar flow. This microfluidic system, however, has not yet been shown to enhance pregnancy rates. Heo and coworkers established a dynamic microfunnel embryo culture system to better mimicking the fluid-mechanical and biochemical stimulation that embryo experienced in vivo [63]. Blastocyst developmental rate was significantly enhanced under dynamic microfunnel culture conditions as evidenced by an increased percentage of hatching or hatched blastocysts and significantly higher average number of cells per blastocyst. Most importantly, preimplantation developmental kinetics and clinical performances of embryos developed in dynamic conditions more closely resemble

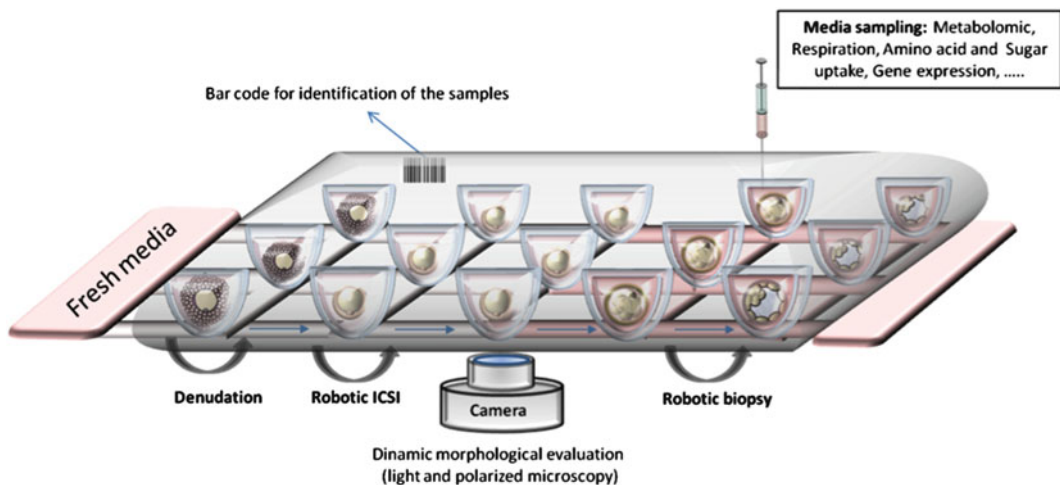
those of the in vivo counterparts. Compared to microchannel culture, dynamic microfunnel system allows to benefit either from fluid mechanical stimulation to the embryo or from retention of a significant amount of embryotrophic factors simultaneously.

These encouraging data, although preliminary, indicate that the microfluidic technology has great potential for improving clinical ART and may represent a solution to meet the mutable needs of embryos, while maintaining an optimal microenvironment during the preimplantation culture.

Taken together, these novel approaches could potentially revolutionize the concept of embryo culture; unfortunately, most of the data discussed above arise from animal models, whereas there are little evidences that these approaches truly benefit also human embryos. Moreover, the implementation of the IVF laboratories with these new technologies would require significant economical efforts.

## 9.4 Integrated Automated System for Embryo Production

Once established, the enhanced culture system can also be integrated with other equipment as a video camera to monitor all steps of the embryo development (Fig. 9.1). Such purpose-designed



**Fig. 9.1** Integrated automated system for embryo production

instruments are already available and provided their value in embryo selection [64, 65]. Further extensions may include various sensors measuring parameters such as embryo-derived biomarkers (metabolomics) or gene expression profiles (transcriptomics). The enormous amount of information derived from the time-lapse imaging together with the biochemical parameters may provide a significant support to select the best embryo(s) to transfer and to compare the efficiency various culture methods.

Eventually, the microchannel system may also be useful to personalize embryo culture according to the individual needs of each embryo to compensate deviations in metabolism [9]. However, caution is suggested while using this approach. It should be considered that embryos are autonomous living beings with proven ability to establish their proper microenvironment even under compromised conditions. On the other hand, their adaptation ability to the ever-changing environment may be limited, and continuous or frequently repeated flushing even with the most sophisticated solutions may cause more problems than benefits. A proper use of the enormous possibilities offered by the microchannel system may help to find the right compromise and to bridge the existing gap between the technology level of laboratory embryology and that of other prominent branches of science.

An ideal system should also reduce risk of mistakes providing secure identification of the biological material during each stage of a patient's cycle. Measures, such as labeling of all lab ware and double-witnessing protocols, are currently employed in IVF laboratory worldwide. Recently, innovative solutions for electronic witnessing that allow automatic recognition and confirmation of sample identity and matching have been developed as an alternative to double witnessing (Fig. 9.2). This is already possible by using Radio Frequency Identification (RFID) technology to track and record patient samples monitoring all critical steps carried out in the laboratory (RI Witness™ Research Instruments, UK). In future, direct tagging of embryos through the microinjection of silicon-based barcodes in the perivitelline space could be considered to minimize mismatching errors during ART procedures.



**Fig. 9.2** Electronic witnessing using Radio Frequency Identification (RFID) technology

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