Transport analysis of bioreactors for the in vitro culture of ovarian tissue

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Summary

Many papers have recently reported on the in vitro culture of ovarian tissue or isolated follicles. Strips of ovarian tissue have been cultured to understand the mechanisms and the hormonal requirements of the activation of primordial follicles, their growth, and development of steroidogenic capacities. Isolated follicles, encapsulated in hydrogel matrices, have been cultured to study how to promote follicle development in vitro and how to exploit the reproductive potential of cryopreserved ovarian tissue of pre-pubertal girls with premature ovarian failure or of women needing immediate cancer treatment. Culture techniques have also been proposed as in vitro models to study folliculogenesis, to screen new drugs, and for toxicological studies. Many publications address the optimization of culture media and supplements provision, and of the matrices used for encapsulating ovarian tissue and follicles. Bioreactors have been proposed which are suited to the small scale of the tissue samples, varying for configuration and operation mode with the aim of enhancing and controlling transport of nutrients, oxygen and biochemical cues to tissue. The broadly differing bioreactor types and operating conditions that have been proposed suggest that the optimization of bioreactor design is often approached in empiric fashion without paying due attention to the interplay of occurring flow and mass transport phenomena and cells metabolism.

In this paper, the theoretical aspects of solutes transport to cells in ovarian tissue or encapsulated follicle preparations are briefly discussed, and used for analyzing the relevant transport features of some bioreactors proposed for ovarian tissue culture. The analysis suggests that a rational and multidisciplinary approach to bioreactor design for ovarian tissue and follicle culture in vitro is highly desirable to exploit the full potential of in vitro culture techniques and to advance knowledge in the field of assisted reproduction.

KEY WORDS: bioreactor, design, follicle, in vitro culture, ovarian tissue.

Introduction

In 1996, it was reported the first successful in vitro culture of ovarian tissue in which murine oocytes were grown from primordial follicles to developmental competence eventually leading to live mice offspring (1). In the following twenty years, many in vitro systems have been proposed to culture fragments (i.e. strips) of ovarian tissue and isolated follicles to grow meiotically competent oocytes starting from follicles at varying developmental stages (2-4). In vitro culture systems have been proposed to achieve three main objectives: 1) to grow developmental competent oocytes starting from the large pool of primordial follicles, possibly from cryopre-
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served human tissue, and use them in assisted reproduction technology; II) to study folliculogenesis and the steroidogenic capacity of ovarian tissue; III) for drug and toxicological screening and research. Obtaining mature oocytes entirely in vitro from cryopreserved ovarian tissue would permit to exploit the full fertility potential of pre-pubertal girls with premature ovarian insufficiency or of women in need of immediate cancer treatment for aggressive malignancies, and to delay and plan pregnancy. Such a technique could also be useful to regulate fertility of commercially important domestic and laboratory (e.g. knock-out mice) animals, and to help prevent extinction of endangered animal species. What makes in vitro culture systems attractive for research purposes, whether to understand the physiology of ovarian tissue or for toxicological screening and research, is that such models permit experiments that cannot be performed in vivo. In fact, they would allow for varying the culture environment and the concentration challenge to which tissue is exposed in highly controlled fashion thus helping identify the mechanisms of action of extra-ovarian factors (e.g. metabolic substrates, hormones, nutrients or toxicants).

The core of any in vitro culture system is the bioreactor. A bioreactor may be defined as a vessel or volume in which many biochemical or biological reactions occur for the presence of biocatalysts, independent of whether they are cells or tissues (5). Bioreactor design is the decisional process with which it is defined the bioreactor configuration (i.e. the bioreactor structure, the way the feed stream(s) is(are) fed to the bioreactor, the biocatalyst culture technique (e.g. whether the biocatalyst is immobilized and how), the way the bioreactor exchanges mass and heat with the environment, and the materials of which the bioreactor is made), the bioreactor geometry (i.e. its geometry and volume), and the bioreactor operating conditions (i.e. the conditions under which the bioreactor should be operated (e.g. T, pH, medium composition, feed flow rates, pressure, etc.) to deliver the expected performance) (6, 7). Some exemplary bioreactors used for the in vitro culture of ovarian tissue or follicles are schematically reported in Figure 1.

From a structural point of view, most of them are modifications of the conventional batch-operated two-dimensional (2D) culture dish to host small three-dimensional (3D) tissue samples, or follicles, in a niche where paracrine signals may accumulate. An issue that has been extensively investigated is the optimization of the biomaterials in contact with ovarian tissue or follicles, or used for encapsulating the isolated follicles (and by doing so optimize the tissue/follicle culture technique). The in vitro culture of strips of fresh human ovarian cortical tissue on cellulose membrane inserts coated with Matrigel® was reported to yield a higher proportion of viable follicles than on uncoated membranes after 7 to 11 days of culture (8, 9), more so when Matrigel® diluted 1:3 with medium was used (9). It has been shown that the in vitro culture of isolated follicles in direct contact with hydrophilic culture dish surfaces (e.g. on surface-modified dishes for tissue culture) causes the shedding of granulosa cells (GCs), that end up adhering at the dish surface, and follicular flattening (1). Coating 2D polymeric membranes or dishes with collagen or poly-L-lysine was reported to prevent flattening of isolated rodent preantral follicles by binding the outer granulosa cells (GCs), which prevents further GC migration away from the oocyte (10-12). Follicle culture on 2D hydrophobic membranes was also reported to preserve the follicle three-dimensional (3D) structure by preventing cell attachment and migration (13). Maintenance of the structural integrity of follicles ensures the connections and the bidirectional exchange of biochemical signals between the oocyte and the surrounding granulosa cells that are essential for follicle growth (14). In vivo, the natural extracellular matrix (ECM) of the ovarian tissue provides for 3D scaffolding of follicle cells but it also provides for the immobilized biochemical cues that regulate cell behavior, differentiation and secretory activity (15). In the effort to mimic the natural ECM functions, natural and synthetic hydrogel matrices have been used for the 3D encapsulation of isolated follicles. Collagen, a natural protein of the ovarian ECM, and alginate, a polysaccharide isolated from seaweed, have mainly been used for the purpose, either as such, or blended with other ECM proteins (e.g. fibronectin or laminin), or modified by grafting on the main polymeric backbone biochemical cues lacking to the native polymer, as was the case of the grafting of the arginine-glycine-aspartic acid (RGD) moiety on alginate (3). The hydrogel chemical composition, physical prop-
The optimization of the culture environment in vitro has been the focus of the majority of the reports. Briefly, there is general agreement that culture media for the in vitro growth and maturation of follicles should supply nutrients, electrolytes, antioxidants, amino acids, energy substrates, vitamins and growth factors. As a result, culture media have been tested ranging all the way from pure serum to commercial salt solutions containing serum or human serum albumin (HSA) as a protein source, to defined base media (e.g. minimum essential medium or Waymouth medium) in the effort to minimize tissue exposure to unknown allo-/xenogeneic biological components. There is general agreement on the fact that supplements, such as hormones and signaling molecules (e.g. FSH and LH), should be better supplied to tissue in a timely pattern that mimics how they appear in vivo as follicles are activated, grow and mature. The optimization of the perifollicular dissolved oxygen concentration has been approached by culturing ovarian tissue and follicles in 2D bioreactors un-
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Theoretical aspects of solutes transport to ovarian cells in vitro

The extent to which the interplay between solutes transport and cellular metabolism influences the distribution of solutes concentration throughout the bioreactor volume strongly depends on how the bioreactor is operated (21). Bioreactors for the in vitro culture of ovarian tissue have been proposed that are operated in batch or continuous-flow mode. In batch operation, culture medium, containing nutrients and biochemical cues, is loaded in the culture dish (vs tube) where it contacts the tissue. Tissue is either laid at the dish bottom, or is held in suspension by hanging on a mechanical support or by fluid-mechanical stirring. In the case of ovarian tissue, batch culture dishes have mainly been statically operated by culturing tissue underneath a stagnant layer of medium. Oxygen is continuously supplied to ovarian cells by passive diffusion from the gaseous phase above the medium. A new batch of nutrients, biochemical cues and metabolic challenges is provided (the latter are timely supplied) as a bolus when fresh medium replaces spent medium. Only in very few cases, batch culture dishes have been operated dynamically, either by recirculating the medium with a pump (22), or by positioning the dishes on a shaker during culture (23), or else by culturing tissue in a batch-operated rotating wall vessel bioreactor (24). In continuous-flow operation, a stream of medium is continuously fed to the bioreactor through its inlet, contacts the ovarian tissue, and leaves the bioreactor. In single-pass mode, fresh medium is continuously fed to the bioreactor, is metabolized by the ovarian cells, and spent medium leaves the bioreactor through its outlet. In recirculation mode, part, or all, of the medium stream leaving the bioreactor is fed back (i.e. recirculated) to the bioreactor inlet, where it mixes with fresh medium before re-entering the bioreactor. It should be noted that the bioreactor classification suggested above may give different results depending on the species considered. For instance, in a continuous-flow bioreactor for ovarian tissue culture indeed medium is continuously fed to the bioreactor, but the bioreactor is operated batch-wise with respect to the ovarian tissue, which is loaded in the bioreactor at the beginning of culture and recovered at the end. In the following, the qualitative concentration profiles in a batch...
and a continuous-flow bioreactor for ovarian tissue culture are briefly discussed with respect to a solute which is metabolically consumed by cells (e.g. nutrients or dissolved oxygen) under the simplifying assumption that tissue covers the entire surface of the bioreactor bottom.

In batch-operated static bioreactors (e.g. culture dishes), medium composition is generally assumed uniform and equal to that in medium bulk (i.e. at a far enough distance from the tissue surface at which concentrations do not change spatially, or at the gas-medium interface in the case of dissolved oxygen). Nutrients concentration in medium is also assumed equal to that in the medium loaded in the bioreactor at the beginning of culture. Different from that assumed, as shown in Figure 2a, to supply the tissue with nutrients and oxygen, nutrients and dissolved oxygen concentration spatially decreases from the bulk medium to the follicle (vs oocyte) surface to an extent that depends on ovarian cells metabolism and on medium and stromal tissue resistance to solutes transport. To be transported to cells, oxygen in the gaseous phase has also to dissolve in the underlying liquid phase (e.g. medium or mineral oil). Oxygen dissolves in the liquid at the gas-liquid interface at a concentration in equilibrium with its partial gas pressure in the gaseous phase, which is much smaller than that for its scarce solubility in the liquids used (much more so in medium). The concentration of consumable solutes generally decays more steeply inside the tissue (than in medium) for the metabolic consumption of stromal and follicular cells and the higher transport resistance of the extracellular matrix (ECM). The concentration of metabolic wastes may be expected to decrease in space in the opposite direction (i.e. from the follicle surface to medium bulk), and to increase in time anywhere in the bioreactor as a result of cells metabolism. Dynamic operation (e.g. by mixing medium with a mechanical stirrer or by flowing medium along or around the tissue) decreases solutes resistance to transport of the medium layer above the tissue, and increases nutrients and dissolved oxygen concentration at the follicle surface with respect to static operation. Nutrients concentration at any position in the bioreactor continuously decreases also in time, during culture, as cells consume them. Any increase of ovarian cell metabolic activity during culture (e.g. as follicles are activated and grow) causes the dissolved oxygen at any position in the bioreactor to decrease also in time and accelerates the decay of nutrients concentration.

As shown in Figure 2b, in continuous-flow bioreactors, dissolved oxygen, nutrients and biochemical cues are generally continuously fed to the bioreactor with the entering medium, only. Then, they distribute in the medium bulk along the bioreactor, are transported from the medium bulk to the ovarian cells in the tissue, and spent medium, depleted of nutrients and oxygen and enriched in waste metabolites, continuously leaves the bioreactor. Ovarian cells metabolism, and medium and stromal tissue resistance to solutes transport, makes nutrients

![Figure 2](image-url)

Figure 2 - Scheme of oxygen concentration profiles that establish in the medium and in a strip of ovarian cortical tissue cultured in vitro in bioreactors operated in different modes: a) static culture dish; b) continuous-flow bioreactor. Please, notice that drawings are not in scale.
and dissolved oxygen concentration at any distance from the bioreactor inlet decrease in a direction perpendicular to the tissue surface from the bulk medium to the follicle (vs oocyte) surface. Depending on the distribution of matter in the bioreactor (hence its fluid dynamics) and on the spatial distribution of cellular metabolic activity in tissue, the nutrients and dissolved oxygen concentration in the medium bulk may decrease in a direction parallel to tissue surface from the bioreactor inlet to the outlet (25, 26). As follicles are activated and grow during culture, the increasing cellular metabolic activity may make nutrients and dissolved oxygen concentration at any location in the bioreactor decrease also in time.

That reported above suggests that the actual dissolved oxygen and nutrients concentration at the follicle outer surface (e.g. perifollicular) (and at the oocyte outer surface) generally is lower than that in the medium bulk, and that its actual value strongly depends on the interplay between cells metabolic consumption in the tissue and solutes transport across medium and stromal tissue. When follicles are cultured in situ inside a strip of ovarian cortical tissue, their activation and growth may benefit of the physiological autocrine and paracrine signals provided by the neighboring follicles and the stromal cells. However, the metabolic consumption of nutrients and dissolved oxygen of stromal cells and the resistance to solutes transport caused by the densely concentrated stromal protein in the extracellular matrix may dramatically decrease the perifollicular solutes concentration with respect to the medium bulk. When a single isolated follicle is microencapsulated in a hydrogel and cultured in vitro, the absence of stromal cells and other neighboring follicles reduces the decay of nutrients and dissolved oxygen concentration inside the microcapsules, and allows for a better control of the follicle microenvironment. However, if the production process causes formation of a poorly permeable layer around the microcapsule, this may act as a selective barrier and hinder the free transport of large molecular weight solutes (e.g. metabolically relevant proteins and biochemical cues such as FSH and LH) from the medium bulk to the outer surface of the microcapsule, and eventually to the follicles. Follicle metabolic processes also lack the physiological paracrine signals that the stromal cells provide in natural tissue.

**Transport analysis of exemplary bioreactors**

The choice of bioreactor design for the in vitro culture of ovarian tissue is often determined by the bioreactor types available in the laboratory and by the operator’s experience. As noted above, ovarian tissue is mainly cultured in vitro either to direct the activation, growth, and ideally maturation of follicles from the primordial stage to the meiotically competent stage, or to study folliculogenesis or drug effects on ovarian tissue. The basic requirement that a bioreactor should fulfil for either application is that it should enable maintenance of the ovarian cells in a viable and functional state for a long enough time for the desired metabolic changes to occur. However, the requirements specific to each application are rather different. In the former, the bioreactor design should timely provide ovarian cells in the stroma or in the hydrogel microcapsule with concentrations and concentration gradients of exogenously administered dissolved metabolic effectors similar to those that in nature determine cells metabolic changes inducing meiotic competence. In the latter, the bioreactor design should permit to challenge ovarian cells with measurable and controlled concentrations of drugs and drug metabolites that vary in time similar to the drug presentation and elimination pattern in the natural ovarian tissue. The general transport analysis suggests that seldom one bioreactor design fulfills the requirements of all possible applications, and may be used to sort out the bioreactor more suitable for a given application among those available. For the sake of the example, in the following, qualitative information is gathered on the main transport features of the bioreactors shown in Figure 1 with the help of the proposed brief transport analysis. Hereinafter, each bioreactor is indicated with the letter used in Figure 1 (e.g. the bioreactor shown in Fig. 1f is indicated as bioreactor 1f).

The broad variability of the physical-chemical properties of the species relevant to ovarian cells metabolism hinders a generalized analysis. In the following, the bioreactors shown in Figure 1 are analyzed with respect to the transport of dissolved oxygen, as representative of low molecular weight (LMW) solutes, to ovarian tissue strips, the most challenging case for transport. Reason for this is that experimental evi-
Batches of the tissue culture dish type are easy to set up and operate, cheap and their operation is generally well established, both in the laboratory and the clinical setting. They all share some of the common features previously discussed, but may differ from one another in terms of oxygen transport. In bioreactors 1a-d, f, oxygen is supplied to the ovarian cells only through the upper and lateral surfaces of the ovarian strips (29-31). This suggests that steep gradients of dissolved oxygen concentration likely establish across the tissue thickness, that the dissolved oxygen concentration profiles in the tissue strongly depend on the shape of the strip at any given gaseous oxygen tension, and that supplying physiological amounts of oxygen to cells anywhere in highly metabolically active strips may be difficult. In the oil overlay drop culture in static batch dishes (Fig. 1c), mineral oil is laid on top of the medium drop to stabilize its shape and to prevent evaporation of the medium water (32, 33). As compared to bioreactors 1a,b, oxygen partitioning in the oil decreases its concentration at the oil-gas interface (although to a lower extent than in medium), and the oil layer above the strip causes an additional resistance to oxygen transport to the tissue. This is partly balanced off by the fact that the surface through which oxygen is transported is greater than that in bioreactor 1b, at given medium volume, although the varying shape of the drop makes it difficult to estimate reliably the actual oxygen-exchanging surface area. It should be noted that bioreactors 1a,b enable tissue culture under thinner, and more finely controlled, thicknesses of medium, at any given medium volume per unit strip.

Oxygen delivery to tissue in bioreactors 1d-c is generally more efficient because oxygen is supplied also through the bottom surface of the tissue strip. The larger oxygen-exchanging tissue surface is expected to result in smoother dissolved oxygen concentration gradients across the tissue thickness and in the enhanced capacity to supply physiological amounts of oxygen also to cells in metabolically active ovarian tissue strips. In the hanging-drop, or inverted drop, batch bioreactor 1d, medium and one strip of tissue is loaded in each well of a multiwell plate, which is then inverted and laid on the periphery of a culture dish so that a drop forms that protrudes from each well into the dish (34-36). Water at the bottom of the dish minimizes water evaporation from the drop. Gravity causes the strip of tissue to fall towards the bottom of the drop at the interface between medium and the oxygen-rich gas. This minimizes the length of the oxygen diffusion path to tissue and medium resistance to oxygen transport to the top of the strip, as compared to bioreactors 1a-c. To reach the bottom of the strip, gaseous oxygen dissolves in medium and diffuses around the tissue strip. Tissue oxygen consumption coupled to the resistance to dissolved oxygen transport from the gaseous phase to the bottom of the strip in bioreactor 1d causes the strip bottom to be cultured at lower oxygen concentrations than in bioreactor 1e, more so in the culture of metabolically active strips. The culture of large number of follicles in bioreactor 1d is also more delicate and labor-intensive. Moreover, it is difficult for the operator to maintain the shape of the hanging drop constant and the position of the strip fixed in the medium drop at any change of medium. This makes control of the oxygen transport resistance of the upper and lower medium layers (hence of the dissolved oxygen concentration at the strip surfaces) awkward and less reproducible than in bioreactor 1e.

Operating a bioreactor in dynamic mode is generally more labor intensive than in static mode, more prone to faults, more expensive to operate, and requires personnel with specific training. Bioreactors 1f-h are exemplary types operated in dynamic mode. Bioreactors 1f,g,h are batch-wise, whereas bioreactor 1h is operated in continuous-flow mode. They also differ in the way oxygen is externally supplied to tissue. Similar to bioreactors 1a,b, in bioreactors 1f,g, oxygen is supplied from the external gaseous phase, either directly through the gas-medium free interface (bioreactor 1f) (22), or indirectly through an oxygen permeable membrane separating the tissue medium from the environmental oxygen-rich gaseous phase (in the rotating wall vessel bioreactor 1g) (24). Continuously flowing medium along tissue surface, or rotating medium to keep tissue in suspension, generates some degree of mixing. This decreases the oxygen transport resistance of the medium layer outside the strip and adhering to it (and decreases...
es the dissolved oxygen concentration decay associated with it), as compared to static bioreactors 1a,b. Similar effects may be obtained by culturing ovarian tissue in orbiting test tubes (37) and in rolling bottle culture (38). High rates of rotation or flow may enhance mixing and cause a significant reduction of oxygen transport resistance external to the strip, and increase of the dissolved oxygen concentration at the strip surface. However, the high mechanical stresses caused by such high rates (and tissue bumping against the bioreactor walls) may damage the ovarian tissue, as was the case of isolated follicles cultured in the rotating wall vessel bioreactor (24). Moreover, thus far the distribution of matter in the (medium) volume of these bioreactors has never been characterized, nor optimized. This is particularly critical because in the proposed bioreactors the location and position of tubing at the site of medium suction and return and of the tissue strips is rather arbitrary (and often variable during culture) with unknown effects on the pattern of medium flow and the distribution of solutes in the bioreactor and around the tissue strip. Under these conditions, it cannot be excluded that shunt flows and/or stagnant zones form, in which medium is channeled without contacting the tissue strip or tissue rapidly depletes oxygen without any possibility for fresh medium to replenish it, respectively. This makes it very difficult to control oxygen transport to tissue and oxygen concentration at the tissue periphery anywhere in the bioreactor, and to prevent possible tissue necrosis for a possible lack of nutrients or oxygen. In continuous-flow bioreactors (e.g. the microfluidic bioreactor 1h), and independent on whether they are of the macro- or the micro-scale, oxygen is supplied only with the medium entering the bioreactor. In these bioreactors, the efficiency of oxygen transport to tissue depends on medium oxygenation prior to entering the bioreactor, on medium flow rate, and on the actual oxygen consumption rate of ovarian cells in the tissue. The actual flow pattern of medium strongly depends on the tissue size and shape, and on how and where tissue is held in position in the bioreactor, as well as on the design of the bioreactor vessel. In the small-scale microfluidic bioreactor 1h, tissue is located in a microchannel directly in the path of the flowing medium. Depending on medium flow rate, the thin gap through which medium flows around the tissue strip may cause the flowing medium to exert significant mechanical stresses on tissue and oxygen depletion in the flowing medium. Also in these bioreactors, the distribution of matter has not been generally characterized. This makes it difficult to estimate the extent to which the actual solute concentration at the tissue outer surface, and its distribution around tissue, differs from that in the stream entering or leaving the bioreactor, which are often the only concentrations that can be measured. It should be noted that the stiff and thin microchannel typical of bioreactor 1h may not accommodate the large size increase of growing follicles, thus making such bioreactors unfit for the purpose. In spite of their downsides, the unique possibility that these bioreactors offer to easily reduce solutes transport resistance external to tissue, to control solutes concentrations at the tissue surface, and to enhance solutes transport to ovarian cells, makes bioreactors 1f-h attractive for studying ovarian follicles or tissue metabolism, as well as tissue response to a drug challenge. Continuous-flow operation may also enable the attainment of a pseudo-steady state and may help minimize the hassle of culturing tissue under time-varying operating conditions. The downside is that these bioreactors require the use of larger volumes of culture medium than batch bioreactors. This may make detection of metabolites produced by the cells more challenging because, at a given metabolic production rate, they dilute in a larger bioreactor volume.

Similar considerations to dissolved oxygen hold true for the transport of other LMW nutrients and cellular metabolites to/from the ovarian cells, possibly but for the delivery route. Care should be used to extend these considerations to the transport of high molecular weight solutes (e.g. hormones and serum proteins) and of lipophilic compounds (both secreted by ovarian cells and exogenous). In fact, depending on the physical-chemical and transport properties of the compound and of the materials used in the bioreactor, separation, adsorption and absorption phenomena may significantly change the actual concentration of such species in the close vicinity of the ovarian cells. In bioreactors 1d, polymeric membranes with maximal pores a fraction of a micron large are laid at the bottom of the insert to hold the tissue strip in the insert. These membranes often
feature a hydrophobic polymeric backbone on which large proteic species with biological activity (e.g. FSH, LH) or from serum may adsorb. Adsorption to the membrane pore surface may hinder the transport of exogenous large compounds to tissue either directly, by removing them from medium, or indirectly, by fouling the membrane pores. In fact, pore fouling reduces the size of the pore channels actually available for the compound transport so that proteins may not freely pass across the membrane wall (39). Adsorption on hydrophobic plastics may also physically remove from medium significant amounts of lipophilic species (or of species with hydrophobic domains in their backbone) in bioreactors (40), in particular those with a high material surface-to-medium volume ratio, such as the multiwell plate bioreactor 1b or the microfluidic continuous-flow bioreactor 1g. In the batch culture of ovarian cells, it has been shown that absorption in the mineral oil overlaying the cells (41, 42) and in the silicon inserts used to minimize the medium volume per unit tissue mass (and maximize the accumulation of autocrine and paracrine factors during culture) (43) may significantly decrease the concentration of steroids (e.g. progesterone and estradiol) secreted by ovarian cells and of growth hormones and supplements exogenously administered (e.g. cholesterol or embryotoxic drugs). Absorption of lipophilic species in mineral oil or the silicon inserts may also considerably change the relative abundance of free fatty acids as compared to that loaded with medium. It should be noted that the extent of lipophilic species absorption strongly depends on the physical-chemical properties of the materials used in the bioreactor and the compounds. For assisted reproductive technology, occurrence of such phenomena may influence tissue susceptibility to freezing, may hinder or cripple tissue capacity to grow, and may confound experimental attempts to optimize the culture medium composition for the in vitro maturation of oocytes. For folliculogenesis or drug toxicity studies, occurrence of such phenomena may prevent from the correct estimation of the perifollicular concentration of cell-secreted species, exogenous hormones, drugs and their metabolites, and may hinder the correct assessment of their effect on ovarian cells.

Conclusions and future outlook

The analysis of transport of exogenous and endogenous biochemical species in every bioreactor compartment and in ovarian tissue may shed light on the interplay between physical transport and cellular metabolic reactions, and on the extent to which this may affect the actual species concentration at the follicle or oocyte surface. The transport analysis may also help find quantitative relationships that permit to estimate the perifollicular (vs perioocyte) concentration of species from their concentration in the medium bulk, as a function of bioreactor and tissue geometry and the operating conditions. This is of utmost importance to timely change environmental culture conditions to guide follicle activation, development and maturation, or to assess correctly drug toxicity for ovarian tissue in a fashion relevant to pharmacological treatments. Unfortunately, the current lack of bioreactor characterization and of information on the transport and metabolic properties of ovarian tissue enables only qualitative considerations on the performance of the proposed bioreactors and hinders their optimization for any application. Such qualitative considerations are sufficient to evidence that significant differences may exist between the nominal culture microenvironment, characterized based on feed, bulk or initial species concentrations, and the actual culture microenvironment near the tissue. This may have contributed to some inconsistent results reported in literature. It also casts doubts on some conclusions on the metabolic behavior of ovarian tissue based on the outcome of in vitro culture experiments carried out under operating conditions characterized in terms of feed, bulk or initial concentrations of metabolically active solutes and proteins. The lack of quantitative information also hinders a thorough understanding of the effects on ovarian tissue metabolism, and fate, of culture in the various bioreactors proposed in literature.

The absence of vasculature and the lack of communication with other bodily tissues is a severe impediment to the in vitro culture of ovarian tissue explants, such as ovarian cortical fragments or isolated follicles. To overcome these limitations and enhance the long-term follicle viability and the number of follicles activated and pro-
gressing to meiotic competence, it is of utmost importance to optimize the bioreactor design for the in vitro culture of ovarian tissue. The lack of quantitative characterization of bioreactor fluid dynamics and transport properties of ovarian tissue, and the incomplete understanding of the physiology of folliculogenesis and of ovarian tissue metabolism makes such a task very challenging.

A feasible approach is to develop mathematical models that provide a quantitative description of transport and metabolic reactions of metabolically relevant species, biochemicals, and metabolites in the ovarian tissue, and mathematical models of flow and species transport in every region of the bioreactor. The former would provide a relationship between solutes concentration at the tissue outer surface and the perifollicular (vs periocyte) concentration at any position in tissue and at any time as a function of the strip morphology and composition, its geometry and shape, and follicle stage. Of the latter, the flow models would provide quantitative information on the flow pattern in the bioreactor and the fluid mechanical stresses exerted by the flowing medium on tissue as a function of bioreactor design and strip geometry and mechanical properties. The solute transport model would provide a relationship between species concentration in the medium bulk and their concentration at the tissue outer surface. Coupling the models of species transport and metabolic consumption (vs production) in the tissue to the models of flow and species transport in a given bioreactor could provide the operator with a precious tool for estimating species concentration in the close vicinity of follicles (or oocytes) in tissue from their bulk (or feed) concentration, at any given bioreactor and strip geometry and operating conditions. In assisted reproduction, this would make it possible to control the follicle microenvironment in in vitro culture, so as to maintain them in a viable and functional state long-term, and guide their progression to meiotic competence.

The methods of biochemical reaction engineering may come useful to characterize the distribution of matter in bioreactors. Yet, tissue metabolism and structure should be quantitatively characterized to develop semi-empirical or theoretical models providing a reliable description of species transport in tissue. It should be stressed that, to be of any use, the developed mathematical models should be extensively validated against experimental measurements.

A successful strategy to the optimization of bioreactor design for the in vitro culture of ovarian tissue for assisted reproduction, or pharmacological drug screening, requires to exploit the collaboration and synergy of researchers with different expertise (e.g. in the fields of embryology, gynaecology, material science, and bio-process engineering) possibly within the framework of large, and ambitious, research programs. It is hoped that the European and national governmental bodies responsible for clinical and scientific development will soon give such an opportunity to researchers active in the field of assisted reproduction techniques by launching large research grants finalized to this purpose.

**Disclosures**

The Authors declare no competing financial interest.

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