Pertinence of Apoptosis Markers for the Improvement of In Vitro Fertilization (IVF).

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Abstract

In assisted reproductive technology (ART), the pregnancy and birth rates following in vitro fertilization (IVF) attempts are still low. Recently, apoptotic markers have been suggested as new criteria for oocyte and embryo quality selection. Many studies have provided evidence that poor oocyte and embryo quality can be associated with apoptosis. The aim of this review is to summarize our current knowledge on the apoptotic process in oocytes and embryos, and focus on the possibility for using apoptotic markers as a reliable and predictive marker to select competent oocytes and embryos during IVF. Moreover, it is currently accepted that IVF failures, linked to poor embryo quality, are, in part, associated with suboptimal in vitro culture conditions. Here, we also review the current state of knowledge concerning how the genetic control of apoptosis during folliculogenesis and pre- implantation embryonic development is affected by in vitro culture conditions during IVF. In the future, identification of apoptotic markers in ART for oocyte and embryo selection should result in the development of new agonistic or antagonistic molecules of apoptosis by medicinal chemistry.

MESH Keywords Apoptosis ; Biological Markers ; Embryonic Development ; Female ; Fertilization in Vitro ; Humans ; Male ; Oocytes ; cytology ; Signal Transduction

Author Keywords IVF ; Apoptosis markers ; Genetic control ; Oocyte ; Embryo ; Microenvironment ; Diagnosis

Introduction

Infertility is a public health issue affecting some 60 000 reproductive-age couples every year in France. Female infertility represents 60 % of these cases. In vitro fertilization (IVF) is a possible solution for many of these couples. Despite the enormous progress made in IVF, many couples, however, are subject to repeated IVF failure and birth rates following IVF are still little more than 15 % per initiated cycle [1]. Though the reasons for this are both subtle and complex, poor quality of the embryos, that is itself conditioned by poor quality of the gametes, is obviously an important criterion. Many studies have provided evidence that poor embryo quality and bad grade (III or IV) can be associated with apoptosis [2 , 3]. Although apoptosis might be induced, or facilitated, on account of the sub-optimal in vitro culture condition of the embryo, its primary cause could also derive from apoptotic signalling already triggered in the oocyte. Therefore, the possibility of using apoptotic markers as reliable markers to select competent oocytes and embryos, which were hitherto selected by " operator- dependent" evaluation of morphological criteria, opens new perspectives in ART centers.

Here, we review the current state of knowledge concerning the genetic control of apoptosis in the oocyte and embryo as well as how this process is affected by in vitro culture conditions.

Apoptosis

Apoptosis is a form of programmed cell death that is indispensable to embryonic development, homeostasis and in the surveillance of aggressions such as infection, uncontrolled cell division or severe cellular damage [4]. During development, cells are produced in excess. Structures specific to each organ are produced by the selective ablation of certain cells by apoptosis. The number of cells in an organ is therefore finely controlled by an equilibrium between cell proliferation and cell death. The classical example is the morphogenesis of the fingers that involves the apoptotic demise of cells in the inter-digital spaces during embryogenesis. Similarly, the developing nervous system comprises a large excess of neurons, over half of which will be eliminated by apoptosis in order to establish correctly connected neural networks. Development of the reproductive system is no exception; for example the Müllerian ducts that develop into the uterus, the Fallopian tubes and the upper vagina are initially present in both the male and the female foetus, but are lost in the male through apoptosis. Dysregulation of apoptosis is associated with a number of human pathologies, such as Huntington's disease, Alzheimer's disease and AIDS as well as playing a preponderant role in carcinogenesis.

The morphological features of apoptosis

When cells die by apoptosis, they progressively acquire a characteristic morphology arising from profound changes in structure and function. These changes, that are very different to those observed in necrosis, allow the dying cell to be detached from its neighbours and

to be eliminated by phagocytosis. The structural modifications include nuclear and cytoplasmic condensation, the alteration of organelles such as mitochondria and lysosomes and the invagination of the plasma membrane and nuclear envelope [5 –7]. Membrane blebbing gives the cell the appearance of a bunch of grapes that can be easily visualised by real-time video microscopy [8]. The sum of these structural modifications leads to the fragmentation of the cell into membrane-bound apoptotic bodies that will be phagocytosed by surrounding cells [5 , 6]. Modification of membrane asymmetry, accompanied by the externalisation of phosphatidylserine (PS), allows the recognition of these apoptotic bodies by phagocytic cells.

It is possible to use a specific ligand of PS, annexin V, to detect and quantify apoptotic cells by immunofluorescence and flow cytometry (Fig. 1) [9]. The apoptotic process also involves the fragmentation of DNA by endonucleases. Genomic DNA is preferentially cleaved in the internucleosomal regions leading to fragments of DNA with sizes that are multiples of 200 base pairs. These fragments can be separated by agarose gel electrophoresis giving a characteristic ladder pattern. The fragmented DNA can also be detected by the histochemical labelling of the free 3′OH ends created by the endonucleases using a technique called TUNEL (**T** erminal deoxynucleotidyl Transferase-mediated d**U** TP-biotin **N** ick **E** nd **L** abelling) [10 –12].

Apoptotic Genes

Two complementary approaches, biochemical analysis and genetical analysis of simple model organisms (notably C. elegans and D. melanogaster), have been particularly fruitful in the description of apoptosis, which is the most abundant and best studied of the cell death mechanisms [13 , 4]. The core enzymatic machinery of apoptosis is composed of a family of cysteine proteases called caspases. To date, 14 caspases have been identified in man; though it is probable that this list is not exhaustive [14 –18]. Caspases are omnipresent in the form of zymogens, the activation of which is subject to a number of regulatory mechanisms [14 –18]. Members of the Bcl2 and IAP (Inhibitor of Apoptosis) families of proteins are fundamental elements in the pathways that control apoptosis [19 –21]. Two major pathways regulate apoptosis (Fig. 2). The extrinsic pathway is activated by the binding of ligands to membrane- bound death receptors of the TNFR family [22]. The intrinsic pathway involves a plethora of signals that converge on the mitochondria via the pro- and the anti-apoptotic members of the Bcl2 family [19 –23]. The extrinsic pathway links into the intrinsic pathway via a pro- apoptotic Bcl2-family protein, Bid [24].

Apoptotic phenomena have been described in the blastocyst and, more recently, throughout the development of the pre-implantation embryo [25 , 26]. The development of the embryos could well depend on an equilibrium between cellular proliferation and apoptosis. Accumulating data indicate that sub-optimal in vitro culture conditions severely affect the survival of the embryos. However, it is also highly probable that the poor quality of the fertilized oocytes, that are already apoptotically compromised, is a major factor in embryonic survival. This underlines the considerable importance of studying the genetic control of apoptosis in both the gametes and the pre-implantation embryos.

Oocyte apoptosis

During the foetal period

The definitive stock of primordial follicles, that will ultimately enter into the follicular growth phase, during adult life, is established during foetal oogenesis. More than 90 % of germinal cells present in the foetus at mid-gestation will have disappeared by birth. Experimental results from animal models confirm that this lost of germinal cells occurs through apoptosis during gonad mitosis, prophase of meiosis I and primordial follicle formation. Apoptotic cells have been identified, by in situ labelling, both in man [27 , 28] and in the mouse [29]. Owing to the limits imposed on the exploitation of human tissue samples, most available data derive from experiments with other animal species. The invalidation of genes in the mouse, especially members of the Bcl2 family and the caspases, have been invaluable in establishing their key roles in oocyte and follicular apoptosis [30 , 31]. The implication of these pathways in the regulation of germ-cell number in the human foetal ovary has largely been established using immunolocalisation techniques. Thus, certain members of the Bcl2 family of proteins, such as the anti-apoptotic Bcl2 and Mcl1 and the pro-apoptotic Bax [28 , 32] in addition to caspases 2, 3,7,8 and 9 have all been detected [33 , 34]. The presence of cleaved, active, forms of the initiator caspases 8 and 9 and executioner caspases 2 and 7 have been confirmed by western blotting; although this is not the case for caspase 3, one of the best characterised executioner caspases [33]. The presence of activated caspase 3 has been observed, however, by immunohistochemistry in all species tested. The number of germinal cells expressing active caspase 3 increases between the 14^{th} and 19^{th} weeks of gestation, but then falls to zero in primordial follicles in both human [33] and mouse [34]. The invalidation of the caspase 3 gene in the mouse suggests that this protease is primordial in the apoptosis of granulosa cells, but not in germinal cells, during ovarian development [35 , 36]. In contrast, the inactivation of the caspase 2 or Bax genes, in the mouse, leads to a dramatic increase in the number of primordial follicles in the post-natal ovary [37 , 38]. The study of the expression profiles of the actors and regulators of apoptosis, therefore, appears to be crucial in order to define the mechanisms and signalling pathways implicated in this physiological process.

A number of trophic factors, identified in the developing ovary, have been shown to reduce the loss of both human [39] and rodent [40] germinal cells in vitro . Although the causes, and the regulation, of this apoptosis are not yet fully understood, recent studies have

implicated the tyrosine kinase receptor TrkB whose principal ligands are the neurotrophins BDNF (Bone-Derived Neurotrophic Factor) and NT4 (neurotrophin 4) [41]. TrkB is a so-called dependence receptor that can transmit two opposing signals: a survival signal in the presence of the principal ligands, and an apoptotic signal in their absence. The implication of TrkB has been confirmed by the invalidation of its catalytic activity in the mouse, the use of specific inhibitors in in vitro culture of human and mouse ovaries and the localisation of neurotrophin 4 mRNA [41].

In the Adult

In the adult human ovary, the individual fate of a follicle (growth/ovulation vs atresia) is tightly regulated by a dialogue of death and survival signalling, including both endocrine factors (gonadotropins) and paracrine factors (e.g. GDF9, BPM15). Only a small proportion of the primordial follicles present at birth progress to ovulation, the others die by apoptosis during the regulated process of follicular atresia.

Two types of follicular atresia can be distinguished. Basal atresia principally affects reserve follicles as they start to grow. This type of atresia, that is observed throughout reproductive life, and that is independent of the menstrual cycle, is characterised by the premature involution of the oocyte and by mild nuclear fragmentation in the granulosa cells [36]. Preantral follicles, and follicles at the start of antrum, show high levels of atresia (≈ 30 %), whereas follicles between the sizes of 0.5 and 2 mm show lower levels (≈ 15 %). In addition to this basal atresia, larger follicles (> 2 mm) are subjected to cyclic atresia. This type of atresia starts with high levels of nuclear fragmentation in granulosa cells, followed by a rapid degeneration of the oocyte. The highest levels of atresia are observed in the mid-luteal phase (73 % for 2 – 5 mm follicles and 100 % for 6 – 10 mm follicles). A number of studies of human and mouse oocyte quality have reported high levels of DNA fragmentation, as analysed by TUNEL, associated with low fecundity [42 –44]. The expression of caspase 3 is greatly increased in pre-ovulatory follicles entering into atresia [45] and activated caspase 3 has been detected not only in the oocyte [34], but also in the granulosa cells of atretic follicles in the mouse ovary [46]. Apoptosis could, therefore, be a means of eliminating defective or fertilization-incompetent oocytes. The description of the apoptotic cascade, as well as its induction and regulation, have, and continue to be, the object of a very large number of studies (Table I). It is probable that the death receptor Fas expressed in the oocytes of primordial and primary follicles in the post-natal ovary, is involved in apoptotic signalling [43]. Fas ligand (Fas-L) is expressed in oocytes of primary, secondary and tertiary follicles, adding weight to the argument that the couple Fas/Fas-L is involved in the regulation of oocyte apoptosis [47]. Moreover, the activation of the Fas pathway during oocyte maturation in vitro increases the incidence of apoptosis of cells of the cumulus oophorus [48] and granulosum [49]. In addition, soluble Fas, a truncated form of Fas that acts as an inhibitor of Fas-induced apoptosis, is found at high concentration in follicular fluid and in the medium bathing the cumulus oophorus/mature oocyte complex [50]. Other factors that induce apoptosis have also been incriminated, including cytokines such as TNF-α and l'IL-6 [51 –53]. These latter may act either by inducing the uncontrolled production of free radicals, or by increasing intracellular Ca^{2+}, that in turn leads to endonuclease activation, either by activating pro-apoptotic genes of the Bcl-2 family (Bax, Bcl_{XS}), or by generating second messengers such as ceramide that lead to caspase activation [54 , 55]. Indeed, the knockout of either Bax or caspase 2 leads to a spectacular increase in the number of primordial follicles [57 , 37]. Nevertheless, these apoptotic signals are not present in the developing follicle in normal conditions. One theory that could explain this is that cell proliferation activates a default apoptotic programme that, unless countered by survival signalling, leads to the inexorable demise of the cell. Here, the principal survival signal is Follicle-Stimulating Hormone (FSH) that is prerequisite to the survival of post-antrum stage follicles. FSH both inhibits the expression of pro-apoptotic genes of the Bcl2 family (Bax and Bcl_{XS}) and stimulates the production of growth factors, such as EGF, TGFα, bFGF and the IGFs, that have a positive effect on the oxidative stress response. In addition, when small antral follicles from rat are cultured in the presence of FSH, they show increased follicular growth, reduced apoptosis and an increased expression of XIAP (X-linked Inhibitor of Apoptosis) [58]. Originally identified in baculovirus, the IAPs are a family of survival proteins that regulate cell fate by modulating post-mitochondrial apoptotic signalling. Several members have been identified in man, including XIAP [59]. A causal relationship between XIAP and apoptosis has been confirmed by the fact that inhibition of its expression induces apoptosis and represses follicular development, even in the presence of FSH. XIAP would, therefore, seem to play a key role in FSH-stimulated follicular development. We have recently demonstrated, by transcriptome analysis, that oocytes also strongly express two other anti-apoptotic factors, Bcl2L10/DIVA and survivin (another member of the IAP family), as well as a pro-apoptotic factor (BNIP1) [60]. The equilibrium between these pro- and anti- apoptotic factors may contribute to the regulation of oocyte apoptosis in the adult.

It is interesting to note that, contrary to the well established anti-apoptotic role of gonadotropins during early antral follicle development, Luteinizing Hormone (LH) and FSH stimulate caspase 3 and caspase 7 activities in in vitro cultures of rat pre-ovulatory follicles, potentialising apoptosis in theca cells but not granulosa cells [61]. This strongly suggests that apoptosis of theca cells is a physiological process that is necessary for gonadotropin-induced ovulation [62].

Although the growing body of literature that has been generated over the past few years has permitted the identification of the major actors regulating apoptotic signalling in oocytes, the reason that it should occur at all remains a mystery. A number of hypotheses might be suggested, such as the selection of oocytes prior to ovulation or the surveillance of their genomic integrity.

Apoptosis in early stage of embryos development

The term early embryo stages refers to the developing from the zygote (resulting from the fertilization of the oocyte) to the moment that it implants in the uterus, at the blastocyst stages, and establishes an intense molecular dialogue with the maternal tissues. Indeed, during this period, zygote migrates along the genital tract to the uterus and the embryo undergoes important developmental stages and cellular differentiation. The apoptotic phenomena, observed throughout the development of the pre-implantation embryo, are necessary for the elimination of genetically abnormal or mutated cells [3]. The degree of apoptosis, that is particularly elevated at the blastocyst stage, however, cannot exceed a certain threshold without leading to the collapse of embryonic homeostasis and developmental arrest. Despite this “natural selection” during the pre-implantation stages, there is still a substantial loss (estimated at 20 to 25 % in vivo) on account of defective implantation [63].

The critical phase of implantation process, occurring between the sixth and the fifteenth day post fertilization, has been little studied because of difficulties encountered in the culture of human embryos from these stages of development. Despite efforts to improve the culture conditions (reduced glucose concentration, amino-acid supplements, growth factors in the culture medium, controlled hypoxia) of early stages of in vitro embryo culture, only 20 – 40% of embryos reach the blastocyst stage after 5 – 6 days post fertilization, and very few of these have the morphological characteristics of implantation-stage embryos.

Embryos are selected essentially on the basis of morphological criteria: number and regularity of blastomeres, rate of development, and embryonic fragmentation percentage [64 , 65] (Fig. 3). The low implantation rate of embryos aged between 2 and 3 days (10 – 12 %) might be explained by uterine hyper-motricity and the inappropriate timing of transfer. Indeed, these embryos are placed in an unsuitable environment because, in vivo , they would still be in the Fallopian tubes, and would not reach the uterus until the blastocyst stage i.e. 5 to 6 days post fertilisation. This is one of the reasons that have led to the in vitro culture of embryos up to the blastocyst stage. In addition to synchronising the embryo to the endometrium, the prolonged culture in vitro also allows the selection of the most competent embryos, with the highest developmental potential, and the elimination of those that are blocked at a stage corresponding to genomic activation (6 – 8 cell stage, D3 post-insemination). Thus, most IVF teams transfer 1 or 2 embryos at the blastocyst stage and achieved better pregnancy success rates per blastocyst compared to embryo transfer on day 2 or 3 [66]. In many embryos, the embryonic genome is not activated and they remain blocked at the 4 – 8 cell stage. Arrested development at this stage is possibly due to genetic factors, apoptosis, maternal age or paternal genome.

In addition, around two-thirds of the embryos obtained show cellular fragmentation > 15%. This fragmentation is observed in both in vivo and in vitro fertilized embryos [63 , 2]. The origin, the role and the mechanism of this fragmentation remain to be clarified. However, there is now little doubt that it does affect embryonic development. Indeed, a number of studies have highlighted a negative correlation between the degree of fragmentation and development to the blastocyst stage; excessive fragmentation being associated with diminished embryonic viability and a low implantation rate [67]. Several hypotheses have been proposed to explain the negative effect of fragmentation on pre-implantation embryonic development. First, the fragments could physically disrupt cell-cell interactions, interfering with embryonic development, especially during compaction, cavitation and blastocyst formation [68 , 69]. Secondly, the fragments could reduce the cytoplasmic volume, depleting essential organelles or polarised domains that are indispensable for correct development [70]. Thirdly, ultrastuctural analysis reveals that blastomeres that are adjacent to fragments show signs of degeneration; probably due to the liberation of toxic substances by the fragments [71 , 2].

On account of the resemblance of these fragments to apoptotic bodies, a number of groups have taken an interest in the role of apoptosis during the development of pre-implantation embryos cultured in vitro [72]. It is probable that the activation of apoptosis in human pre-implantation embryos prevents the transmission of damaged DNA. This apoptosis is preponderant after compaction, mainly at the morula and blastocyst stages, initially amongst the embryonic stem cells (cells of the inner mass) and later throughout the foetus [70]. In embryos with low, or no, fragmentation, 7–8% of the cells of the inner mass undergo apoptosis. Two hypotheses have been proposed. In the first hypothesis, the number of cells in the inner mass of the developing blastocyst is maintained at equilibrium by regulated proliferation and cell death [73]. The fact that embryos with a low morphological quality, and a reduced number of cells in the inner mass, have a lower rate of apoptosis than embryos that are morphologically perfect argues in favour of a role for apoptosis in the maintenance of cellular homeostasis. The second hypothesis proposes that, in the developing foetus, apoptosis eliminates cells with an altered genome. Indeed, aneuploid cells can appear in the inner mass. Until recently, these fragments were thought to be structures that are independent of the blastomeres, and simply derived from the mass of embryonic cells. This notion, however, is currently under debate. Several studies have reported a fragmentation of blastomeres as well as trophoectodermal cells [74 , 75]. Importantly, a recent study has shown that severe fragmentation, equivalent to the loss of at least one blastomere, at two days post fertilization, is associated with a drastic decrease in the rate of formation of blastocysts at six days post fertilization (12% vs 60 % in non-fragmented embryos) [76].

Human early embryos that are marked by an excessive fragmentation on day 6 post fertilization, or that have an arrested development, display a high proportion of cells undergoing apoptosis (over 15 % of the cells), characterized by cytoplasmic and nuclear fragmentation, condensed chromatin, fragmented DNA and phagocytosis. These characteristics have been observed for the most part by classical

microscopy techniques and have not been confirmed by biochemical markers such as TUNEL or annexin V binding [70 , 77 , 78]. Whether embryonic fragmentation is associated with the activation of an apoptotic cascade is, therefore, a valid question. The ratio of the expression of transcripts of pro-apoptotic (Bak, Bax, Bid, Bik, Bad) and anti-apoptotic (Bcl2, Bcl_{XL} , Mcl1, Bclw) members of the Bcl2 family is invariable throughout normal embryonic development in both man and mouse (Table I) [79 , 80]. At the protein level, only Bax is constitutively present, the other members of the family show variable expression levels throughout development, with some data in the literature being contradictory [80 , 81]. Bcl2 expression, however, appears to be restricted to specific cells of the inner mass, whereas Bax is localised in the cytoplasm of the blastomeres, the cells of the inner mass and the trophectodermal cells. Similarly, an increased expression of Bax has been described in human blastocysts with poor quality morphology [72 , 75], whilst Bcl2 has been reported to be over-expressed in mouse blastocysts with good morphology as compared to blastocysts with a fragmented morphology [79]. It would appear, therefore, that blastocysts with a high degree of fragmentation are characterised by disequilibrium between pro- and anti-apoptotic members of the Bcl2 family of proteins [79 , 72 , 82]. Caspase 2 and 3 transcripts are detectable at all developmental stages of the human pre-implantation embryo [82 , 76]. Increased caspase 3 activity, as well as increased expression of Harakiri, a pro-apoptotic protein of the Bcl2 family, has been reported in fragmented embryos as of the 4-cell stage [76].

Finally, survivin, a member of the IAP family, plays a key role in embryonic development. Survivin is highly expressed throughout embryonic life and is absent from differentiated tissues. It is re-expressed, however, in most common cancers and in tumour-derived cell lines [83]. The anti-apoptotic function of this protein is associated with its capacity to bind certain caspases (especially caspases 3, 7 and 9). Recently, alternative transcripts have been shown to give rise to proteins with different (and sometimes opposing) apoptotic or anti-apoptotic effects [84]. Survivin appears to act during mitosis. It associates with the mitotic spindle and appears to be regulated by the absence of tension at the kinetochores. Transgenic embryos, lacking survivin expression, die in utero from massive apoptosis [85]. Similarly, down regulation of survivin expression, by antisense RNA, provokes metaphase arrest and various anomalies, depending on the model studied. In the early mouse embryo this is translated by developmental arrest at the morula or blastocyst stage, followed by apoptosis [86]. In addition this apoptosis can be inhibited by inhibitors of caspases 3 and 9. Survivin is, therefore, an essential anti-apoptotic gene, that is expressed throughout all stages of development of the human pre-implantation embryo and that can protect embryos from apoptosis by inhibiting apoptotic pathways implicating caspases.

Despite some controversy over the role of apoptosis in inducing fragmentation, the altered expression of apoptotic regulatory proteins does seem to be linked to embryonic viability.

Micro-environment and apoptotic signalling

We have described above the state of our knowledge concerning the genetic control of apoptosis in the oocyte and the embryo, and will now examine the consequences of culture conditions on folliculogenesis and the development of the early embryo development.

It is currently accepted that IVF failures, linked to poor embryo quality, are, in part, associated with suboptimal in vitro culture conditions [74 , 78]. Apoptosis in early embryos might be provoked by the lack of maternally derived factors, such as essential growth factors and cytokines [87 –89]. However, the mechanisms that trigger apoptosis in suboptimal in vitro culture conditions have not yet been clearly identified. Ideally, an experimental model should take into account the three-dimensional (3D) structure of the tissue. Classical cell culture, however, obliges cells to adapt to physical and environmental constraints that are very different to those encountered in vivo . Indeed, cell behaviour has to adapt to the unnatural rigidity of the support and to the lack of other cells above and below. Within the organism, cells are organised into tissues and bound to the extracellular matrix that is composed of molecules such as collagen, fibronectin and laminin. The spatial constraints of 3D cell culture aim to recreate these conditions, such that the cells correctly perceive and interpret the biochemical and biophysical cues of the microenvironment; especially those related to their binding to the extracellular matrix and neighbouring cells and their response to growth factors [90 –93]. In the context of 3D culture, cells conserve the characteristics of a tissue, whilst remaining amenable to experimentation. The importance of 3D culture is especially crucial in the study of complex cellular systems, such as epithelia. Indeed the composition of the extracellular matrix, as well as its topographical organisation are essential elements contributing to the survival, migration and differentiation of epithelial cells that are otherwise unable to polarize correctly and differentiate [94]. Epithelial cell polarisation, that depends on binding to the extracellular matrix, via integrins, and to neighbouring cells, via adherent and tight junctions, both affects, and is affected by, intracellular signalling pathways including the PI(3)K pathway [95 , 96]. One of the effectors of this pathway is the serine/threonines kinase, Akt that constitutes a signalling node that regulates cell survival [97]. The substrates of Akt include several components of the core apoptotic machinery.

In conclusion, an understanding of the molecular mechanisms involved in cell death decisions necessitates the use of experimental models that recapitulate major aspects of physiological conditions, such as the extracellular matrix and growth factor composition.

Impact of the microenvironment on folliculogenesis

Culture medium and microenvironment

A number of studies have highlighted the importance of the culture media employed in in vitro maturation, as they affect not only the maturation of the oocytes, but also the subsequent development of the embryo. For instance, the use of basal media (bicarbonate buffered physiological saline containing pyruvate, glucose and lactate) with low concentrations of non-essential amino acids and vitamins, but lacking purine precursors (glutamine, glycine, aspartic acid) and hypoxanthine (responsible for meiotic arrest), and supplemented with human serum seems to significantly increase the survival and maturation of oocytes [98].

For over a decade now embryologists have recognized the importance of preserving the microenvironment [99]. Follicle culture, for instance, has given way to the culture of fragments of ovarian cortex as numerous studies have reported that the growth and development of the oocytes and follicles are better when the integrity of the oocyte/granulosa cell/follicular component interactions is preserved. Indeed, follicle survival is significantly increased when the ovarian tissues are only partially dispersed [100]. In these conditions it is possible to maintain inter-follicular interactions, as well as the action of various factors present in the follicular liquid. However, it is difficult to maintain the three dimensional nature of the tissue in vitro and the follicles degenerate after a few days in culture. The culture of follicles or ovarian tissues on a monolayer of extracellular matrix does allow the maintenance of follicular structure over a longer period of time [100], but the culture systems suffer from the lack of extracellular matrix contact on the upper side of the biological sample.

Hormones and Growth Factors

Primordial follicles present in the ovary represent the stock of follicles for the reproductive lifetime. As the follicle is characterised by rapid growth, intense interaction between follicle cells and growth factors, hormones and steroids is necessary in order to transmit elements indispensable for the maturation of the oocyte. Notably, cumulus oophorus cells secrete, in an FSH dependent manner, glycolytic intermediates (lactate, pyruvate …) as nutrients for the germline. Indeed, the oocytes, themselves, have little, or no, glycolytic activity [101] and increased glycolytic activity in cells of the cumulo-oocyte complex is associated with the complete maturation of human oocytes in vitro [102] (Fig. 4). The hormone FSH is already used to supplement most culture media used for the maturation of oocytes in vitro [102]. Protocols, however, remain to be standardised. Indeed, the concentrations of FSH vary between laboratories. In certain animal species, treatment with high doses of exogenous gonadotropins affects the pre-implantation period as well as post implantation development. Studies of cumulus cells-oocyte complexes in vitro have shown that the metabolism of pyruvate, but not that of glucose, is affected by high doses of FSH. This example highlights the urgent need to standardise protocols for the in vitro maturation of oocytes [102].

The mechanisms permitting the consumption of glucose by cumulus oophorus cells, following gonadotropic stimulation, is currently well understood and involves the activation of the PI(3)K/AKT (phosphoinositide-3-kinase/V-akt murine thyoma viral oncogene) [102 , 103]. This signalling pathway plays a determining role in the suppression of both spontaneous and gonadotropin-stimulated meiotic re-entry in oocytes [102]. Indeed, the low levels of Akt activity found in the cumulus, as well as those observed immediately after the collection of the oocte in its follicle, are necessary for meiotic arrest. Increased Akt activity favours the production of progesterone by the cumulus cells, which in turn favours meiotic resumption and survival signalling (Fig. 4). The pharmacological inhibition of the PI(3)K pathway, blocking the activation of Akt, increases caspase-3 activity and the proportion of apoptotic cells in the cumulus oophorus, and affects meiotic resumption stimulated by porcine oocytes [104]. The use of basal media for the culture of cumulo-oocyte complexes, however, does not allow the maintenance of Akt activity, which decreases drastically within a few hours of culture [104]. It is probable that the loss of survival signalling in the cumulus cells-oocyte complexes provokes the apoptosis of cells in the cumulus oophorus. As interactions between these cells and the oocytes are necessary for embryonic development, decreases in their numbers have a negative effect on development rate [105].

A number of factors are involved in follicular progression and growth; notably the tyrosine kinase receptor cKit, that is synthesized by the oocyte, and its ligand, KITL that is present at the surface of the follicle cells. Mice with naturally occurring KITL mutations form primordial follicles that are incapable of progressing to the primary follicle stage. All of the stages from the formation of follicles through their progression up to the secondary follicle stage are independent of hypophyseal gonadotropins, and are regulated instead by intra-ovarian paracrine factors. Of these, three are members of the TGFβ (Transforming Growth Factor) super family: AMH (Anti-Mullerien Hormone), that appears to drive primordial follicles into the following stages, GDF9 (Growth Differentiation factor) that is implicated in stages posterior to the primary follicle and BMP15 (Bone Morphogenetic Protein) that affects the proliferation of follicle cells [106 –110]. GDF9 is expressed very early during follicular development, and is secreted by oocytes within growing follicles [111]. The addition of GDF9 to the culture medium enhances survival and follicular progression up to the secondary follicle stage in cultured ovarian slices [112]. In this same model, other growth factors, such as IGF-I, IGF-II and insulin have been shown to be oocyte survival factors [113 –115].

Transcriptome studies, along with the completed genome sequences of several species (man, mouse, cow, pig etc .), have led to the identification of novel transcripts expressed in the developing ovary [116 , 117]. This should allow the improvement of ovary and early embryo culture conditions in the near future.

The impact of the microenvironment on the early embryo development

Studies with mice have demonstrated that in vitro culture increases cell death in the embryos to a degree that is dependent upon the culture medium [78 , 118]. There is now a good deal of evidence that the cellular microenvironment influences the degree of fragmentation in early embryo stages. For example, embryos cultured on a monolayer of feeder cells (e.g. fibroblasts) have a lower degree of fragmentation than those cultured alone [119]. Likewise, embryos cultured in small volumes, or those expressing growth factors and their receptors, have a lower incidence of fragmentation [120 , 121]. Thus, in order to take into account nutritional (modification of glucose metabolism) and environmental (passage from the Fallopian tubes to the uterus) changes of the developing embryo in vivo , two different culture media are used sequentially in most IVF centers today: one for culture up to the 4-cell stage, the other for culture up to the activation of the genome and compaction. Most mammalian cells cultivated in the absence of serum (or other extracellular signalling molecules), however, undergo apoptosis. In most cell types, the proteins necessary for the execution of the death program are constitutively expressed, but are maintained in an inactive state by extracellular survival signals. Although few data are available concerning the exact composition of the fluid surrounding the embryo in vivo , evidence is now accumulating as to the role of growth factors, of both maternal and embryonic origin, in the development of the pre-implantation embryo. Notably, the addition of growth factors, such as TGF-α, IGF-I, or insulin, to the culture medium is beneficial to the development of the embryo, increasing the formation and number of blastocyst cells (60% of embryos at the blastocyst stage in the presence of IGF-I vs 35% without IGF-I). In addition, the presence of IGF-I or TGF-α strongly represses the number of apoptotic nuclei in the human blastocyst [72 , 98 , 118 , 89]. Other growth factors, such as LIF (leukaemia inhibitory factor) and HB-EGF (Heparin Binding EGF) increase blastocyst formation by 24 and 30 % respectively [122 , 123]. Further studies will be necessary, however, before these results can be extrapolated in terms of implantation and clinical pregnancy rate.

Apoptotic markers and pre-implantation potential

Taken together, the above review of the literature suggest that apoptotic markers might be of predictive value in the selection of competent oocytes and early embryos of high quality. With this in mind, the results of Malamitsi-Puchner et al [49] are of particular interest as they underline the correlation between the level of soluble Fas in the follicular fluid and/or the cumulus cells-oocyte complex with both the morphological quality of the pre-implantation embryos and the clinical pregnancy success rate. Indeed, these authors demonstrate that high soluble Fas levels, in the follicular fluids, are a reflection of oocyte maturity. In addition, low levels of soluble Fas in the cumulus cells-oocyte complex are only observed following fertilization of the oocyte, when the morphology of the pre-implantation embryo is perfect or in the case of a successful clinical pregnancy [49]. However, this study, albeit promising, involved only a small number of patients and needs to be verified on a larger scale in order to confirm the predictive potential, for IVF, of soluble Fas levels in the follicular fluid and/or the cumulus cells-oocyte complex.

Other studies suggest that telomere length in the oocyte is a predictive factor of cytoplasmic fragmentation in the embryo [124]. Telomeres that are too short are known to lead to genomic instability, with unrepairable damage to DNA that can lead to apoptosis, suggesting that shortened telomeres in the oocyte might be responsible for increased apoptosis in the human pre-implantation embryo. However, the inverse correlation between telomere length, as measured by Fluorescent In Situ Hybridization (FISH), and cytoplasmic fragmentation has only been observed as of the 3rd day after fertilization, the moment at which the pre-implantation embryos are transferred into the uterus [124]. Taking into account the technical difficulties and complexity (24 to 48 h) of molecular cytogenetic techniques, such as FISH, it is difficult to envisage that it can be a pertinent diagnostic tool in the evaluation of oocyte and embryo quality. In addition, recent studies indicate that up to 26 % of FISH analyses involving oocytes are artefactual [125 , 126], introducing an element of doubt as to the reliability of the technique.

Finally, classical apoptotic markers (nuclear fragmentation, annexin 5 labelling, caspase activation), as described above, do not currently appear to be very promising. Data in the literature do not yet give a clear indication as to whether embryonic fragmentation is indeed associated with activation of the apoptotic cascade. Although alterations in the expression of protein regulators of apoptosis (Bcl-2 family members and IAPs) do appear to be associated with the viability of embryos, the evidence in the literature remains contradictory [70 , 77 , 78 , 80 , 81 , 88]. Thus, there is still a need to identify novel markers, especially those involved in early events within the apoptotic signalling pathways. The identification of a genetic signature as a reliable way to predict the fertilisability of the oocyte and the quality of its pre-implantation embryonic development remains a crucial goal. Identifying gene expression profiles is the first indispensable step in the identification of potential molecular markers of the expression of abnormal genes in the oocytes and the pre-implantation embryos.

Although this type of strategy has already been applied to cumulus cells, oocytes and human supernumerary embryos [116 , 117 , 127 , 128], the samples were in each case biologically stimulated or super-ovulated and, therefore, far from being in a physiologically normal environment. Using a recent technology, laser dissection microscopy, Arrazota et al [128] isolated primordial oocytes from sections of rhesus monkey ovary and uncovered 84 novel genes implicated in the survival and maturation of oocytes. It is also difficult to draw specific conclusions from such global data and further work will be required to validate the predictive value of the candidate genes in order to develop novel diagnostic genetic tests for oocyte and embryo quality.

Conclusion and perspectives

Despite much progress in IVF over the past decades, more than 50% of human embryos cultured in vitro do not reach the blastocyst stage on account of developmental defaults. This is due, in part, to defective oocyte maturation, for which suboptimal culture conditions are probably a contributing factor. Little is known of the molecular events driving early embryonic development and transcriptome and proteome studies should lead not only to major advances in our understanding of the first week of human life, but also to the identification of pertinent and reliable markers of oocyte and embryo quality.

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Fig. 1

Principle of the annexin V apoptosis detection. (A) After initiating apoptosis, cells translocate the membrane phosphatidylserine from the inner face of the plasma membrane to the cell surface. Once on the cell surface, phosphatidylserine can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for phosphatidylserine. (B) Structure of phosphatidylserine-bindig annexin V. Annexin A5 core (PDB code 1A8A) domains are intracellular phosphatidylserine-binding domains.

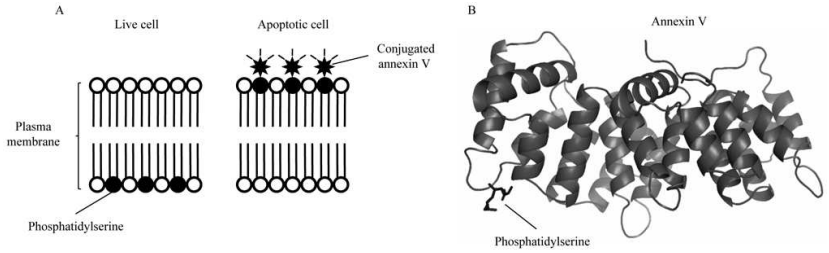

Fig. 2

Schematic representation of the two major transduction pathways of apoptotic signalling. The first one, called the extrinsic pathway, is activated by the binding of ligands to membrane-bound death receptors of the TNFR family. The second pathway, called the intrinsic pathway, involves a plethora of signals that converge on the mitochondria via the pro- and the anti- apoptotic members of the Bcl2 family. The extrinsic pathway links into the intrinsic pathway via a pro- apoptotic Bcl2-family protein, Bid.

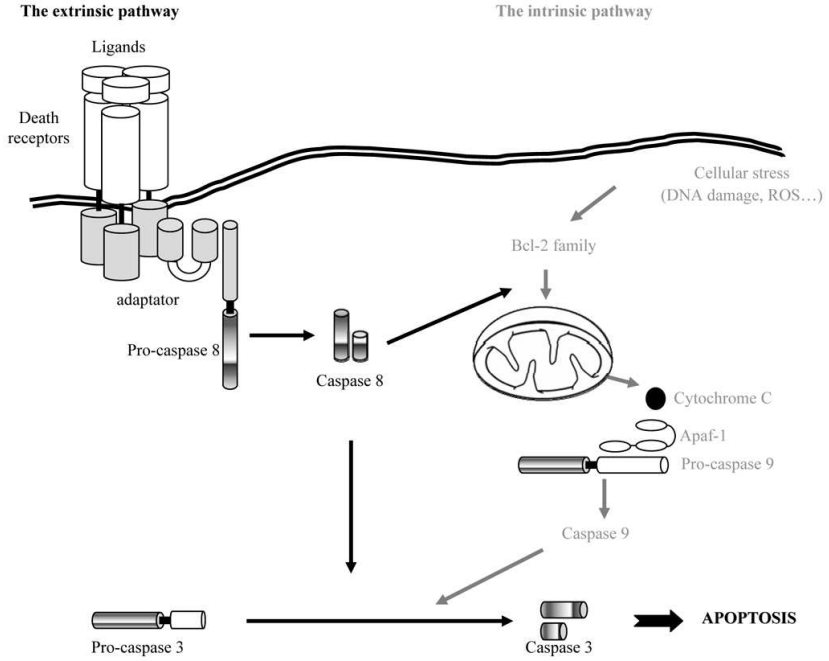

Fig. 3

Morphological aspect of early embryo developement. Left, normal embryonic development caracterised by few or no fragmentation; Right, abnormal embryonic development with fragmentation.

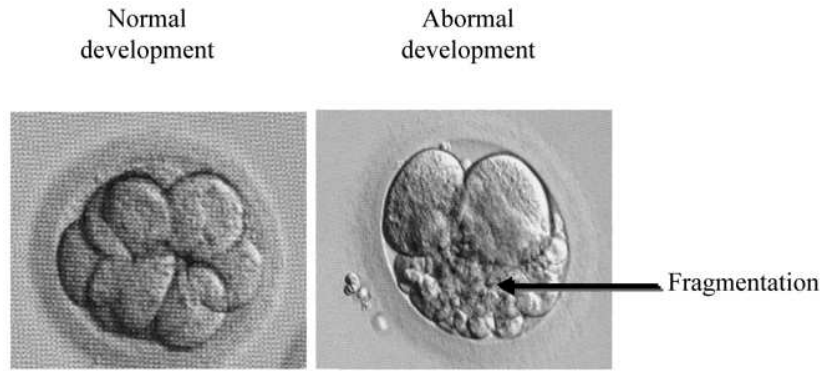

Fig. 4

Key role of the PI(3)K/Akt signalling in the cumulo-oocyte complexe survival. The PI(3)K/Akt signalling pathway in cumulus cells plays a determining role in the suppression of both spontaneous and gonadotropin-stimulated meiotic re-entry in oocyte as well as in oocyte survival.

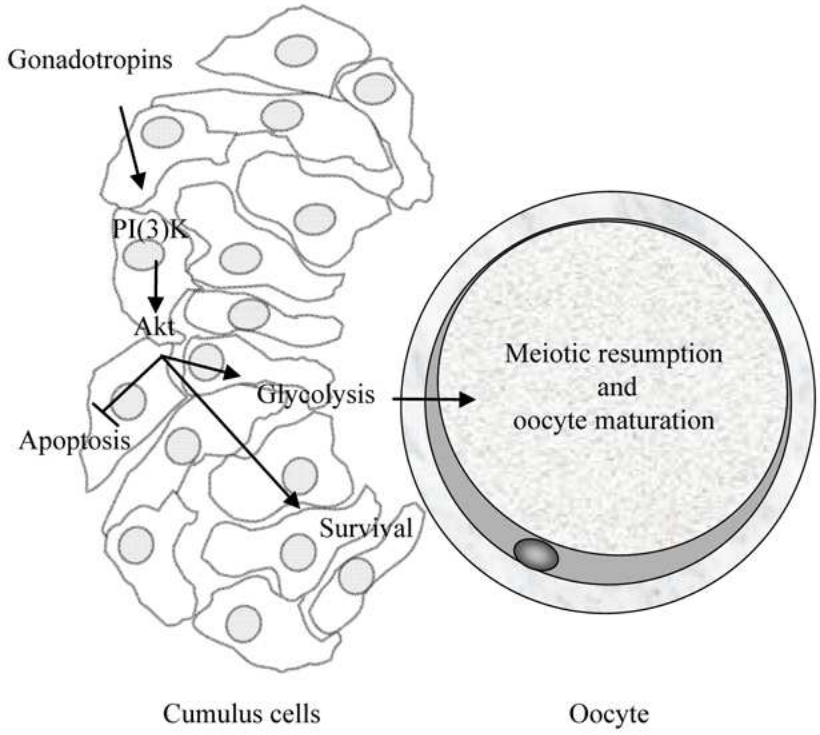

Table I

mRNA expression of the Bcl-2 family and caspases during human pre-implantation embryo development.

		Ovary	Oocyte	Early embryo development stage			
				zygote	2 cells	8 cells	Blastocyst
Bcl-2 family							
Pro-apoptotic members:	Bak	+/−	n.d	+	+	+	+
	Bax	+/−	+/−	+	+	+	+
	Bid	n.d	n.d	−	+	+	−
	Bad	n.d	n.d	+	+	+	+
Anti-apoptotic members:	Bcl-2	+/−	+	+	+	+	+
	Bcl-xL	+/−	+/−	+/−	−	+	−
	Bcl-w	n.d	n.d	+	+	−	−
Caspases							
Caspase 2		+	+	+	+	+	+
Caspase 3		+/−	+	+	+/−	+	+

This table shows the data of several publications and the contradictory results. +: detected, −: not detected, +/−: detected and not detected, n.d: not determined.