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Preimplantation development of the mammalian embryo

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Summary. - The bases for the differentiation of an adult individual are founded during the early development of the mammalian embryo before its implantation in the uterus. After the first cleavage, which is under maternal genome control, the segmentation continues under the control of the embryonic genome. At this time polarization and flattening of the blastomeres and the formation of specialized junctions among them, characterize the foundation of two different cell populations. These will give rise to the inner cell mass and the trophectoderm, which are finally segregated during cavitation. The last step of the preimplantation development is the blastocyst hatching from the zona pellucida, that prevented the implantation until now. During the preimplantation period the embryo may be exposed to environmental chemicals via mother genital fluid and teratogenic effects have been reported during this period. Even if the differentiation of the blastomeres begins very early in the development, the embryo seems to conserve its developmental plasticity, suggesting that the mechanisms of this teratogenicity are eventually to be found at DNA level.

Key words: preimplantation embryo, blastocyst, trophectoderm, inner cell mass.

Riassunto *(Lo sviluppo preimpianto dell'embrione di mammifero).* - Durante lo sviluppo embrionale di mammifero in fase preimpianto vengono poste le basi per il differenziamento di un organismo adulto. Dopo la prima divisione, che è sotto il controllo del genoma materno, la segmentazione continua sotto il controllo del genoma embrionale. In questo secondo periodo alcuni eventi, come la polarizzazione e l'appiattimento dei blastomeri e la formazione di giunzioni cellulari specializzate, iniziano il differenziamento dei blastomeri in due popolazioni di cellule differenti. Queste daranno origine alla massa cellulare interna e al trofoectoderma, che saranno poi segregati durante la fase che porta alla formazione della blastocisti. L'ultima fase dello sviluppo preimpianto consiste nello sgusciamento della blastocisti dalla zona pellucida che l'aveva avvolta sino ad ora impedendone l'impianto. Effetti teratogeni sembra che possano essere indotti anche durante questo periodo, ma i meccanismi alla base di questi effetti non sono ancora del tutto chiari. Infatti, sebbene nell'embrione di mammifero il differenziamento dei blastomeri cominci precocemente, durante il periodo di preimpianto l'embrione sembra conservare una notevole capacità regolativa. Un probabile meccanismo andrebbe ricercato a livello genomico.

Parole chiave: sviluppo preimpianto, blastocisti, trofoectoderma, massa cellulare interna.

Introduction

The preimplantation development of the mammalian embryo lasts from the formation of the zygote, after the fertilization, until the implantation of the embryo in the uterus. The understanding of the series of events that characterize this period of development is highly improved with the embryo *in vitro* culture. This knowledge is the basis for a good comprehension of early development toxicology and teratology.

In this review we will discuss the development from the one-cell zygote to the blastocyst stage prior to its implantation in the uterus. On the basis of mouse development this will be analysed in its three principal phases:

- the earliest development from the fertilized egg to the 2-cell zygote, which is under maternal genome control;
- the cleavage from the 2-cell stage until the formation of the mouse blastocoele (cavitation), with the formation of a morula, the phenomenon of compaction, and the beginning of the embryonic genome control,
- cavitation and the formation of a fully expanded blastocyst with two distinct cell populations, the inner cell mass (ICM), which is the precursor of the proper embryo and part of its extraembryonic membrane, and the trophectoderm (TE), which will give rise to the extraembryonic structures [1].

Early cleavage

The fusion of two highly polarized cells, the sperm and the egg, initiates a series of cellular transformation [2]. Initially the egg completes the second meiotic division and forms the female pronucleus. Prior to syngamy the two haploid pronuclei duplicate their DNA and begin the first mitotic division resulting in the formation of two doughter cells (the blastomeres), each containing a diploid nucleus and half of the mother cell cytoplasm [3]. Consequently the cleavage leads to the progressive reduction of cell volume and the maintainance of the

whole embryo volume. The cleaving embryo is hold in the zona pellucida that prevents it from adhering to the oviduct wall [4-6].

Fertilization, DNA replication in the 1-cell zygote and the first cleavage leading to the 2-cell stage seem to be under the control of maternal genome in the mouse embryo [7-10]. The embryonic genome is either inactive or its activity is irrelevant to the next developmental events [8, 11, 12].

Several experiments on the mouse embryo demonstrated that the switch from the maternal to embryonic control is at the 2-cell stage: Levey *et al.* (1978) [13] showed that the synthesis of hnRNA and of mRNA occurs only from the 2-cell stage onward. Moreover the activity of RNA polymerase II has not been detected in the 1-cell zygote, while it has been found in the 2-cell embryo [14]. Experiments using inhibitors of RNA transcription as actinomicyn D [15-17], have shown a partial resistence by the early cleavage embryo, suggesting a lack of transcription during this period. Furthermore α-amanitin, a specific RNA polymerase II inhibitor, has no effect on the 1-cell zygote [17, 18]. Particularly, in the mouse embryo the genomic activation seems to occur in two phases during the 2-cell stage, with a limited activation between 18-21h post-insemination (p.i.) and a major activity between 26-29h p.i. Most of the maternal mRNA may be inactivated over a period of 29-48h p.i. [9].

Furthermore, the change in the mRNA populations (maternal and embryonic) is accompanied by marked change in the qualitative pattern of protein synthesis [19-22]. These changes seem to be due to either translational activation of pre-existing maternal mRNA [7, 9, 23-25], or to post-translational modifications and polypeptide turnover [12, 26, 27], or to translation of newly synthesized mRNA [9].

In other mammals the timing of the change of genomic control varies from 8- to 16-cells stage morula [9]. Particularly in human preimplantation embryo the activation of embryonic genome occurs at the 4-8-cell stage morula [28].

Cleavage

Cleavages in mammalian embryos are among the slowest occurring in the animal kingdom, about 12 to 24h apart [29]. The divisions are asynchronous, thus the embryo does not increase from 2- to 4- to 8-cell stage morula, etc. but it increases gradually (Fig. 1) [29].

Table 1 indicates the timing of the different developmental stages for primates [30] and for the mouse [31].

During the cleavage period two important phenomena that are fundamental for the subsequent morphogenesis, occurs: the compaction and the foundation of two different populations of cells that will give rise to the inner cell mass (ICM) and the trophectoderm (TE) of the blastocyst.

Compaction

The compaction normally occurs at the 8-cell stage morula [32-35] (Fig. 1 c, d) and it is associated with three fundamental events: polarization of the blastomeres, flattening of cells against each other and formation of specialized intercellular junctions.

The polarization occurs at the late 8-cell stage through a radial axis: the surface microvilli, homogeneously distributed until this stage, become restricted to an externally facing apical pole [32, 36, 37], and ligand binding sites are apically located as well [32, 38, 39]. In the cytoplasm the actin and myosin microfilaments appear to concentrate beneath the apical surface [40-44]. Microtubules and mitochondria orientate parallel to the basolateral membrane [36, 45]. Between the nucleus, which assumes a basal position [46], and the apical pole a column of endocytotic vesicles appears [33]. All these polarization features of individual blastomeres seem to be a consequence of the asymmetrical contacts between the cells at the 8-cell stage [39, 47, 48]. A series of *in vitro* experiments on isolated blastomeres demonstrated that the ability to induce polarity develops during cleavage from the 2-cell stage [47]. Furthermore the polarity once achieved, is stable and conserved during the next cleavage divisions [47]. At this stage the segregation of two cellular populations (polar and apolar) begins, these are the precursors of the future TE and ICM, as discussed below.

The cell flattening that occurs at compaction, to maximize the cellular contacts, does not seem to influence the polarization, as pointed out by Pratt *et al.*, with a series of inhibitors of flattening, as 7-ketocholesterol, concanavalin A, tunicamycin and a Ca-depleted medium [34]. Cell flattening is not required therefore for the transmission of the induction cue for the polarization [34]. The most important consequence of flattening is the extension of the cellular contacts and it is therefore implied in the third event of compaction: the formation of specialized cell junctions, which are tight junctions and gap junctions [38, 49-53]. Johnson and Ziomek [47] and Goodall and Johnson [54] demonstrated that the polarization at the stage of 8-cell morula occurs when no gap-junctions mediated transfer can be detected between the blastomeres. Thus gap-junctions are unlike to transmit the inductive signal for polarization [34, 47].

Flattening and specialized junctions are lied rather than to the origin of polarization, to the maintainance of polarity in the next division and to the formation of the blastocoele [34, 45].

Foundation of two cellular populations

Two populations of blastomeres become positionally distinct during the fourth cleavage, that leads to a 16-cell morula: the outside cells greater and polarized and the

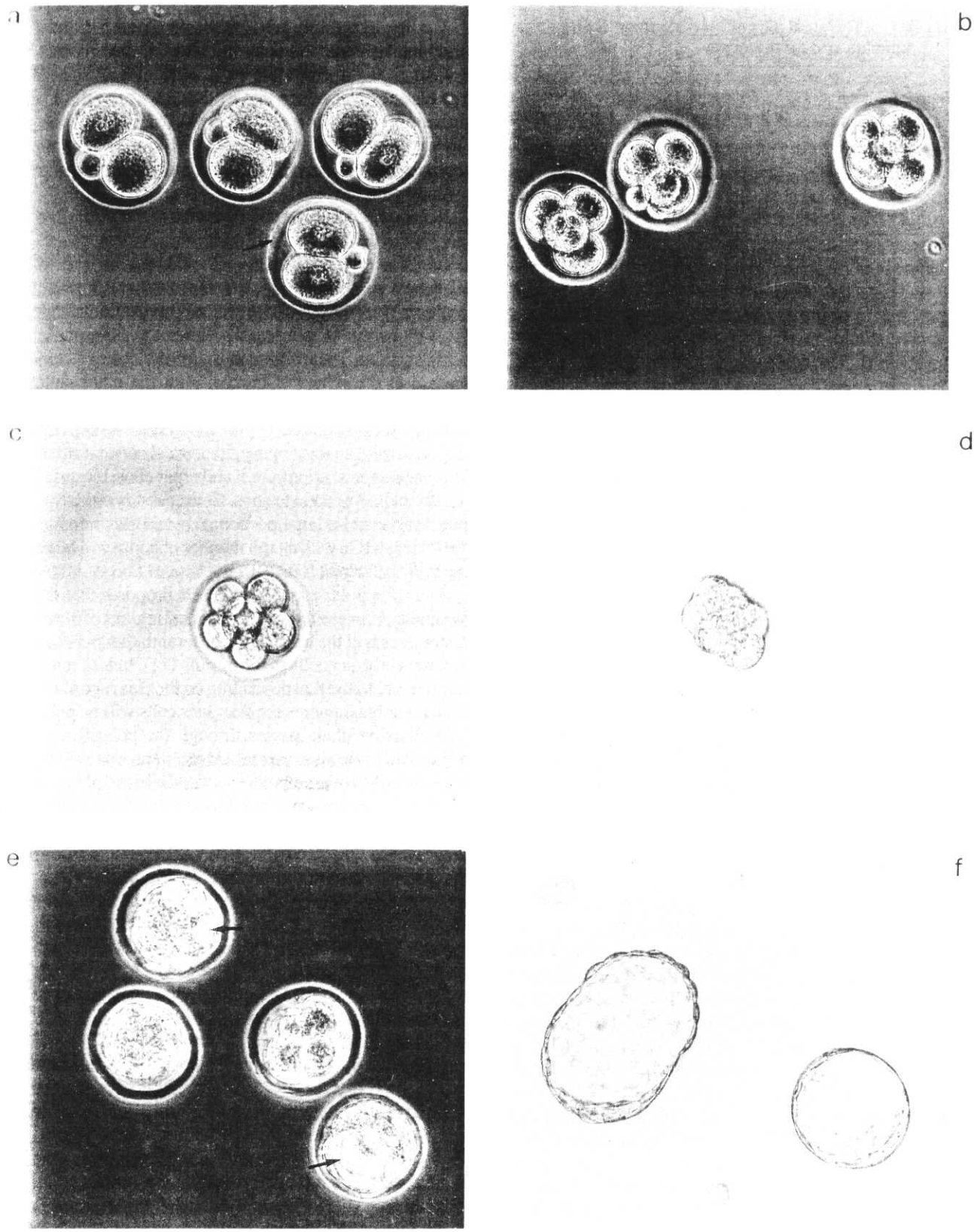


Fig. 1. - Different stages of mouse preimplantation development of embryo cultured *in vitro* from the 2-cell stage (x200). a) 2-cell stage at about 36h p.i. The zona pellucida is present (arrows); b) 4-cell stage at about 15-18h of culture; c) 8-cell stage at about 20-24h of culture; d) compacted morula at about 28h of culture; e) morula in cavitation at about 48h of culture. The vacuoles of the nascent blastocoel are visible (arrows); f) fully expanded blastocyst and hatching blastocyst at about 72h of culture.

Table 1. - Timing of different developmental stages for primates and for the mouse

Developmental stage	Primates h post ovulation	Mouse h post mating
1-cell	24	12
2-cells	36	36
3-4-cells	48	48
8-cells	72	60
16-cells	96	72
Blastocyst	4-6 days	3.5-4 days

inside cells smaller and apolar [32-35, 38, 39, 47, 55-59]. The differentiation arises from the achievement of polarization at the 8-cell stage morula, as pointed out before. These two populations are the foundation of the ICM and TE as demonstrated by the similitudes between the clusters of inner cells in 16-32 cell morula and the ICM [60]. Furthermore, Johnson (1981) [61] has shown that the inner cells have the same uniform alcaline phosphatase activity distribution as the ICM, and that the outer cells have a non uniform distribution of this activity like the TE. Finally, Balakier and Pedersen (1982) marked the cell lineages with horseradish peroxidase (HRP), injecting it in the blastomeres of the 2-, 8- and 16-cell stage mouse morula [62].

Two mechanisms have been proposed for the generation of the inside outside populations in the mouse morula, as reviewed by Johnson (1981) [61]:

a) a positionally instructive mechanism which has brought to the microenvironmental hypothesis of Ducibella and Anderson [36], which postulate that microenvironmental differences arise by the inclusion of some blastomeres among other blastomeres. Consequentially an enclosed cell receives the signal to differentiate into an ICM precursor, contrary to an outside cell, which, in lack of this signal differentiates into a TE precursor. Ducibella suggests that a difference in the fluid microenvironment in the inside parts of the morula may occur as a consequence of the formation of tight junctions between adjacent outer cells. Alternatively, he proposed that the inner cells might perceive their different microenvironment by the cell contact with the encircling blastomeres [36, 63];

b) a lineage mechanism which lead Johnson [61] to formulate his polarization hypothesis based on the idea that the cell itself contains the specification for its proper proximate development. Inner and outer cells are different from the moment of their formation and the differences arise from previously generated cytoplasm and membrane radial organization which is conserved during cellular divisions from 8-cell stage to 16-cell stage. Therefore it is the cellular inherence of differences rather than the cellular perception that is important [61].

The first hypothesis does not seem to fit with the real development: Pederson demonstrated that one morula microsurgically injected into the blastocoelic cavity of a large blastocyst develops into a normal blastocyst [64]. Furthermore, *in vitro* cultures of 2/16 polar-apolar couplets demonstrate that the apolar cell maintains its properties even deprived of its microenvironment and enveloping context [61].

Recently, it has been demonstrated that a positional signal is transmitted between blastomeres to determinate the axis of polarity and it involves the calcium-dependent cell adhesion glycoprotein "ovomorulin" (also called Ecadherin) [59, 65-69]. Indeed, the neutralization of this glycoprotein leads to abnormal polarization. In response to this signal a first reorganization of the cytoskeleton adjacent to the points of contact with the other cells occurs. Then this local change spreads from the contact point and an apical zone, with the surface microvilli associated with an underlying subcortical concentration of microfilaments, microtubuli and organelles, forms far from the cellular contact zones. Consequently, with few points of contact a large portion of cytocortex remains unaffected, while with a high number of contacts a large pole will be formed [59, 70].

Thus, the polarization hypothesis proposes that the asymmetry between the apical and basal regions of 8-cell blastomeres is at the basis of the differentiation between the inner and outer cells of the morula [71]. Indeed it has been demonstrated that depending on the cleavage plane in the polar blastomeres the doughter cells will be polar if the division plane passes through the polarity axis (perpendicular to the morula surface); and one will be polar and the other apolar if the division plane is perpendicular to the axis (parallel to the morula surface), i. e. the basal part of the polarized blastomere will give an apolar cell and the apolar part, with the microvillous pole will give rise to a polar cell [72-74]. Furthermore, Sutherland *et al.* have recently reported (1990) that the plane of division may be oblique to the surface and may give rise to two polar doughter cells if it passes through the apical pole so that the two doughter cells inherit parts of it or it may give rise to one polar and one apolar cell if it segregates the apical pole [72].

On the basis of this hypothesis Garbutt (1987) proposed that the earlier formed blastomeres contribute preferentially to the inside cells population at the 16-cell stage [75-77]. This proposal was confirmed by Pickering *et al.* (1988) who demonstrated that the pattern of contacts during the first part of 8-cell stage exerts influence on the proportion of differentiative divisions (polar/apolar doughter cells) [70]. The more extensive the contact area is, the less of the surface will have microvilli, i.e. a smaller pole. Furthermore, with a greater pole the probability that the division plane could cut it is higher than that with a smaller pole. Consequently, the early forming and flattening blastomeres will make more

extensive contacts and thus, they will have a smaller polar area and the divisions will be preferentially differentiative. In conclusion, the early forming blastomeres allocate more progeny to the inner cells mass progeny [70, 72, 75, 78].

The allocation of cells to the TE population and the ICM population in the human embryo seems to be like the mouse one, as it has been shown by Hardy *et al.* (1989) [79].

The polarization model is in contrast to the plasticity of the mammalian development, because it leads to the conclusion that the early development is more determinative than instructive. But even if the foundation of the two cells lineage occurs at the early stage of morula, this determination is not inflexibly instructive [61].

We expected that the totipotency is conservative until the 8-cell stage embryo, as demonstrated by Kelly [80], but it has been shown that the 16-cell embryo blastomeres are still totipotent. Indeed, experiments on aggregates of only polar or apolar cells from a 16-cell stage morula have shown that the latter give rise to normal blastocysts [59-61, 81-84]. At the stage of 16-cells a second line of regulation occurs, if isolated the two groups of cells behave as early 8-cell morula blastomeres and polarize consequently to asymmetric contacts [59].

Cavitation

During cavitation the embryo develops a blastocoele, the fluid filled cavity essential for the formation of the blastocyst. Cavitation begins at the stage of 16-32-cell morula [85] (Fig. 1 e, f). The 2 cellular populations formed in consequence to polarization at the 8-16-cell stage, as cavitation proceeds, become the trophectoderm and the inner cell mass. Wiley has proposed two purposes for cavitation of the mouse embryo:

- to facilitate the development of the outer layer of blastoderm into the first epithelial layer (TE);
- to create the conditions for the differentiation of the second epithelial layer which appears in the embryo, i.e. the primitive endoderm, with the formation of a new free surface on the ICM facing the blastocoele [85].

Three models for cavitation have been proposed: 1) the secretion cavitation model [86, 87]; 2) the transporting cavitation model [36, 88] and 3) the metabolic cavitation model [89].

1) At the 16-cell stage just before the beginning of cavitation refracting cytoplasmic droplets appear at the basolateral borders of the outer blastomeres. These droplets decrease during the coalescing of fluid accumulated between blastomeres [86]. The theory proposed is that the droplets contain the nascent blastocoele fluid [86, 87, 90]. Additionally microtubule depolarizing agents have been reported to inhibit cavitation [86]. However it has been demonstrated that the contents of these droplets are lipidic whilst the blastocoele fluid is an aqueous solution [87, 88, 90, 91].

2) During compaction tight junctions at the apical ends of the outer blastomeres appear [36]. These junctions form a permeability seal which isolates the inner blastomeres from the external environment [36, 92]. These observations lead Ducibella and Anderson [36] to formulate their transport cavitation model according to which the permeability seal is a prerequisite of cavitation. This and the transport of ions and water across the outer blastomeres are responsible for the origin of nascent blastocoele fluid [36, 91, 93]. However, McLaren and Smith [94] have shown that zonular tight junctions in the 16-cell stage morula are still permeable to proteins when the blastocoele has already formed. Furthermore the embryo does not increase in volume before the blastocoele is 1/3 of the diameter of the embryo, thus it is improbable that the embryo could take up extraembryonic fluid [85, 95]. In addition this model does not explain the function of the cytoplasmic droplets observed at the beginning of the formation of blastocoele [85].

3) Wiley proposed the metabolic cavitation model taking account of the observation made in the two previous models and considering the following facts [84]:

- at the beginning of cavitation the ion transport enzyme Na^+/K^+ ATPase are located on the basolateral membrane of the outer blastomeres [96];
- the lipid catabolism [97] and the ATP utilization [98] arise;
- the droplets contain the neutral lipids that are catabolized in the mitochondrial pathway of β-oxidation which produce ATP [89];
- the mitochondria are collocated in the outer blastomeres with the lipidic droplets [89].

This model proposes that the juxtaposition of mitochondria, lipidic droplets and Na^+/K^+ ATPase located on the basolateral membrane are responsible for the production of blastocoele fluid. The ATP produced by the β-oxidation is utilized by the Na^+/K^+ ATPase to pump Na^+ out of the cell into the intercellular spaces and the water passively follows the sodium [85].

At the end of the cavitation the blastocyst is formed with about 64 cells (in the mouse embryo). At this point we can distinguish the inner cell mass and the trophectoderm that encloses the former and the blastocoele.

Until the formation of the blastocyst the totipotency of the cells, i.e. their capability to differentiate into either ICM or TE, has been demonstrated [59-61, 80, 82]. Winkel and Pedersen (1988) have shown that the polar trophectoderm recruits cells from the ICM during blastocyst growth and expansion [99]. Indeed ICM cells retain their totipotency even after their allocation within

the blastocyst [60, 61, 82-84, 99]. It seems that the restriction in potency of the ICM occurs after the blastocyst expansion [60, 82, 100-102] and the allocation process becomes complete by the time of implantation [99]; whereas, the TE potency seems to be restricted earlier, coinciding with the sixth cell cycle and blastocoele expansion [103-105].

Hatching

Until the formation of the blastocyst, the embryo is contained in the zona pellucida, that prevents the embryo from adhering to the oviduct wall [4, 56]. So it is necessary for the subsequent implantation phase that the blastocyst eliminates its zona pellucida. Thus, the embryo hatches from the zona in the uterus, at the fourth day of development, by lysing a small hole in it and squeezing through this hole as the blastocyst expands (Fig. 2).

Perona and Wassarman (1986) have shown that a trypsin-like proteinase, called "strypsin", is involved in hatching of mouse embryo *in vitro* [106]. Thus, mouse blastocysts themselves are responsible for initiating the hatching process, even if *in vivo* experiments have shown the existence of uterine proteinase that dissolve the zona pellucida following the hatching [107-111]. Perona and Wassarman suggest that the strypsin is located to cells of mural trophectoderm, which make contacts with a limited region of the zona pellucida via cytoplasm processes. Such situation would permit a localized proteolysis. However, Yamazaky and Kato (1989), recently, demonstrated that there is no particular site of shedding of the zona pellucida and that strypsin is uniformly distributed throughout the tophectoderm [112, 113].

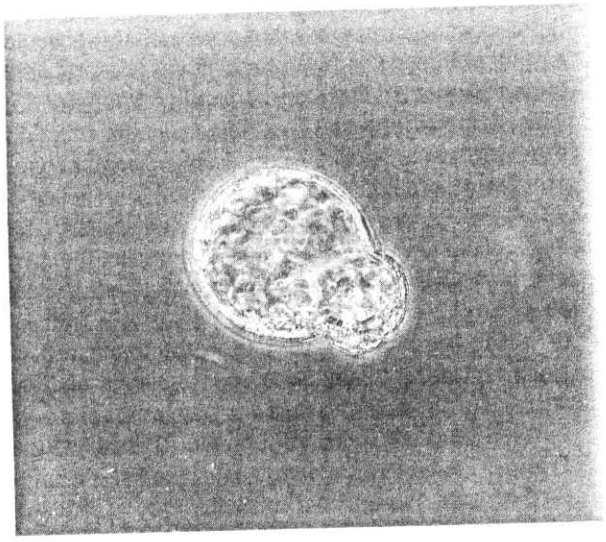


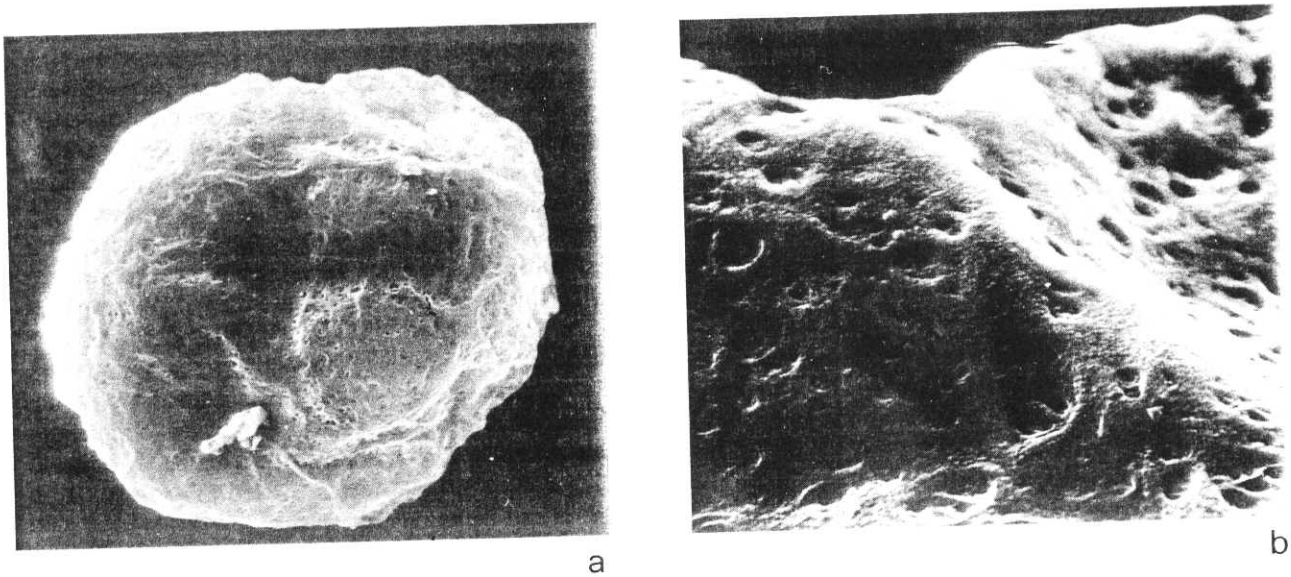
Fig. 2. - Hatching blastocyst of mouse. The zona pellucida is visible (x200).

Nutrition

During its preimplantation development the embryo passes down the oviduct to the uterus, surrounded by the zona pellucida. The zona is a highly porous glycoproteic coat (Fig. 3) permeable even to large molecules [114-116] and viruses [117, 118]. Thus the embryo is in contact with the oviduct and uterine milieu [119].

The maternal environment seems to be permissive and it acts only to permit or retard the developmental programme. Indeed the uterine secretions do not seem to contain regulators of embryonic development [11].

During cleavage the embryo does not utilize maternal nutrients but those from the stocks in the blastomeres. However, it has been shown, using *in vitro* culture of



a b

Fig. 3. - Scanning electron microphotography of the porous zona pellucida of mouse blastocyst. a) x800; b) x5000.

preimplantation mouse embryo, that lactate and pyruvate are necessary for the first and the second cleavage division [120] and glucose is utilized by the embryo from the third cleavage division onward [121-126]. The oviduct fluid is a complex mixture of constituent derived from the plasma and of specific proteins synthetized by the oviduct epithelium [127, 128]. Generally the concentrations of nutrients in the oviduct fluid are considerably below their plasma concentrations, suggesting that their transport across the oviduct is by diffusion rather than active transport [127]. The most abundant macromolecules derived from the blood are albumin and IgG [127]. The immunoglobulines seem to have a role in the defence against microorganisms [129], while the albumin may contribute to the amminoacidic pool of the embryo [130]. Furthermore, the electrolytes maintain a stable osmolarity and a rather high pH (7.5-8), due to a high bicarbonate concentration. Compared to maternal plasma concentration the potassium level in the oviduct is higher while the sodium concentration is lower [88, 127, 131]. The oxygen tension is low at about 21-60 mmHg [132]. Finally, in the uterus the fluid is abundant in nutritive substrates, cofactors or substrate-ligands as retinol [133], sterols [134-136] and ions [11, 133]. At this level the embryo has developed to blastocyst stage and an early transport from the mother to the embryonic blastocoele begins. Beier and Maurer (1975) have reported that the fluid of rabbit blastocyst contains uterine proteins [137].

Conclusions

It is evident that the preimplantation embryo may be affected by environmental chemicals via mother genital fluid.

Until recent years it was thought that the effect on preimplantation embryos depended on the number of cells killed or inhibited: above a certain proportion the embryo dies, below this proportion the remaining cells multiply to replace those lost and subsequent development is normal, as proposed by Austin (1973) with the "All or nothing law" [138]. Recently some investigations on fetal outcome *in vivo*, exposing the mothers to various chemicals at the preimplantation period, have pointed out that fetal malformations could arise [139-141]. Furthermore a dose-dependent embryolethality has been shown in drugs treated pregnant mouse during the preimplantation period [142].

As we have shown, even if the foundation of two cell lineages, i.e. the beginning of the differentiation, occurs very early in the mammalian development, the latter conserves its plasticity. Thus the teratogenic effects observed, lied to the preimplantation period, could be explained at genomic level. Indeed, Pampfer and Streffer (1988) have shown that the irradiation of mouse zygotes by X-rays, which increases the specific-locus mutations number in the mouse oocytes, causes malformations [141]. Other drugs known to induce chromosome aberrations in different test systems, like ethanol, have a teratogenic effect after a treatment during the preimplantation period [143]. Furthermore the induction of micronuclei in the blastomeres of rat embryo and of malformations in the rat fetus by anticancer drugs may suggest that the genotoxic effects, observed during the preimplantation period of these drugs may induce the teratogenic effects observed at term [144]. Moreover, the Sister Chromatide Exchange (SCE) tests on culture of preimplantation mouse embryo have shown the possible DNA-lesions due to drugs as alcohol, caffeine, and cyclophosphamide [140-143].

These observations lead to the conclusion that the teratogenic effects observed at term after a chemical treatment during the preimplantation development may be due to DNA-damage, but it is not yet clear. Therefore, further studies at molecular level on the preimplantation mammalian development are necessary for a better comprehension of the problem.

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