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SPECIAL ARTICLE

The Important Factors in Preimplantation Embryo Development

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According to the limitation of the ethics, law and the resources of human embryo for studying, the knowledge of human embryo after fertilisation was adverse to obtain. The first report of the studying of preimplantation embryo which demonstrated the anatomy and morphology of preimplantation and early preimplantation stages by using the hysterectomy specimens was performed by Hertig.[1] However, these knowledge was still difficult to obtain, until the organisation of modern technique of in vitro fertilisation (IVF) for the management of infertile couples for 25 years ago. This useful technique offers a lot of knowledge of the human embryos, including the early stages human embryogenesis and the factors related the human embryogenesis; nevertheless, the progression of knowledge is still slow. A few number of embryos left for studying are obtained because the major aim of IVF creating embryos by IVF technique is for the therapeutic, not for researches.

In the U.K., the controlling of IVF was approved by the Human Fertilisation and Embryology Authority and many policies were established, including research on preimplantation human embryos feasibly only under licence, not more than 14 days after fertilisation, and for the aim of improving update in infertility treatment, obtaining knowledge about the aetiologies of congenital disease and miscarriages, development of more effective contraceptive methods, or development of tools for searching abnormal gene or chromosome in preimplantation embryos.[2]

In many centers, the overall pregnancy rate by IVF and embryo transfer (ET) is not high with the result of 21-34%.[3] The biochemical pregnancy is the pregnancy detected by a rising of serum human chorionic gonadotrophin (hCG) and were found about 45% of cases. Only 34% of the biochemical pregnancies can progress to the stage of clinical pregnancy which confirmed by the detection of sonographic fetal cardiac activity and result in 285 of an implantation rate and 22% of an overall fetal development rate per embryo transferred.[3] The advance knowledge of the embryo development as well as the best embryonic selected method will increase the success rate of therapeutic IVF. The left human embryos donated are very advantageous for the research propose in the present.

Preimplantation Embryonic Development

Fertilisation starts after the sperm nucleus passes through the oocyte, the oocyte finishes the second metaphase of meiosis and the extrusion of the second polar body from the oocyte giving rise to the definite oocyte. The chromosome of the sperm and the definite oocyte are enclosed by nuclear membranes to form transient and female pronuclei; and then they fuse with each other to form a single diploid nucleus fertilised zygote. The fertilised zygote then gives rise to a series of the same mitotic cell division called as a

cleavage within 24 hours. The division is not correlated with cell expansion; therefore, they subdivide the large zygote into a plenty of smaller daughter cells called blastomeres. The cleavage is still enclosed the zona pellucida if it does not enlarge in size. Cleavage divisions spend about 20 hours along, and then completes the second division within 40 hours after fertilisation resulting in four equal blastomeres. In three days, the embryo composes of six to twelve cells, and by four days of 16 to 32 cells, of which the stage is called a morula due to a small mulberry-like appearance.

The morula then passes a reorganisation process called as compaction with the formation of an inside-outside polarity. Compaction provides the flattening and differential adhesion of blastomeres and separates central blastomeres (called the inner cell mass) from peripheral blastomeres (called as the trophectoderm). The inner cell mass will become the embryo, and the trophectoderm will become the placental membranes. By the fourth day of the development, the morula, which comprises of about 30 cells, begin to absorb fluid into intracellular vacuoles within the blastomeres and then collect between the cells. The major cell-to-cell adhesion structures or tight-junctions start to develop between the trophectoderm cells. A blastocoele or the blastocyst cavity forms within the morula represents of the increased hydrostatic pressure of the fluid inside the internal cavity and then the embryo is then called a blastocyst. The inner cell mass forms a compact mass at one side of the blastocoele (embryonic pole of the blastocyst). The trophectoderm is regulated into a thin, single-layered epithelium and the opposite of the embryonic pole is called the abembryonic pole.

By the third and the fourth day of development, the morula travels to the uterus. By the fifth day, the zona pellucida is piercing a hole using an enzymes which gives rise to the blastocyst to hatch out. By the sixth day, the implantation process begin by the adherence of the nacked blastocyst to the uterine endometrium.

According to a lot of restrictions, the timing of development stages in utero is not accurate enough. In order to maintain the embryonic developmental process as similar as in the human nature, the observation can be performed only once a day to avoid disturbing the embryos. For this reason, the timing of cleavage divisions is not precisely measurable.

By the sixth or seventh day, only half of normally fertilised human embryos reached the blastocyst stage due to the rest half of human embryos arrest at all stages between the 2-cell and the morula stages.[3]

Meiosis II begins and the second polar extrudes after fertilisation. The development of pronuclei and DNA replication occur about 3 to 10 hours and 8 to 22 hours after fertilization respectively.[2] The time of pronuclei breakdown and first cleavage division are very different between embryos with the former of 27 to 30 hours and the latter of 29 to 32 hours after fertilisation respectively. The cleavage divisions to the 4-, 8- and 16-cell stages are detected at 45, 69 and 93 hours after fertilisation respectively. Transferring of the embryos by the second or the third day during the 2- to 4- and 8-cell stages in normal embryos is generally accepted. Compaction starts at the 16-cell stage (by the fourth day) in normal human embryos and at 8-cell stage in the mice. The growth of blastocysts finish by the fifth day both in vitro[4] and in vivo.[5]

● Cleavage-Stage Arrest

The aetiology of cleavage-stage arrest in the development of human embryos has not been demonstrated in the present. The numbers of blastocyst stage of human embryos which succeed by IVF technique in vitro differ very much between 0 to 82%.[2] These various results depend on a various factors; these include the detection time of the embryos, the variety in media used for culture, the criteria of subjective morphological evaluation of blastocysts, and the inclusive criteria for early, expanding and fully expanded blastocysts.[2]

In summary, the few number of human embryos can reach to the fully expanded blastocyst stage in vitro due to those restriction; and the mean embryonic numbers from IVF techniques compares with those from

in vivo fertilisation which collected by uterine lavage are 34% and 29% respectively.[2,5]

The Implantation Factors in Culture Conditions

● Culture Media

According to studies of mouse embryos in vitro, there are some circumstances that the culture media for human embryo IVF are not ideal for human embryo culture. These include high incidence of development arrest,[2] inconsistent cleavage, cytoplasmic fragmentation, high incidence of polyploidy, anucleated and multinucleated cells.[6] The reports from many cultures,[2,7] demonstrated the variant survival rates of embryos in different media; demonstrating that both extrinsic and intrinsic factors affect the survival of embryos.

The purpose of research in human embryos use is to explain reasons why a large numbers of cleavage-stage is arrested prior to reaching blastocyst formation in human embryos. The investigations include different culture systems to search for appropriate conditions that will maintain human embryogenesis in vitro to reach at least the blastocyst stage; and the methods used to detect the embryos that will survive and to continue development later. The efficiency of cleavage stage human embryo development depends on the gross morphology and the rate of cleavage.[7] The healthy embryos with regular, spherical blastomeres and no fragmentation can reach to the fully expanded blastocyst stage more than those with poor morphology. The too slow or quick cleaving embryos give rise to the poor developmental process. The embryo that cleaves to the 4-cell stage by the second day, can often develop the blastocysts on their five days after fertilisation in vitro. The miscarriage after transfering to the uterus may result from the abnormally developed blastocysts.[8]

Many studies reported that the different blastocyst formation rates depend on the different culture media, Tyrode's 6 (T_6) and Earle's balanced salt solution (EBSS) and simple media seem to be insufficient for human embryo culture.[2,4,7] The complex media are prepared by adding essential factors; i.e. hypoxanthine, glutamine, vitamins and essential amino acids to the basic salts of simple media. A study supported that a high morulae or blastocysts formation rate of 85% can be obtained from Ham's F_{12} media.[7] The 60% of blastocyst formation rate can be obtained from alpha-minimal essential medium (α-MEM) conataining 1% human cord serum (HCS). Human tubal fluid medium (HTF) contains more potassium, more calcium, and less glucose than T_6. T_6 contains 10% patient serum and gives rise to less successful pregnancies than HTF.

The uncertain efficiency and proportional variety of the serum adding which may be beneficial or disadvantage lead to the presence of synthetic serum substitues and commercially prepared serum products; and more studies are on going to evaluate its efficacy.[2] The poor human development in a serum-free medium was reported.[9] The study of human embryo development in vitro using a complex, serum-free medium indicates that the serum is unnecessary for that development.[10] However, T_6 medium containing 10% patient serum gives rise to better blastocyst formation rate and its viability than those.[7] Yet, many growth-promoting and protein factors affecting the human embryogenesis are also studied by CSFM. The examples of those factors are insulin, insulin-like growth factors (IGFs), epidermal growth factor (EGF), colony-stimulating factor-1 (CSF-1), platelet-derived growth factor (PDGF), thioredoxin and superoxide dismutase, ethylenediaminetetraacetic acid (EDTA) and leukaemia-inhibitory factor (LIF).[7]

● Group Culture and Micro-Drop Culture

The mammalian embryos secreting factors that have autocrine effects on their development have been demonstrated.[11] Human preimplantation embryos cultured in microdrops or in grouped media will produce transforming growth factor-α (TGF-α), IGF-II, PDGF, and platelet-activating factor (PAF).[11] Group culture was demonstrated to promote cleavage rate, embryo morphology,[12] and pregnancy rate after the embryo transfer.[13]

● Co-Culture with Feeder Cells

The higher human implantation rates after embryo transfer using co-culture with feeder cells were reported.[2] These cells include Vero cells derived from African green monkey kidneys, foetal bovine fibroblasts, bovine oviductal epithelial cells, human ampullary cells, homologous endometrial cells, homologous cumulus cells, granulosa cells and ovarian cancer cells.[2] It is believed that feeder cells play roles in rescueing the delayed cleavage embryos; and the production of growth-promoting fetuses and the elimination embryo-toxic factors are the results of the similar impacts. Its disvantages are a high cost, time-consuming method,[14] and a chance to contact a disease from the contaminated cultures.

The Important Factors Affecting Human Embryo Viability

● Morphology

Shulman has established embryo scoring system for evaluating embryo viability.[15] This system provides embryo morphology, cleavage rate, symmetry of blastomeres, appearance of the cytoplasm, and the oegree of extracellular fragmentation. The best predictor of preimplantation embryo development and viability is cytoplasmic fragmentation, where as the cleavage rate, blastomere symmetry and cytoplasmic granularity are less important.

The half of human embryo poses cleavage-stage arrest in vitro and the rest can carry on to the blastocyst stage with poor implantation rate in vivo after embryo transfer. It was described that different embryonic and maternal factors cause the poor development of some blastocysts in vivo.[15] The abnormalities of trophectoderm and inner cell mass numbers provide abnormal morphology, unicorporates blastomeres, unseen inner cell mass, small numbers of total cells, a high number of cell death, a small number of good morphology of inner cell mass leading to implantation failure or biochemical pregnancies (a primary raise of hCG levels without foetal development).[3] More studies are needed to prove the accurate causes of a few cell numbers of blastocysts, either their own inherent defects or the inappropriate conditions.

● Embryo Metabolism

The most essential energy substance during the early cleavage stage in preimplantation mammalian embryo is pyruvate,[16] while those during the blastocyst stage is glucose.[16] Lactate, amino acids, and fats are the less essential substances during the development. The aetiology of the changing from a pyruvate-based to a glucose-based metabolism is still unknown. However, the high glucose uptake may relate with many circumstances. Firstly, the embryonic genome is activated during the 4- to the 8-cell stage, together with the raise of function of ribosomes and uridine uptake.[3] A precursor in many macromolecules of the cell components is glucose; while ribose moieties for nucleic acid biosynthesis, glyceral phosphate for phospholipids formation and complex sugars are for mucoproteins and mucopolysaccharides. For this reason, the essential cause of the raise in glucose demand is the alteration of embryonic genome.[17] Secondly, the change from pyruvate to glucose uptake happens simultaneously with the first cells differentiation, forming the trophectoderm and inner cell mass.[17] Thirdly, the metabolism of embryo is rising due to a lot of ATP requirement of embryo during compaction and blastocoele formation process.[17] Fourthly, the change of substance uptake happens at the same period of embryo travelling from the fallopian tube into the uterine cavity.[17]

It was postulated that the blastocyst formation and the embryonic genome activation require glucose uptake because the cease of glucose uptake appears simultaneously to the cleavage-stage arrest embryo.[17] Furthermore, a pyruvate uptake rate of the cleavage-stage arrest is 10-30% lower than that of the normally developed embryo.[3]

● Embryo Secretions

The mammalian embryos secrete essential factors are established; these include autocrine and

paracrine effects in order to contact with their mothers. The hCG secretion in vitro is time-dependent without the correlation with the cleavage-rate or morphology of the embryos.[18] Therefore the morphology of the evaluation of embryo is impossible with the hCG levels usage. The detection of pregnancy specific β-1 glycoprotein (SP-1) is very early in the first day after fertilisation without a definitely secreted-patterns. Hence, this is useless for evaluating the quality of human blastocysts.[19] The CSF-1, interleukins 1 and 6 (IL-1, IL-6), tumour necrosis factor-α (TNF-α) and TGF-β are produced by the 2- to 8-cell stages of human embryos[20] and TGF-α and IGF-II by the morula and blastocyst stages.[21] Interferon-α may act in connecting between the embryo and corpus luteum without the detection in the medium.[22] Other factors such as PDGF, α-immunoreactive inhibin and immunosupressive factors are still unknown for the actual action.[2] The production of PAF by human embryos in utero is detected as early as in the 1-cell stage. This may have a role in human embryogenesis but needs more study before stating it as a marker.[23]

The Important Factors in Cytogenetic Studies

● Chromosome Abnormalities in Cleavage-Stage Embryos

There are many chromosomal abnormalities; such as mosaic, non-mosaic, and chaotic which are found in the left embryos after transfer with the high rate of greater than 46%.[24,25] This may be assumed that the genetical abnormalities lead to the arrested embryo in vitro are due to the low incidence observed of congenital abnormalities in IVF pregnancies (1.4%); with the same incidence in spontaneous conception (1.8%).[25] Monosomies which have very low incidence in early clinically detected pregnancies, are found in preimplantation embryos as usual as in trisomies.[26] For these reasons, monosomies tend to be one of the most essential causes of cleavage stage arrest in vitro with an incidence of 10%.[26] The chromosomally abnormality embryos can obtain the implantation stage with a high incidence of miscarriage.

The normal morphologic embryos poses the lowest incidence of chromosome abnormalities (23%);[26] while the highest incidence (74.5%) is found in polypronucleus, delayed and arrested development[27] and 90% of the incidence in the poor morphologic embryos.[28] Furthermore, it also correlates with extracellular fragmentation,[29] multinucleated blastomeres[30] and developmental arrest.[29]

According to the production of the essential protein for compaction prior to the fully activation of the embryonic genome, the compaction process can continue without the embryonic genome activation.[3] At the 8-cell stage embryo, the transcriptional activity enhancement is related to the embryonic genome activation; however, its absence can lead the embryo to the normal morulae.[31] The recent study reported that the embryonic genome activation is one of the essential factors for blastocyst formation,[32] particularity for the vectorial fluid transport mechanisms and the effective permeability seal development.[32] In conclusion, the absence of embryonic genome activation may lead to the morula-stage arrested in the human embryos.

Important Factors in Cytochemical Studies

● Growth Factors and their Receptors

The preimplantation mammalian embryos have autocrine growth factors receptors and paracrine factors receptors which are produced by the mother. These are supported with many animal studies but very few studies on human embryos.[33] TGF-α receptor and EGF receptor (EGF-R) occur in the 4-cell stage human embryos;[33] EGF, TGF-α, EGF-R, IGF-I and IGFR-I receptor occur in the 8-cell stage embryos and blastocysts;[33] CSF-1 receptor appears for all the period of preimplantation human embryonic development.[34] The growth factors and their receptors activities are very essential for the development and implantation during the preimplantation of human embryos. The preparation of the good quality culture conditions requires the knowledge of their accurate

function in human embryogenesis.

● Cell Adhesion Molecules

The cell adhesion affects the blastocyst cell lines differentiation.[35] The adhesion to the endometrium in implantation during early embryonic development stage requires the extracellular matrix (ECM) proteins.[35] The human oocyte surface expresses integrin α-5; whereas laminin, fibronectin and integrin β1 which are detected during early embryonic development.[35] The human oocyte and the 2-cell and blastocyst stage embryos poses many other adhesion molecules, such as, integrin, cadherin, immunoglobulin-like, and the selectin families.[36] The presence of sex integrin subunits (α3, αV, β1, β3, β4 and β5) are steady throughout preimplantation development regardless of the embryo morphology, whereas the other five subunits (α2, α4, αL, β2, β7) appear in some oocytes. The oocytes and early embryos also express the adhesion molecules including cadherins and L-selectin.[36] A small numbers of studies are carried out for cell adhesion molecule function. The dissolution of the zona pellucida requires fibronectin or laminin which is important for hatching of the blastocyst.[35] There was a study that the adding fibronectin or laminin into the culture medium will give rise to the blastocyst hatching rate.[35] In summary, the presence of specific ECM proteins, integrin and proteolytic enzymes are essential factors for human embryogenesis. The most important factor for promoting the human embryos to survive in vitro is through its contact with the ECM proteins.

Important Factors in Molecular Studies

● Expression of Growth Factor and their Receptors

The RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) methods are used to detect the presence of messenger RNA (mRNA) for particular growth factors or their receptors. These methods are beneficial to confirm immunocytochemical studies and have identified mRNA for EGF, TGF-α and EGF-R in oocytes, the 4-cell, the 8-cell and blastocyst stage embryos;[37] and CSF-1 receptor during preimplantation embryo development.[34] The mRNA for IL-6 and its receptor were demonstrated in blastocysts, but not in cleavage stage embryos.[2] TNF receptors (TNF-Rp80) were identified in stages greater than the 6-cell stage and TNF-Rp60 appear in the 6-cell and blastocyst stages.[2]

● Patterns of Polypeptide Synthesis

The presence of transcriptional inhibitor α-amanitin, polypeptide synthesis and cleavage stops at the 4-cell stage.[38] These can be defined from the obvious changing in polypeptides synthesised patterns between the 4- and 8-cell stages, affecting the embryonic genome activation.[38] The switch from maternal to embryonic development controlling occurs at the similar period with the highest rate of development arrest. Hence, the failure of embryonic protein synthesis does not correlate with the cleavage-stage arrest.[39] The gene activation of the embryo are dependent on the advanced age and the number of completed cytokinesis cycles.[39] The arrested embryos with poor morphology gives rise to more variations and numbers of polypeptide synthesis patterns than the normally embryo with round blastomeres and the absence of fragmentation. There is however, no definite summary that abnormal development is related with the polypeptide synthesis differences; even though the increase of abnormal development was identified in the raising of abnormal polypeptide synthesis patterns.[39]

● Embryonic Gene Expression

The regulation of embryos at the post-transcriptional level by preserved maternal mRNA gives rise to embryonic gene expression activation during the 4- and 8-cell stages. Maternal mRNA is absent after the switching processes from maternal to embryonic development regulation.[2] Nevertheless, some embryonic gene expression activation is identified prior to the main activation at the 4-cell stage.[2] The study of gene expression patterns at all stages of development and gene demonstration can proceed by RT-PCR method; while only a few studies can define

the accurate maternal or embryonic cause of transcripts.[2] Semiquantitative RT-PCR is used to detect the maternal and embryonic transcripts origin; and also reassures that the stored maternal mRNA is decreased during the activated embryonic transcription.[2]

Conclusion

In vitro fertilisation plays a major role in the study of the preimplantation embryos development. Many on going studies in preimplantation human embryos are relevant to improve culture conditions and to find the best method to select the good quality of embryos that have chance to survive and implant. A lot of factors involved in the improvement of the preimplantation human embryonic development are identified. The restriction in human studies, however including the few numbers of the left embryos from the use in therapeutic IVF, has delayed and limited the understanding. The studies of animals, which are compared with these in humans, are very helpful to understand the human embryogenesis. The overall success rate of IVF and embryo transfer is still low, although many studies have been performed. More studies are needed in order to increase the pregnancy rate after therapeutic IVF and more knowledge of the important preimplantation embryonic factors is needed. The precise timing of preimplantation development of each stage in vitro is undetected because the only one time a day of observation is allowed to avoid disturbing the embryos. The accurate timing of embryonic development can be possible by using continuously recorded camera observation. Furthermore, the abnormal embryonic development and cleavage-stage arrest can also be detected by this method. The left over of excess embryos from therapeutic IVF are very useful to study under approval of the law and ethics.

Low levels of glucose, hypoxanthine, glutamine, vitamins and essential amino acid are observed as the essential energy resources. Growth factors and cell-adhesion molecules are also the important factors in preimplantation embryo development leading to the promotion of cleavage and implantation rates. The co-culture with feeder cells method may improve cleavage rate but it is expensive and poses high risk of disease contamination. Embryo morphology is a subjective technique but is simple and effective to assess the human embryos at present. The association between cleavage-stage arrest and the high incidence chromosome abnormalities is also important in preimplantation embryo development. Pyruvate, glucose and lactate uptake are good objective factors to assess the preimplantation human embryo development. The studies of growth factors, their receptors and cell adhesion molecules will disclose the important growth enhancement and helps in finding out new predictors for embryo viability assessment. Most studies at the present are done in vitro. However, in vivo studies will be possible if there is available method and will lead to the real understanding in human preimplantation embryo development in the future.

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