



## Illustrating the Past, Present and Future Perspective of Human Embryo Culture Media

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### ABSTRACT

Culture media has an important role in *in vitro* embryo development and in determining the success rate of assisted reproduction technologies. Extensive research during the last few decades has brought about major advancements in the culture media of *in vitro* fertilization (IVF). Studies on the composition of the mammalian oviductal fluids and the uterine fluids have improved our understanding of the evolving metabolic needs of the preimplantation embryo. This has led to the gradual development of human IVF media, from simple salt solutions to highly complex defined media. Regarding the ideal composition of IVF culture media, two different philosophies have come up “back-to-nature” or sequential culture principle and “let-the-embryo-choose” or one-step culture principle. Sequential culture closely mimics the physiological conditions of the female reproductive tract, while the monoculture system employs simultaneous use of all components in an optimal concentration. Besides the role in viability maintenance, culture media can also take up a new role as a therapeutic agent in the future. The improvised media apart from maintaining the viability may work on improving the quality of gametes/embryos obtained from poor prognosis patients. This review would discuss the progressive evolution of embryo culture media based on studies on pre-implantation embryo physiology and lastly will hypothesize the future of this technology.

### Introduction

Despite intensive studies on human IVF, elucidation of the optimum metabolic requirements of the human embryo remains a work in progress. Embryo transfer at the blastocyst stage has more physiological synchronization with the

naturally occurring time of embryonic implantation and increases the receptivity of the endometrium for *in-vitro* developed embryos (Wirleitner et al., 2010). So, presently most IVF labs are moving towards an extended culture of embryos to the blastocyst stage. However, the adoption of extended embryo culture has entailed new challenges on

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best culture media formulations. Since the establishment of IVF technology, a large number of media have been developed but the formulation of an optimal culture media that can support extended embryo culture as well as maximize embryo viability is the need of the hour. An advantage of extended embryo culture is it helps to segregate embryos based on their developmental competency while also maximizing embryo viability so that the surplus embryos can be cryopreserved (Lane and Gardener, 2007). To improve pregnancy outcomes, many assisted reproductive technology (ART) clinics end up transferring a few blastocyst embryos in the current IVF cycle whilst cryopreserving the rest for the frozen embryo transfer (FET) cycle. Therefore, the culture media in addition to its potential to produce the best quality embryos should enhance the sibling embryo's cryotolerance ability so that they can survive freezing or vitrification (Lane and Gardener, 2007). To culture, a higher number of blastocyst-stage embryos having the maximum inherent ability for the current IVF cycle and cryopreservation, demands justified scrutiny of the embryo culture system. Usually, the selection of an embryo culture media is based on its ability to produce one or two highest-grade embryos that can result in a successful pregnancy. But such a culture system should be selected that can produce a greater number of viable embryos from the entire cohort, which can result in successful pregnancy both fresh and FET cycle. The embryos generally experience stress unique to the *in-vitro* culture environment and additionally, certain unknown factors present in the culture media also have retarding effect on the developmental potential of the embryo (Fauque et al., 2007; Yao and Asayama, 2016; Ramos et al., 2019). Therefore, all IVF scientists must have a clear understanding of the function of each component of the medium so that the culture media selected has minimized environmental stress on the embryo.

Based on demonstrable research on the composition of mammalian oviductal and uterine fluids and preimplantation embryo metabolism, currently, two different strategies are used for the culture of human preimplantation embryos- the "back to nature" or sequential culture method and 'let-the-embryo-choose' or one-step culture method (Sepúlveda et al., 2009; Yao and Asayama, 2016). The sequential culture method mimics the female reproductive tract and uses two media in sequence (Sepúlveda et al., 2009). While the monoculture medium does not mimic the *in-vivo* environment, rather constitutes a medium that has an appropriate concentration of each component that remains the same throughout the culture period (Lane and Gardner, 2007). The following review, in the first part, discusses preimplantation embryo physiology vis-à-vis the evolution of human embryo culture media. The second

part gives a complete update on the detailed functions of media components to know what components are essential or detrimental. And lastly, the study has hypothesized a future gaze on culture media formulation to assist poor prognosis of IVF patients.

## Pre-implantation embryo physiology

The mammalian embryo is completely different from somatic cells concerning their metabolic requirements, biosynthetic pathways, and also likely their epigenome (Zander-Fox and Lane, 2012). And that is why the culture media developed for the *in-vitro* culture of somatic cells is also not adequate for the development of preimplantation embryos (Brinster, 1965). But initially, as there was not much information regarding the metabolic requirement of mammalian embryos, studies on pre-implantation embryo physiology became the basis upon which research for the development of embryo culture media began. The preimplantation embryonic stage is the progressive development of the fertilized zygote from the 2PN stage to the blastocyst stage that is marked by several key morphological, physiological, and genetic changes all of which are critical for the development of the early embryo (Gardner, 2018). Broadly the preimplantation embryonic stage is considered in two distinct developmental stages- pre-compaction stage and post-compaction embryo stage (Fig 1; Zander-Fox and Lane, 2012).

## Pre-compaction stage embryo

The pre-compaction stage of the embryo is a very dynamic and crucial period that starts post-fertilization during which the relatively quiescent oocyte develops to the pronuclear stage embryo under the genetic control of maternally derived transcripts and then further develops to cleavage-stage embryo having its genetic control (Lane and Gardner, 2007). After fertilization, the embryo undergoes extrusion of the second polar body, fusion of the maternal and paternal pronuclei at the one-cell stage, followed by activation of the embryonic genome, and a series of mitotic or cleavage divisions that result in the formation of a compacted morula. (Ramathal et al., 2015). The first few divisions are named as restrictive mitoses during which the cells (known as blastomeres) divide into daughter cells smaller in size, which is required to restore the cytoplasmic: a nuclear ratio that remains abnormally high in the fertilized oocyte. The first of these divisions occur around 18 h after fertilization, with subsequent divisions occurring approximately every 12 h (Gardner, 2018).

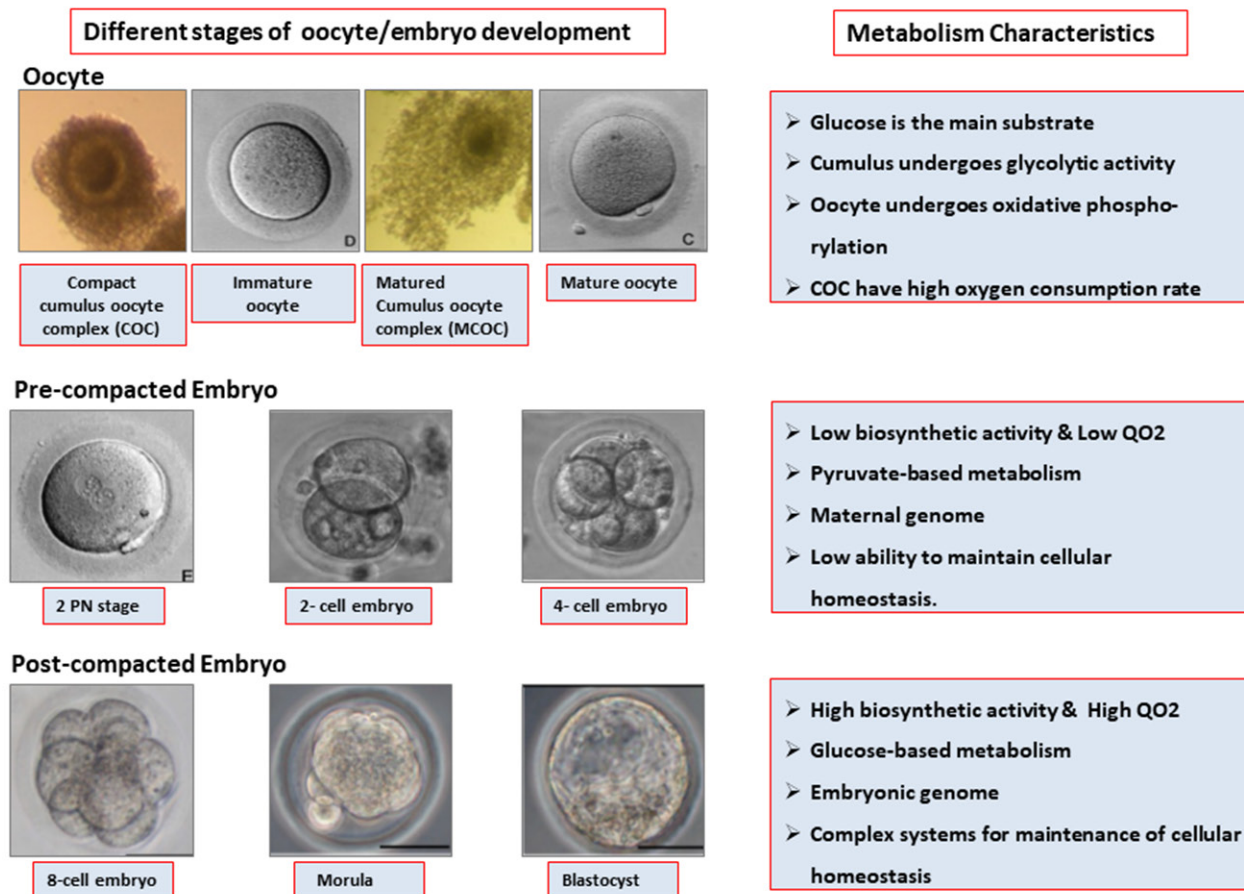


Fig. 1. Dynamics of pre-implantation embryo physiology (Yadav et al., 2014; Choi and Xiaoming, 2015; Qu and Bishop, 2012 under Creative Commons Attributes License (CC By) and Goud et al., 1998 reproduced with permission from).

**Pre-compacted embryo metabolism:** The early-stage embryo exhibits a unique metabolism primarily based on low levels of oxidation of pyruvate with little or no ability to metabolize glucose (Gardner, 2018). At this stage, the early embryo is characterized by low oxygen consumption and very little energy demand. The dire consequence is the rise in ATP/ADP ratio that leads to allosteric inhibition of the glycolytic enzyme phosphofructokinase which explains why the embryo relies entirely on mitochondrial oxidation to produce energy via oxidative phosphorylation of pyruvate (Gardner, 2018). That is why for the early-stage embryos, pyruvate and lactates are the preferred energy substrates, together with specific amino acids, such as aspartate (Baltz, 2013). Analysis of the human oviductal fluid (where the early embryo resides naturally) shows a similar trend that it contains pyruvate (0.32 mM) and lactate (10.5 mM) and low levels of glucose (0.5 mM) (Gardner, 2018). However, for the synthesis of glutathione for protection against oxidative stress, very low levels of glucose are taken up by the embryo which is metabolized by the pentose phosphate pathway for the maintenance of the cell's reducing power (Gott et al., 1990).

**Pre-compacted embryo homeostasis regulation:** No active transport systems are present in the early stage embryos for regulation of intracellular pH which possibly develop after 6-8 h of fertilization and become completely functional until around 10 h post-fertilization (Leese et al., 2001). This is the reason the cleavage stage embryo is more sensitive to stress, as many of the salient homeostatic mechanisms that are normally present in all somatic cells to defend against changes in pH, osmotic stress, and reactive oxygen species (ROS) are absent here (Lane, 2001; Fig 1). In the mother's body, the oviductal fluid maintains the pH gradient according to the early embryo's need and regulates oxygen levels between 5%-8% that are favorable for oxidative-based metabolism (Gardner and Lane, 2017).

## Post-compaction stage embryo

The pre-implantation embryo after 8 cell stage undergoes gradual compaction to form a morula, during which flattening of the blastomeres takes place to maximize cell-cell contact that results in the polarization of the cells (Lopes

and Mummery, 2007). Following compaction, the process of cavitation begins and the compacted embryo transforms into a blastocyst where an outer layer of trophoblastic cells (TE) surrounds the blastocoelic cavity containing blastocoelic fluid and an eccentrically placed mass of cells called the inner cell mass (ICM; Biggers et al., 1988).

**Post-compacted embryo metabolism:** Around the time of compaction, the embryonic genome activation completes and there is dramatically increasing in the level of biosynthesis (Gott et al., 1990). As the embryo undergoes further cell division and the cell number increases there is a concomitant increase in energy demand, resulting in a low ATP/ADP ratio. This contributes to a positive allosteric effect on PFK and thereby facilitates a high flux of glucose through glycolysis shown in Fig 1 (Gardner, 2018). But though the embryo now shifts to glucose-based metabolism, the blastocyst does not oxidize all of the glucose, rather exhibits aerobic glycolysis (that is lactate is produced from glucose even when there is sufficient oxygen for its complete oxidation). As glucose is the key player for all cellular biosynthesis, aerobic glycolysis ensures the availability of glucose for the synthesis of other cellular derivatives-triacylglycerols, phospholipids, and precursors for complex sugars (Gardner, 2018). At this stage in vivo, the embryo descends to the uterus and the uterine fluid composition mirrors a similar trend (Fig 2), enriched with relatively high levels of glucose (3.15 mM), and low levels of pyruvate (0.1 mM) and lactate (5.87 mM) (Gardner et al., 1996).

### Post-compacted embryo homeostatic regulation:

With the onset of embryonic genome activation at 8 cell stage in humans, the homeostatic systems become highly developed (Biggers et al., 1988). As blastomeres compact and become polarised, the cytoplasm forms two zones: apical and basal, with the ion transport systems and tight junctions formed apically and gap junctions formed basally. Further, the next cleavage plane perpendicular to the axis of polarity results in two kinds of cells- the outer cells (TE layer) and the ICM (Lopes and Mummery, 2007). The  $\text{Na}^+/\text{K}^+$  ATPase and tight junctions maintain the ionic gradient across the TE cells and promote water accumulation in the embryo resulting in a fluid-filled cavity known as blastocoel through the process of cavitation (Lopes and Mummery, 2007). The embryo then referred to as blastocyst has an outer trophoblast and inner ICM.

Thus keeping in view the above findings, the culture media developed for the early embryo must be supportive for the embryo's metabolic needs and homeostatic functions (Van Winkle, 2001). Studies depicting the biology of the preimplantation embryo unfurled the dynamic environment of the maternal tract. Further, analysis of the human female reproductive tract showed that it contains the same nutrients that mirror the changing nutrient preference of the developing embryo (Leese et al., 2001; Gardner, 2018). The nutrient content of mammalian oviductal fluid comprises high concentrations of pyruvate and lactate and low concentration of glucose similar to the metabolic requirement of the pre-compaction embryo,

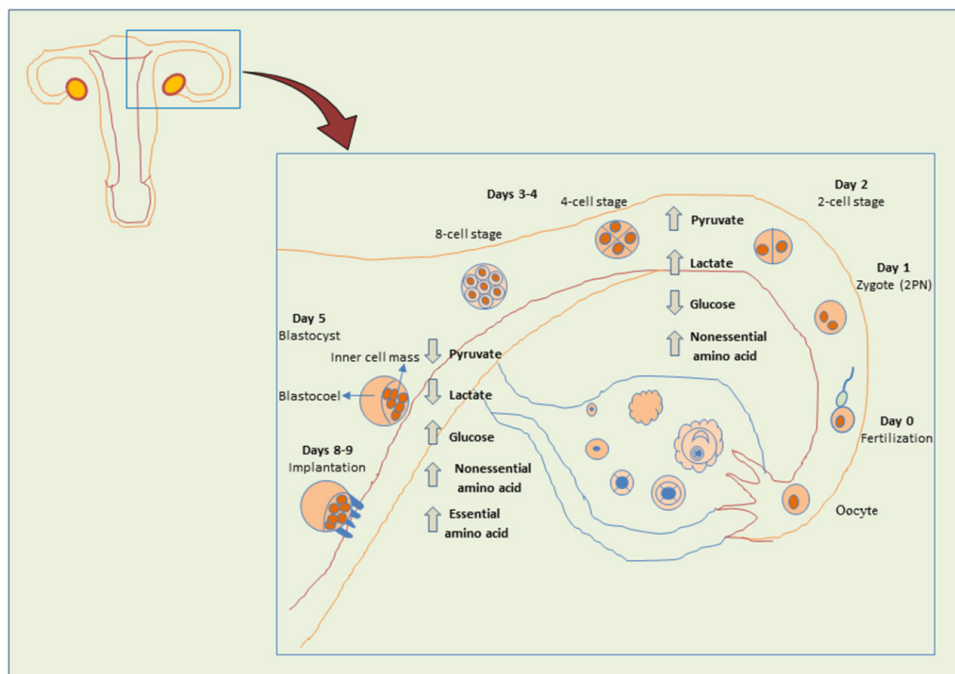


Fig. 2. Nutrient contents of the female reproductive tract and pre-implantation embryo development.

while the uterine fluid contains low levels of pyruvate and lactate and a high concentration of glucose, which is also similar to the metabolic requirement of post-compaction embryos (Fig 2).

## Evolution of embryo culture media: from past till present

The culture of tissue samples was first started using biological media but was soon replaced by chemical media due to varied reproducibility. In 1940, the *in-vitro* culture of mammalian embryos began, which used chemically defined media, but at that time most of the culture media were ill-defined. The culture media used then basically comprised of different salts and were initially designed to support the culture of somatic cells eg. modified Earle's balanced salt solution, Ringer's balanced salt solution, Tyrode's balanced salt solution, etc. However, as studies on pre-implantation embryo physiology unfolded new findings, the embryo culture media underwent a gradual evolution from simple media to complex media, sequential media, and finally monoculture media. The major setback in the development of human IVF culture media was the inability to conduct experimental studies on the human embryo due to strict ethical jurisdictions. As a consequence, the majority of knowledge concerned with the mammalian pre-implantation embryo has been extrapolated from studies performed on animal models.

## Landmarks of mammalian embryo culture

Whitten in 1956 used Krebs-Ringer bicarbonate solution with glucose, bovine serum albumin (BSA), and antibiotics and successfully cultured 8-cell mouse embryos collected from the fallopian tube to the blastocyst stage (Whitten, 1956; Yao and Asayama, 2016). At that time, it was harder to culture embryos retrieved before the 8-cell stage, but subsequently, he demonstrated that adding lactate to the medium enabled the progress of embryos from the 2-cell stage to the blastocyst stage. (Whitten, 1957; Yao and Asayama, 2016). Next, Brinster discovered that the 2-cell-stage mouse embryos did not utilize glucose as an energy source rather required either pyruvate or lactate. He developed brinster's medium for ovum culture (BMOC2) in mice and showed that it resulted in improved blastocyst yield from 2-cell embryos when both pyruvate and lactate were added to the media compared to their single-use (Brinster, 1965; Yao and Asayama, 2016). However, further studies found that without glucose the post-compacted embryos

could not develop into the blastocyst stage (Brinster, 1965; Brown and Whittingham, 1991). In 1968, finally, Whitten and Biggers were successful in culturing 1-cell embryos to blastocyst stage using a medium containing BSA, pyruvate, lactate, and glucose (Whitten and Biggers, 1968; Yao and Asayama, 2016). But using this medium they could only culture fertilized eggs from F1 hybrid mice while most embryos from other strains showed developmental arrest at the 2-cell stage, which was later known as 'the early embryonic developmental block' and was noted in the majority of other laboratory animals (Whittingham, 1968; Bavister, 1995). To overcome the developmental block, some studies used a co-culture system and found that the addition of EDTA prevented premature glucose utilization and alleviated the block (Gardner and Lane, 1993; Baltz, 2013). It was noted that developmental block coincided with the embryonic genomic activation (EGA) at different stages in different species. The presence of glucose and phosphate in culture media was attributed to being the cause for the developmental block in early-stage embryos (Schini and Bavister, 1988; Chronopoulou and Harper, 2015). In summary, the above studies on the animal model provided an overview for developing IVF culture media for human embryos.

## Evolution of media systems for human embryo culture

As the human embryos have the plasticity for asynchronous transfer on day 2/3, conventionally, during early days of IVF, embryo transfer was practiced on day 2 or 3 (Ahuja et al., 1985). But the real reason was due to lack of knowledge regarding the physiology and metabolism of preimplantation embryos, they could not be cultured beyond 8 cell stages. Further, the culture media used then were the ones designed for tissue culture which were either simple salt solutions or complex media. Simple media mainly consisted of balanced salt solutions with added carbohydrates (eg. KRB solution, BMOC2 media), and many components presently known to be essential for embryo growth were lacking (such as amino acids, growth factors) (Yao and Asayama, 2016). Later, embryos were grown in complex tissue culture media (eg. Ham's F-10, MEM, or TCM-199) which though contained additional nutrients, some components such as hormones, hypoxanthine, and trace elements were found to be detrimental for the embryo development (Pinsino et al., 2011). The world's 1<sup>st</sup> IVF baby was born using Ham's F10 media with homologous human serum or BSA (Edwards, 1981). Based on the bovine and human oviductal/uterine fluid composition, two research groups Menezo et al. and Quinn et al.

developed IVF media known as B2 medium and human tubal fluid (HTF) medium respectively (Menezo et al., 1984; Quinn et al., 1995). The HTF medium was developed and first commercial media that became a gold standard for the culture of human embryos transferred on day 2/3 of culture. Further, phosphate and glucose were removed from the HTF media, as it caused the developmental block in embryos and the modified media was known as Basal X1 HTF. Studies showed that inorganic phosphate in culture medium significantly disrupted mitochondrial organization in hamster embryos. While the presence of glucose in early-stage mouse embryos inhibited HGPRT activity and produced more superoxide due to hypoxanthine breakdown (Ludwig et al., 2001; Guerin et al., 2001). So glucose was substituted by glutamine and EDTA was added in Basal X1 HTF medium (Yao and Asayama, 2016). Another culture media named preimplantation stage 1 (P1) medium was introduced that contained citrate and taurine in place of glutamine and EDTA. But in all these media the blastocyst formation rate was not satisfactory (Yao and Asayama, 2016). Subsequent studies on animal models further clarified the changing needs of the embryo, in terms of energy substrate and amino acid requirement, at different stages of embryo development. Experiments showed that the cleaving embryos needed pyruvate and

lactate as an energy source and only non-essential amino acids (NEAA) as osmolytes and intracellular buffers to regulate homeostasis and cell signaling. But post compaction, glucose was essential for development into the blastocyst-stage embryo and both NEAA and essential amino acids (EAA) were required for ICM development (Gardner et al., 1996; Lane and Gardner, 1997).

Gardner's group analyzed the composition of the human oviduct and uterine fluids and found that the concentrations of energy substrates and amino acids corresponded with the changing nutritional requirements of the embryo (Gardner et al., 1996; Gardner and Lane, 1997). Based on these findings, Gardner et al. formulated the new generation culture media called G1/G2 sequential media, which is composed of two culture media that need to be used in sequence. G1 features the oviductal fluid that supports in-vitro development of the fertilized oocyte to the 8-cell stage, and G2 mimics the uterine fluid and nurtures the development of the 8-cell stage embryos to blastocysts stage. This was a precious discovery and a milestone in the era of human IVF (Gardner et al., 1998). The popular sequential systems, that are today in widespread use are Quinn's series in the United States and Cook from Australia, and the MediCult/Origio from Europe (Table 1A).

**Table 1.** Commercially available human IVF culture systems

A. Sequential media used in embryo culture

Sl. No.	Medium Name	Culture Period	Company Name
1	Cleavage K-SICM	Day-1 to Day-3	Cook Medical
	Blastocyst K-SIBM	Day-3 to Day-5 or 6	
2	Quinns Advantage® Cleavage	Day-1 to Day-3	Cooper Surgical
	Quinns Advantage® Blastocyst	Day-3 to Day-5 or 6	
3	FERTICULT™ IVF Medium	Day-1 to Day-2	FertiPro
	FERTICULT™ G3 Medium	Day-3 to Day-4	
4	IVC-TWO™	Day-0 to Day-3	InVivoCare
	IVC-THREE™	Day-3 to Day-5	
5	ECM®	Day-0 to Day-3	Irvine Scientific
	MultiBlast®	Day-3 to Day-5	
6	EmbryoAssist™	Day-0 to Day-3	Origio
	BlastAssist™	Day-3 to Day-5	
	ISM1	Day-0 to Day-3	
	ISM2	Day-3 to Day-5	
7	G-1™ PLUS	Day-1 to Day-3	Vitrolife
	G-2™ PLUS	Day-3 to Day-5	
	IVF™	Day-0 to Day-3	
	CCM™	Day-3 to Day-5	

**B. Single-step media used in embryo culture**

Sl. No.	Medium Name	Culture Period	Company Name
1	GM501	Day-0 to Day-5 or 6	Gynemed
2	SSM™	Day-0 to Day-5 or 6	IrvineScientific
3	global®	Day-1 to Day-5 or 6	LifeGlobal

On the contrary, another team of researchers, Lawitts and Biggers explored the sophisticated simplex optimization software to assess multiple components and their interactions and they came up with a new concept known as one-step protocol or monoculture technique for human embryo culture (Lawitts and Biggers, 1991; Yao and Asayama, 2016). Applying the principle of simplex optimization, the optimal concentration of 10 components (NaCl, KCl,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ , lactate, pyruvate, glucose, BSA, EDTA, and glutamine) were assessed using blastocyst formation rates *in-vitro* for the response (Lawitts and Biggers, 1991; Yao and Asayama, 2016). Based on this an optimally enriched culture media called Simplex Optimization Medium (SOM) was designed which was further modified with the addition of potassium and amino acids and came to be known as mKSOMAA, that provided a one-step protocol (so-called global medium) to culture human zygotes to the blastocyst stage (Lawitts and Biggers, 1991; 1992). Based on the parent formulation, presently three one-step human embryo culture media are commercially available: Gynemed GM501®, Lensahn, Germany; Global®, LifeGlobal, U.S.; SSM™, Irvine Scientific, U.S. (Table 1B).

## Culture media components

The *in-vitro* culture environment does have a significant impact on the genetic, epigenetic, and metabolic makeup of the embryo (El Hajj and Haaf, 2013). To minimize the stress upon gametes and embryos in IVF, the components of the culture media require scrutiny. Concerns regarding culture media components, their potential effects, and the regulation of embryo culture media have been highlighted previously by the Scientific and Clinical Advances Advisory Committee (SCAAC). To evaluate the efficacy of different embryo culture media, it is important to understand the culture media components and their interaction with the developing embryo.

**Carbohydrates:** As stated earlier, at different developmental stages the pre-implantation embryo relies on different energy sources to generate ATP. Pyruvate and lactate are the early embryo's primary sources of energy as they cannot metabolize glucose (Morbeck et al., 2014). Pyruvate is directly utilized by Krebs's cycle while lactate must be first reversibly converted to pyruvate by lactate dehydrogenase

to enter into the TCA cycle. But it is not only their absolute concentration but also the ratio of these two carbohydrates (L:P) in the culture medium that influences embryo viability (Morbeck et al., 2014). To be compatible with the needs of the early embryo, the G1/cleavage-stage media has high concentrations of pyruvate and lactate with low levels of glucose (Tarahomi et al., 2019). Following compaction, as cellular biosynthesis increases and there is also an increase in the respiratory capacity, the metabolism shifts to glucose-based metabolism (Gruber and Klein, 2011). So, for the development of the post compacted embryos, the G2/blastocyst stage media has a high concentration of glucose with low levels of pyruvate and lactate. As glucose is the key anabolic precursor for all cellular biosynthesis, the embryo partly uses it for energy production through aerobic glycolysis (Warburg effect), while the rest is diverted for the synthesis of other derivatives like- glycolipids, glycoproteins, and complex sugars (Gardner, 2018). The mean concentration of glucose in cleavage-stage media and blastocyst stage media is found to be 0.37 mmol and 2.57 mmol respectively (Tarahomi et al., 2019). However, the shift in energy metabolism presents a challenge for the monoculture media to meet the diverse demands of the embryo. Based on the simplex optimization process, single-step culture media contain 0.2 mM glucose and follow consistent lactate to pyruvate ratio for embryo development. Contrary to the claim that glucose has an inhibitory effect on precompact embryos, the rationale used is the embryo selectively chooses the energy substrate that is within the tolerable range and any adverse effect due to glucose could be effectively alleviated by the addition of amino acids (Biggers and Summers, 2008; Morbeck et al., 2017).

**Amino Acids:** Amino acids are important regulators of preimplantation development, utilized by embryos at different rates at different stages of development. Before embryonic genome activation, AAs contribute as an energy source and also carry out other important functions as buffers of internal pH, osmolytes, antioxidants, chelators of heavy metals, and biosynthetic precursor molecules (Gruber and Klein, 2011; Zander-Fox and Lane, 2014). NEAA along with glutamine is present in relatively high levels in the oviductal fluid that stimulate the growth of the early cleavage embryo (Lane and Gardner, 1997; Steeves and Gardner, 1999). After compaction both EAA and NEAA are required for the development of the

embryo. Both stimulate the development of ICM of blastocyst while NEAAs and glutamine help trophectoderm formation and hatching from the zona pellucida (Devreker et al., 2001; Gruber and Klein, 2011). Therefore, both phases of sequential media (G1 and G2) contain NEAAs for chelation and osmolality maintenance while EAAs are added only to the second phase of culture media (G2 media) when the metabolism of the embryo becomes more complex similar to somatic cell (Table 2). However, one undesired effect of amino acids, particularly glutamine, in the culture medium is that they spontaneously break down to ammonium at 37°C which has a significant effect on the cellular health of the blastomeres (Zander et al., 2006; Lane and Gardner, 2003). In this regard, the two-step sequential culture developed by Gardner gives the option

to remove the accumulated ammonium (Lane and Gardner, 1994) while in monoculture media, the possible option explored is replacing glutamine with its stable dipeptide that results in significantly lower levels of ammonium production (Biggers and Summer, 2008).

**Chelating agent-EDTA:** EDTA in culture media functions as a ligand and chelating agent, to sequester metal ions which would otherwise cause peroxidation resulting in oxidative stress. Further, the addition of EDTA also alleviates the 2-cell block in embryos (Gruber and Klein, 2011). It inhibits premature glycolysis decreasing aerobic metabolism and ROS production and it also blocks the conversion of hypoxanthine to xanthin that produces superoxides (Gardner and Lane, 1993; Guerin et al., 2001). However, EDTA at 0.1 mM/L has been shown to decrease ICM development post

**Table 2.** Essential components of commercial embryo culture media

Components	Sequential media G1 Media	Sequential media G2 Media	One media system
Salts & Ions	Sodium Chloride	Sodium Chloride	Sodium Chloride
	Potassium Chloride	Potassium Chloride	Potassium Chloride
	Magnesium Sulfate	Magnesium Sulfate	Magnesium Sulfate
	Calcium Chloride	Calcium Chloride	Calcium Chloride
	-	Sodium Dihydrogen Phosphate	Sodium Dihydrogen Phosphate
Non-Essential AA's	Alanine	Alanine	Alanine
	Asparagine	Asparagine	Asparagine
	Aspartic Acid	Aspartic Acid	Aspartic Acid
	Glutamic Acid	Glutamic Acid	Glutamic Acid
	Glutamine	Glutamine	Glutamine
	Glycine	Glycine	Glycine
	Proline	Proline	Proline
	Serine	Serine	Serine
	Taurine	Taurine	Taurine
Essential AA's		Arginine	Arginine
		Cysteine	Cysteine
		Histidine	Histidine
		Isoleucine	Isoleucine
		Leucine	Leucine
		Lysine	Lysine
		Methionine	Methionine
		Phenylalanine	Phenylalanine
		Threonine	Threonine
		Tryptophan	Tryptophan
	Tyrosine	Tyrosine	
	Valine	Valine	
Antioxidant	Sodium Citrate	Sodium Citrate	-
	EDTA	-	EDTA

Added with: *pH indicator: Phenol red, Antibiotic: Gentamicin sulfate, Buffer: Sodium bicarbonate, Energy substrates: Glucose, Sodium pyruvate, Sodium lactate, Dipeptide: Alanyl-glutamine*

**Note: Ingredients written in italics at the bottom of the Table are common to all three types.** NEAA: non-essential amino acids, EAA: essential amino acids.



compaction (Gardner et al., 2000). The reason for such biphasic effect is- the glycolysis inhibitory effect of EDTA is needed until compaction only, as after that the embryo requires glycolysis for energy production, and the presence of EDTA then inhibits blastocyst development (Hewitson and Leese, 1993). Therefore, EDTA is omitted from G2 sequential media designed for blastocyst stage embryo but monoculture media have optimized EDTA concentration to be 0.01mM/L that has no deleterious effect on embryo development as indicated in Table 2 (Biggers and Summers, 2008).

**Macromolecules:** During the early days of IVF, the patient's serum was added to embryo culture media to provide multiple beneficial factors but was soon replaced by human serum albumin, as it contained many undefined components (Gardner, 2008). In the female reproductive tract, the embryo is exposed to a variety of macromolecules such as albumin and glycosaminoglycans (hyaluronic acid) (Gardner, 2008). Albumin is a carrier for lipids, hormones, vitamins, and minerals and also has multiple functions like- maintaining cell membrane stability, chelating toxic components, and regulating capillary membrane permeability (Tarahomi et al., 2018). Because of its above functions and its promotive effect on in-vitro embryos, it is a component of the embryo culture media in place of serum (Lane and Gardner, 2003). Another physiological attribute found alternate to albumin is hyaluronic acid or hyaluronan which is present in the female reproductive tract and the human embryos also express the receptor for it (Fouladi-Nashta et al., 2017). The inclusion of hyaluronate in culture medium has been shown to increase the rate of embryo implantation and fetal development in mice and human embryos (Gardner, 2008; Bontekoe et al., 2009). Additionally, bovine, hyaluronan, and albumin in culture medium improved the blastocyst development and cryosurvival rate of embryos (Lane et al., 2003, Fouladi-Nashta et al., 2017). As most IVF clinics now transfer fewer embryos in a fresh cycle, for effective cryopreservation of the surplus embryos both albumin and hyaluronan are included in the culture media for better efficacy.

**Growth factors:** The preimplantation embryos are naturally exposed to a varying number of growth factors (GFs), some of which are secreted by the embryo itself and some by the maternal endometrium. The GFs mediate a cross-talk between the maternal tissue and the embryo that promotes the development and implantation of the growing embryo (O'Neill, 2008). The addition of various GFs to human embryo culture media has demonstrated optimistic results showing accelerated development, increased blastocysts rate, and cell number (Sjoblom et al., 2005; Richter, 2008; Kawamura, 2012). Surprisingly, the propositions which render GFs to be beneficial are reasons for

caution too. Under a natural condition in the maternal reproductive tract, GFs regulate cell growth and differentiation and mediate anti-apoptotic function. But this well-known effect translates into a disadvantage in-vitro, as apoptosis is required during embryogenesis to serve as an extreme repair mechanism to deal with abnormal cells (Harper et al., 2012). Therefore, the effect of GF on embryo health needs to be accessed when they are taken as a part of IVF culture media. In this regard, certain GFs have received considerable research attention like- Insulin and insulin-like growth factor (IGF), transforming growth factor-beta (TGF- $\beta$ ), and granulocyte-macrophage colony-stimulating factor (GM-CSF). IGFs which have a role in cell proliferation is found in the reproductive tract and their receptors have been detected in the 8-cell murine embryo (Henemyre and Markoff, 1999). Insulin and IGF ligands added to culture media have been shown to increase blastocyst development and ICM cell numbers in mice and additionally, it has also been shown to protect against oxidative stress (Guler et al., 2000; Makarevich and Markkula 2002). TGF- $\beta$  produced by preimplantation mouse embryos promotes cell growth, differentiation, and formation of extracellular matrix (Paria and Dey, 1990). Studies have demonstrated an increased rate of blastocyst development, upon the addition of TGF- $\beta$  to embryo culture media (Paria and Dey, 1990; Paria et al., 1992). The epithelial cells of the oviduct and uterus produce GM-CSF which regulates cell proliferation and differentiation (Zhao and Chegini, 1994). Both cell lineages of the blastocyst express GM-CSF receptor and studies have shown that culture medium with GM-CSF increased glucose transport to embryos and improved blastocyst development and hatching rates (Robertson et al., 2001). Following these promotive results, currently in human ART, it has become common to use culture media supplemented with IGF or GM-CSF (Yao and Asayama, 2016).

**Vitamins:** Vitamins added to the culture media acts as antioxidants that alleviate the detrimental effect caused due to deliberate secretion of ROS into the medium. Folic acid promotes intrinsic antioxidant production and also contributes to DNA, RNA, and protein synthesis and regulate proper epigenetic activity post-fertilization (Laanpere et al., 2010). Adding folic acid to the culture medium has recently been shown to increase fertilization rate and improve the developmental competence of bovine embryos (Baghshahi et al., 2021; Saini et al., 2022). As an essential epigenomic event, after fertilization DNA demethylation occurs to establish full developmental potential. But this process becomes error-prone during the in-vitro culture of embryos leading to faulty epigenetics (Chu et al., 2021). In mouse and bovine IVF embryos, the addition of vitamin C and cobalamin into culture media is reported to rescue

in-vitro embryonic development by correcting impaired active DNA Demethylation (Zacchini et al., 2017; Chu et al., 2021). Further, moderate dosages of vitamins C and E added to culture media have been shown to reduced oxidative damage and improve blastocyst development rate in mouse embryos (Wang et al., 2003). Therefore, currently, optimum concentrations of the following vitamins: ascorbic acid, folic acid, cyanocobalamin, and tocopherol are used in ART.

**Antibiotics:** To protect embryos from microbial contamination, antibiotics are routinely added to the culture media (Lemeire et al., 2007). Commonly used antibiotics are Gentamycin (50µg/ml), Streptomycin (100 µg/ml), and Penicillin (100U/ml). Gentamycin and Streptomycin work by inhibiting bacterial protein synthesis while Penicillin functions by disrupting the integrity of the cell wall through inhibition of peptidoglycan synthesis. Penicillin has no direct toxic effects on preimplantation embryos, however, the aminoglycosides show some toxic effects (Lemeire et al., 2007).

**pH and buffers:** pH is an important cellular function required to regulate an embryo's intracellular homeostasis. The pH of culture media is regulated by the CO<sub>2</sub> concentration and the bicarbonate concentration present in the media. But additionally, the intracellular ions, interactions between amino acids and other zwitterions, and proteins also regulate the pH of the media (Swain, 2012). Studies have proposed a pH range of 7.4 and 7.2 to be acceptable for embryo culture media (Swain, 2012) which means pHe, the extracellular pH of the medium. The intracellular pH of the cytoplasm (pHi) should be 0.1 unit less than the pHe so that embryo can use this gradient to drive transport mechanisms. The pHi of human embryos before cleavage is 7.12 and so the pHe needs to be within 7.2-7.3 (Philips et al., 2000). The two buffers commonly used in commercially available IVF culture media are the HEPES buffer, and the MOPS buffer. The pKa value of both buffers is 7.2 which is close to the zwitterionic buffers and the pHi of embryos. Both buffers are reported to be widely used in IVF handling but HEPES is preferred as it supports mammalian embryo development and can also chelate heavy metals (Mash et al., 2003). To ensure that the culture medium maintains constant pHe the types and makes of the incubator should be thoroughly validated.

**Medium osmolarity:** Regardless of extracellular and intracellular osmotic perturbations, all cells need to maintain a constant volume and so do the gametes/embryos need, which depends upon the osmolarity of the culture medium. *In-vivo* the embryos are exposed to a physiological osmolarity of >360 mOsmol (in oviductal fluid

(Borland et al., 1980). But most of the commercially available ART culture media have a lower osmolarity of about 250-300 mOsmol. The reason is as NaCl concentration is increased up to 290 mOsmol, the protein synthesis in embryos gets disrupted and the development of the embryo gets severely impaired (Biggers et al., 1993; Baltz, 2013). That is why extracellular AA, such as glycine, alanine, betaine, proline, and hypotaurine are added that act as organic osmolytes, which not only protects the preimplantation embryo against hypertonicity but also promotes embryo development (Biggers et al., 1993; Baltz, 2013).

## Prospective future research on embryo culture media

To date, the function of embryo culture media has been to alleviate stress and maintain gamete/embryo's viability whereas in the present human IVF culture media has excelled in terms of increasing pregnancy rates to manifolds. But can there be a new role assigned to culture media as a therapeutic device! Yes, such a culture media therapeutically designed, apart from its role in maintaining the inherent viability of gametes/embryos, may also help in improving the quality of gametes and embryos. The present embryo culture medium mainly functions to maintain the innate potentiality of the embryo till they are transferred to the reproductive tract. Enumerating the success rate of IVF globally, studies report that more than 85% of the woman under the age of 35, succeed to conceive within 12-18 months of IVF treatment, which is a quite favorable outcome. This means most likely, in these patients as the gametes are healthy and have high inherent viability, the culture media functions best to retain it, providing maximal outcomes. But what about the increasing number of patients where the inherent competency of the gametes is poor or compromised? For example women of advanced maternal age, women with obesity, pathologies such as polycystic ovarian syndrome, diabetes, etc.

## Probable mechanism of cellular damage in aged oocyte

Previous research has demonstrated that as maternal age increases significant perturbations occur in oocytes: mainly mitochondrial impairment occurs and the level of ROS increases. The increase in ROS level induces oxidative damage which potentially affects lipids, proteins, and DNA in the oocyte as indicated in Fig 3 (Eichenlaub-Ritter et al., 2010).

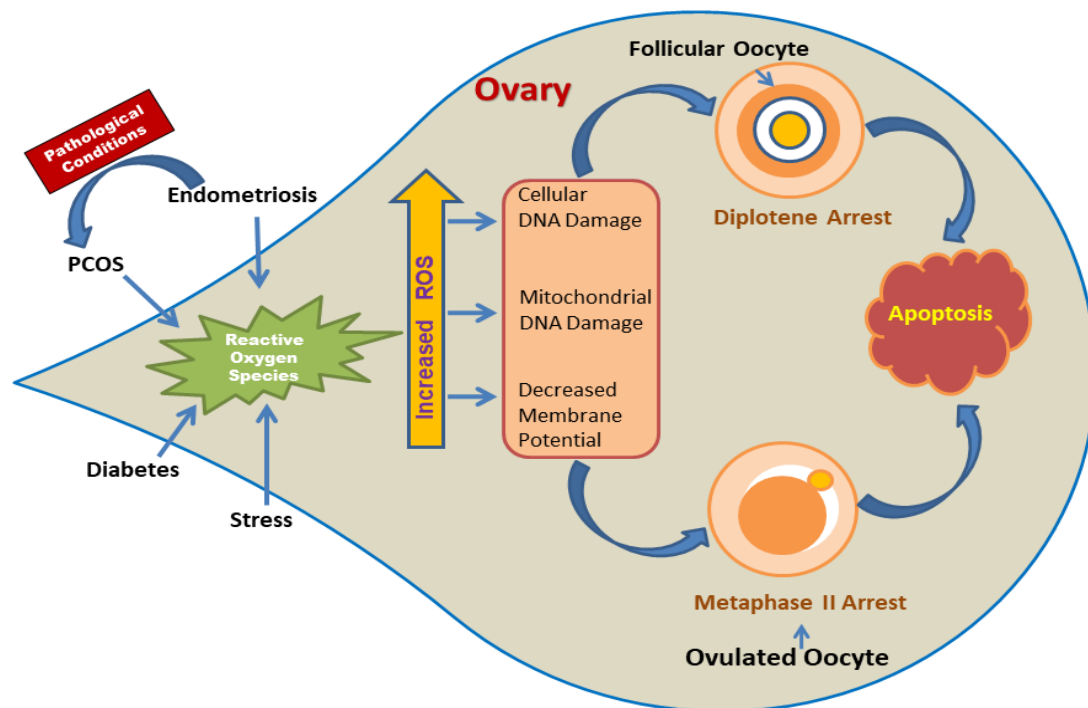


Fig. 3. Reactive oxygen species affecting oocyte ageing

The reason for the elevated ROS level is 'the leaky mitochondria' due to which high energy electrons transported during the electron transport chain are leaked into cytoplasm that forms ROS such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^{\cdot-}$ ), and hydroxyl free radical ( $OH^{\cdot}$ ) (Tarin et al., 1998). These ROS damage the oocyte in several ways: inducing lipid peroxidation, protein inactivation via the formation of disulfide bonds, and breakage of DNA strands (Gutteridge and Halliwell, 1989; Lopes et al., 1998). Besides, ROS even affects mitochondrial DNA (mtDNA) which may result in mtDNA deletions (Guerin et al., 2001). As mitochondrial metabolism is the main source of energy generation for oocytes and embryos, damage to mtDNA may result in decreased ATP generation hampering blastocyst viability and implantation potential (as ATP generation is a positive marker for implantation, Fig 4; Blerkom et al., 1995; Eichenlaub-Ritter et al., 2004). In addition, decreased energy generation has also been found to impair chromosome alignment and spindle activity resulting in increased rates of aneuploidy, in particular, trisomy in advanced age women undergoing ART (Wilding et al., 2005). Advanced maternal age is also associated with decreased antioxidant levels that result in further oxidative stress. Thus, the increase in ROS coupled with decreased antioxidant levels, at advanced maternal age may cumulatively result in cellular damage that can subsequently decrease oocyte/embryo viability (Fig 4).

## Reactive oxygen species quenching by improvised culture media

As discussed, aging contributes to oxidative stress of oocytes or vice-versa which results in mitochondrial function impairment and an increase in ROS levels. And this finally decreases ATP production due to impaired mitochondrial oxidation and severely compromises the quality of blastocyst. Therefore, as a protection measure, antioxidants addition to culture media may be tried which can act as ROS scavengers to rescue mitochondrial function, and improve ATP production. Presently, the ART culture media contain some antioxidants like- pyruvate and low levels of amino acids, but their current concentration is mostly set based on their function as a nutrient rather than as an antioxidant. Coenzyme Q10 (CoQ10) or ubiquinone-10 is a part of the respiratory chain complex, which also has antioxidant activity as it prevents electron leakage in mitochondria that results in ROS production (Hernández-Camacho et al., 2018). CoQ10 in its reduced form of Ubiquinol oxidizes free radicals and decreases their levels in the aged cell (Bentinger et al., 2007). As CoQ10 is water-insoluble, its addition via nano-particle to bovine in-vitro culture media has shown to improve cleavage rate, blastocyst development, and cell numbers (Stojkovic et al., 1999). Further, CoQ10 supplied as culture media adjuvant in mice has also

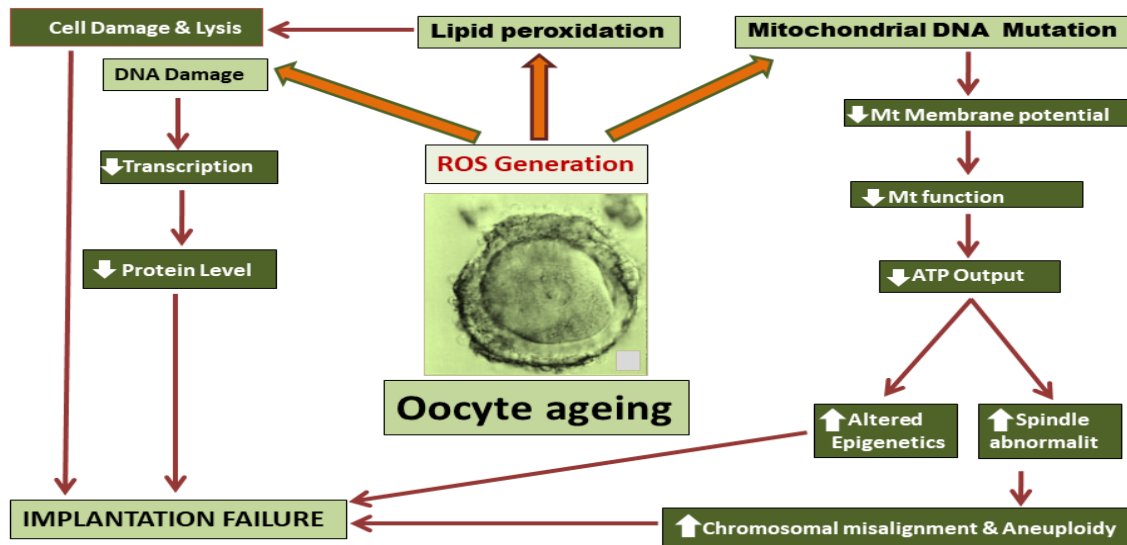


Fig. 4. Proposed mechanism of cellular damage in aged oocyte

been demonstrated to ameliorate the quality of post-ovulatory aged oocytes by suppressing DNA damage and apoptosis (Zhang et al., 2019). Few studies have shown that certain synthetic analogs such as Coenzyme Q2 (CoQ2) and idebenone when used in in-vitro culture, reduced rates of lipid peroxidation, stimulated mitochondrial electron flow, and increased oxidation of Complex II (Briere et al., 2004; Imada et al., 2008). Furthermore, a recent study in mice has shown improvement in oxidative stress and embryo quality in advanced paternal age using idebenone in-vitro (Nikitaras et al., 2021). Thus, the addition of antioxidants like- CoQ10, CoQ2, and idebenone to culture media, may be tried which can help quench ROS and can stimulate mitochondrial OXPHOS function to ultimately improve oocyte viability.

## Therapeutic culture media to improve oocyte quality

Recent clinical studies have shown that the follicular fluid milieu in obese women is different from women with normal BMI, as follicular fluid from these women exhibits increased levels of insulin, lactate, triglycerides, leptins, and C-reactive proteins, all of which are reasons for poor oocyte quality and decreased fecundity (Robker et al., 2009; Song et al., 2020). The dysfunction caused at the cellular level is first, the mitochondrial activity is affected with abnormal morphology, cristae structure, and poor membrane potential. Secondly, the rich mater-

nal metabolite environment causes oxidative stress in oocytes/embryos leading to endoplasmic reticulum stress where protein misfolding occurs associated with metabolic disorders (Lane et al., 2015; Fleming et al., 2018). Studies in mice models show that mice fed with a high-fat diet exhibit increased levels of intracellular lipid within the oocyte, reduced mitochondrial membrane potential and decreased blastocyst development, and decreased blastocyst cell number compared to control mice (Minge et al., 2008; Wu et al., 2010). Such high-fat diet-fed obese mice showed impaired oocyte meiotic maturation, disrupted spindle morphology, and reduced oocyte polarity which is all assorted for poor pregnancy outcomes (Hou et al., 2016). These studies depict that similar to advanced maternal age, the oocyte of obese women also undergoes damage, and likewise, in females with pathologies such as polycystic ovarian syndrome, endometriosis, and diabetes, alterations in follicular fluid milieu occur, which give rise to oocytes with altered physiology or molecular make up shown in Fig 3 (Qiao and Feng, 2011; Goud et al., 2014; Da Broi et al., 2018). Now the question is: Can such oocytes with altered physiology develop into viable embryos to give successful pregnancies? The altered oocytes with weak inherent ability when cultured with a universal culture media, can't develop to healthy embryos equivalent to those produced from normal good quality oocytes. But the possibility can be tried, only if the culture media could be modified to an extent that it can supplement the deficiencies. Therefore, 'customized culture media' needs to be designed (Fig 5) that can replenish the shortcomings,

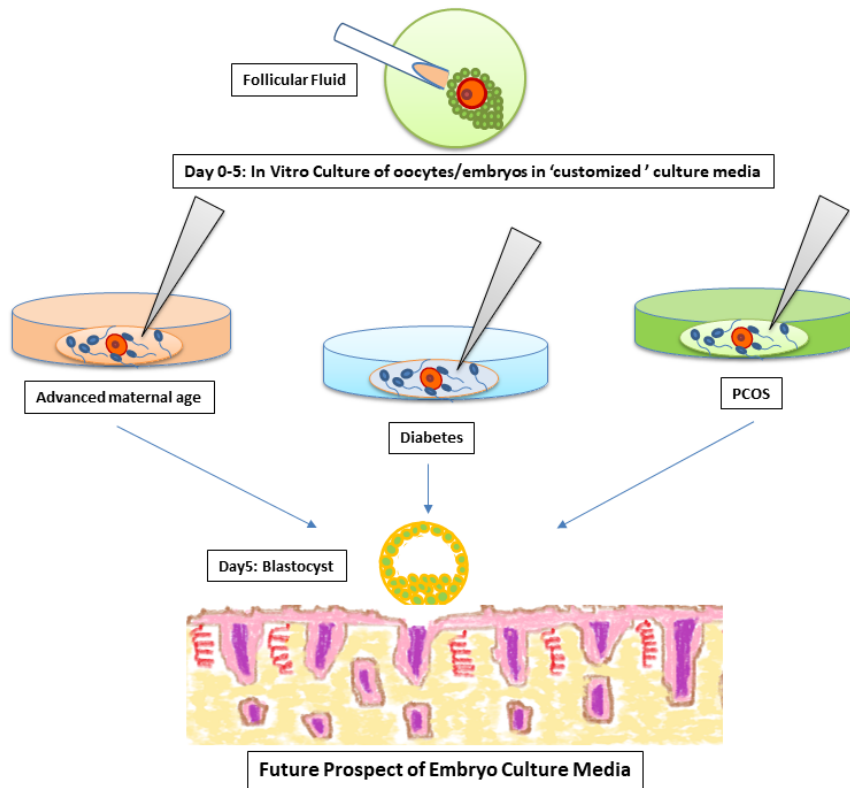


Fig. 5. Application of cultomised embryo culture media

so that the oocyte and embryo with compromised inherent viability may be rescued by reversing the perturbations induced by the suboptimal niche. Generation of customized media should be focussed for further research which would be a tailor-made media, to address the lacking suboptimal conditions of each patient. However, to develop efficient customized media, the molecular details of the altered follicular fluid milieu need to be understood first.

## Conclusion

In a single frame, the present review discussed pre-implantation embryo physiology vis-a-vis the evolution of embryo culture media and finally hypothesized the future of this technology. Studies demonstrated that unlike somatic cells the mammalian embryo is unique in its development and also in its metabolic requirement. This led to the development of culture media specific to embryo culture which could minimize stress and meet the stage-specific nutrient requirement of embryos. With progressive research unfolding new facts about preimplantation embryo physiology, the embryo culture media evolved and lastly, two different culture media *i.e.* sequential culture media and monoculture culture media, have been developed.

Both the media are now widely practiced in ART laboratories and around the world, the IVF results have increased manifolds. However, the present-day media formulations are primarily focused on maintaining oocyte's innate potentiality rather than improving it. Hence, it is the need of the hour to move from current culture conditions and try to design therapeutic media, so that, it can pave the way for ontogenetical improvement of the poor-quality oocytes retrieved from women with advanced maternal age or other pathologies. However, before we go for rescue-based 'customized culture media', a molecular understanding of the factors that alter the competence of the oocyte/embryo is essential.

## Conflicts of interest

The authors declare no conflicts of interest.

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