Trends, Fads and ART!

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Abstract Morphological selection techniques of gametes and embryos are of current interest to clinical practice in ART. Although intracytoplasmic morphologically selected sperm injection (IMSI), time lapse imaging morphometry (TLIM) or quantification of chromosome numbers (PGS) are potentially useful in research, they have not been shown to be of statistically predictive value and, thus, have only limited clinical usefulness. We make the point that morphological markers alone cannot predict the success of the early embryo, which depends on the correct orchestration of a myriad of physiological and biochemical activation events that progress independently of the maternal or zygotic genome. Since previous attempts to identify metabolic markers for embryo quality have failed and there is no evidence that the intrinsic nature of gametes and embryos can be improved in the laboratory, embryologists can only minimize environmental or operator induced damage while these cells are manipulated ex vivo.

Keywords Trends · Fads · Embryo selection · IVF

Introduction

The role and obligation of ART professionals is to achieve a live birth by applying the most minimally invasive technique,

while taking into account the cost and the time required to achieve success. The causes of infertility are many, often multiple within a couple, and the clinician needs to tailor each cycle by selecting the most appropriate protocol for ovarian stimulation, while the embryologist, by evaluating the patients gametes, decides which of the many laboratory technologies available will be most efficient.

New laboratory technologies appear regularly and are often introduced without evidence of effectiveness. General comments in clinical papers such as “*Recently randomized trials have indicated that...xxxxxx.....improves IVF success rates*” may be misleading as they give the impression that the technique under study would be useful for all patient groups. Since the birth of the first IVF baby in 1978 in Oldham, UK [1] ART has developed and spread across the globe as a recognized medical practice leading to the birth of millions of babies. However, as shown in a recent review, using data from the United States Society for Assisted Reproductive Technology (SART) and the Centers of Disease Control and prevention (CDC), overall birth rates remain low [2]. In a retrospective analysis of over 500 IVF cases, Patrizio and Sakkas [3] concluded that only approximately 5 % of fresh oocytes produced in assisted reproductive cycles actually lead to the birth of a baby. This study included all patients in a USA University program in groups below 35 years old, from 35 to 42 years old, over 42 years old and also included oocyte donors. It is not clear whether the 95 % loss of in vitro produced oocytes reflects natural gamete or embryo wastage or if it can be improved by changing or applying new laboratory based technologies. The purpose of this review is to discuss present and past trends in assisted reproductive technologies and in particular whether morphological selection techniques may help in improving live birth rates.

Capsule This review discusses trends and fads in ART and, in particular, whether the morphological selection techniques, IMSI, TLIM or PGS, improve live birth rates.

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Background to in vitro fertilization

Oogenesis is a period of growth and synthesis where all the elements required to support meiotic and early mitotic cycles, from mRNA through to mitochondria, are deposited in a polarized fashion in this enormous cell. The spermatozoon undergoes a re-modelling to prepare it for interaction with the female gamete, to power it with an activating trigger and to provide the division centre for the newborn. At fertilization, ionic messengers trigger a massive and global re-organization of the oocyte leading to meiotic resumption and early cleavage divisions [4]. Thus the myriad of processes that occur in the first 48 hrs of life utilize stored material, mainly maternal, that progresses without de novo expression of the maternal or zygotic genome.

In a stimulated cycle, embryologists are presented with a cohort of oocytes, hopefully with 70–80 % of them in metaphase II. However, in parallel to nuclear maturation, other maturation events progress in the cytoplasm that give the oocyte the ability to interact with the spermatozoon. These were first studied and defined by Delage in 1901 [5]. Nuclear and cytoplasmic maturation are often asynchronous in nature and this may be exaggerated by the forced recruitment of follicles during super-ovulation in the human [6]. Today we know that cytoplasmic maturity is, in part, synonymous with the metabolic status of the oocyte which continually changes, whether the oocyte is fertilized or left in culture medium (aging). Thus our newly collected MII oocytes appear synchronous with regards to nuclear maturation but are poised at various degrees of metabolic repression [7] and, by today's criteria, the best an embryologist can do is to inseminate or inject them at a pre-established time. All cells are dynamic and changes in the cytosol, such as membrane trafficking, are continual. A good example is the tread milling process in the actin cytoskeleton where there is a constant addition of actin sub-units to one end of a filament, with the constant removal at the other end resulting in the apparent movement of the filament across the cytosol [8]. Another non-genomic variable in oocytes is mitochondrial activity which decreases with maternal age and has been suggested to be both the cause of sub-optimal development and a limiting factor in longevity [9, 10]. Oocytes from aged women also have reduced stores of maternal mRNA, which are often depleted before zygotic transition is reached [11, 12].

Super-ovulated oocytes in a cohort are in fact physiologically different from each other; each with its own potential and time course for development. This variability is compounded by its partner gamete, the spermatozoon which, as we have mentioned, has the non-genomic roles of activating the oocyte and delivering the centrosome. Oocyte activation appears to be triggered by a soluble sperm-borne factor [13], which may be a phospholipase [14] and since gametes are heterogeneous populations of cells it is not surprising that the activating capability will vary from one spermatozoon to another [15, 16]. In fact, it is well known that time of entry of fertilized oocytes into S-phase in the bovine is dependent on the fertilizing capacity of the bull used

[17, 18]. Sub-optimal spermatozoa, used in either IVF or ICSI, may trigger meiosis re-initiation, but inefficiently, either quantitatively or temporally, which may lead to embryonic arrest [19]. The key oocyte activation event, the release of massive intracellular quantities of Ca^{2+} , is not sufficient or indicative of sperm entry as shown by the use of parthenogenetic agents and by pharmacologically modifying spermatozoa [20]. Finally, defects in cell division in the early embryo may in turn be due simply to a sub-optimal centrosome delivered by the paternal gamete [21].

In conclusion, activation, or meiotic resumption, of the oocyte is a series of cascading events, the entirety of which, depends on the intrinsic characteristics of the individual gametes and when and how they interact. Notably, all of these events occur in the absence of maternal or zygotic gene expression. Sub-optimal activation may lead to abortive or sub-optimal development, while successful activation triggers the zygote to a successful transition at the 2–4 cell stage, when the new genome will take over. If oocytes have defective signalling pathways this will lead to a myriad of structural defects, including cytoskeletal deficiencies, which may lead, amongst many other effects, to aneuploidy. Thus, aneuploidy in the oocyte, or for that fact in the spermatozoon, is the endpoint or cellular expression of a series of major cytoplasmic defects, reflecting total cell disarray. In contrast, embryonic arrest may occur without chromosomal disarray or signs of apoptosis, due to the effect of ROS on cellular signalling pathways [22].

Clinical concerns

Patients may be broadly divided into good prognosis and bad prognosis depending on maternal age and number of oocytes produced, and there appears to be a non-linear relationship between the number of oocytes produced and live birth rate with a maximum efficiency at approximately 15 oocytes [3, 23]. Taking data from the Italian Istituto Superiore di Sanita register for PMA for 2010, of 44,365 cycles of IVF/ICSI the average age was 36.34 years and the mean number of oocytes collected was 6.6 (Ministro della Salute, Italy, 2011). At the Centre for Assisted Fertilization (CFA) in Naples, Italy and IVF Zurich in Switzerland a good prognosis patient has been arbitrarily defined as being under 38 years of age and producing more than 10 oocytes. In the month of May 2014, of 54 patients AT CFA, Naples, only 12 produced ten or more oocytes, with only five being below the age of 38, whereas at IVF Zurich, good prognosis patients declined from 30 % in 2007 to less than 20 % in 2014 (unpublished data). In many countries, such as Italy, Germany and Switzerland, legislative restrictions also come into force, with the concept of selecting an embryo bordering on eugenic selection, being outright illegal, or frowned upon. In Switzerland for example, not more than three zygotes may be cultured in vitro by law, almost precluding discussion on the utility of techniques to

select the best embryo, which also applies to countries such as Japan that favour natural ovulation cycles.

Thus, it would not be practical to compile international guidelines indicating who may benefit from advanced selection techniques, and in any case it would appear that many patients worldwide produce too few oocytes to justify expensive selection technologies. In situations where selection may be considered and/or offered we may imagine two alternative strategies: first the transfer of selected blastocysts/embryos hoping for a rapid pregnancy or second the progressive transfer of all blastocysts/embryos until implantation is achieved. The latter may be slower and involve freezing, however it is less invasive and more cost effective. It of course does not take into account the physiological and emotional toll that multiple transfers and potential failures could have on the patient.

The use of pre implantation genetic selection (PGS) as a routine clinical practice is the topic of much debate with many advocating its use [24, 25] and others criticizing the treatment as being ineffective [26–28]. Whatever the stance, PGS has not been shown to be of statistically predictive value and therefore at present is of limited clinical use, however there are strong associative data in the literature meriting further investigation to prove its usefulness in a clinical setting. From a basic science viewpoint, there are several issues to be resolved. First, whether biopsy of the polar body, early blastomeres or trophectoderm may in itself have negative short-term or long-term effects on the offspring as well as being inaccurate [29, 30] Second, what is the impact of mitotic errors and instability on early human development [31] and lastly whether, as suggested for large animal embryos, abnormal cells may be tolerated by the human embryo, sequestered into the trophectoderm or indeed eliminated by cell death mechanisms [32]. Finally, we would like to point out that, in any case, perfect euploidy is not a guarantee for development and that embryos may also block in development due to non-genomic effects and probably single gene defects. Considering that numerical variation in chromosome numbers is a gross morphological expression of cell demise it may be opportune to dedicate research resources to studying and improving meiotic maturation rather than classifying defective embryos.

Another laboratory technique gaining popularity is time lapse imaging morphometry of early human development (TLIM). Here, despite the evidence that these machines do not lead to an improvement in pregnancy rates [33–36], their use has been driven commercially into a “must” for the disconcerting patient/clinician. Several clinics initially reported an improvement in embryo survival/quality in time lapse incubators which is most likely due to an improvement over their existing incubators/gas supply/ambient air [37]. Morphological changes in early development indicate little if we think of the many thousands of biochemical and physiological pathways in action at this moment in life. Although we dispute its use as a clinical tool, it could be useful in a research setting.

For example, it appears to predict the probability that an embryo will reach the blastocyst stage [33], and may be of help, using non-vital markers, to gain insight into the role of polarity in early human embryogenesis.

Light is toxic to embryos [38, 39]. Time lapse companies suggest that the energy emitted from the light emitting diodes (LED) that are used for continuous illumination of the embryos under observation is less toxic than the intermittent light emitted by conventional microscopes. Considering that embryos *in vivo* are not exposed to any light source and we do not know the effect of these energy sources on biomolecules, caution would be prudent. Any light source, especially continuous, will generate ROS. This is compounded by the poor protection against the production of ROS in all commercially available culture media, caused by the autocatalytic (spontaneously auto-generated formation of ROS, in presence of oxygen) peroxidation of lipids as demonstrated by Martin Romero [40].

Whereas both PGS and TLIM are subjects for current conjecture, IMSI (intracytoplasmic morphologically selected sperm injection) may have surpassed its phase of popularity. By using computer-enhanced digital microscopy and several morphological parameters, MSOME (Motile Sperm Organelle Morphology Examination) allowed the identification of more subtle morphological abnormalities in spermatozoa, for example the presence of small vacuoles and abnormal proportions of the sperm head [41]. Using an enhanced magnification (5000×) spermatozoa are selected according to MSOME parameters and then injected as per routine ICSI. The elimination of spermatozoa with more subtle abnormalities quickly gained popularity, and was reported to increase pregnancy rates [42–44] while others criticised its validity [45, 46]. Analogous to both PGS and TLIM, IMSI is an expensive technique and to date has not been proven to be of benefit to the patient.

Morphological markers alone cannot define the viability of an embryo. Several attempts have been made to classify embryo viability by measuring products released by the embryo into the culture medium. Metabolic profiling of the culture medium, based on the measurement of simple metabolites such as lactose, was proposed commercially 2 years ago but interest appears to have dissipated [47] and this technology again revolved around expensive equipment. Other ideas were to measure the secretion of factors such as HLA-G histocompatibility antigen, class I, G, also known as human leukocyte antigen G (HLA-G). At least 20 publications from peer review journals were dedicated to the measurement of the hLA-G secretion in pre implantation embryos as a marker of embryo quality and developmental potential. In a careful analysis it was demonstrated that the values reported amounted to 125 % of the total protein content of the embryo [48] - subsequently all mention of hLA-G disappeared from the literature. A similar story occurred with Embryonic Platelet-activating Factor

(PAF), where it was calculated erroneously that the embryo produced 30 % of its dry weight per day [49].

There is no evidence that the intrinsic nature of gametes and embryos can be improved in the laboratory and embryologists can only reduce to a minimum any environmental or operator induced damage while these cells are maintained ex vivo. Environmental damage is unavoidable ranging from fluctuations in temperature and pH to VOCS. Possibly the most important environmental factor to be overlooked is the culture medium. Commercial production of culture medium has also been a driving force in the industry leading to much controversy and potentially dangerous situations. First, the concept that essential amino acids impair the pre implantation embryo was fostered [50]. This led in part to the widespread commercial production of culture medium without methionine (which is involved in imprinting through the formation of S adenosyl methionine, [51, 52]) leading to possible imprinting defects and potentially autistic syndrome disorders [53].

One rationale for removing essential acids was thought to be their role in the accumulation of ammonia [50], evoking a possible negative role on imprinting. In fact, this has been demonstrated to be incorrect [54] since in in vitro culture condition, ammonia forms ammonium carbonate and bicarbonate, highly unstable salts, immediately generating CO₂ and NH₃ which are eliminated by the flow of gas. The model used to demonstrate ammonia toxicity is incoherent. Ammonium chloride, NH₄CL, is an acidic highly stable salt, which is never formed in in vitro culture conditions. Second, the embryo is perfectly capable of recycling ammonia by transamination especially with pyruvate which leads to the release of alanine.

Finally, if we are to improve conditions we must move away from the commercially facile, but unrepresentative, mouse as a reference assay for human culture medium (or indeed generally as a model for the human embryo) [55, 12]. Mouse and human embryos differ enormously in their tolerance to osmolarity of the medium [56], in their ability to regulate the endogenous pool of amino acids [52] and the way they regulate imprinting [57]. To date the main requirement of legislators and industry for the commercialization of human embryo culture medium is that it “passes” the mouse embryo toxicity test. We suggest that the rabbit and bovine are more representative models, that, together with discarded human embryos, would provide superior data relative to that presently gained from the mouse model.

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