7

Third Millennium Assisted Reproductive Technologies: The Impact of Oocyte Vitrification

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1. Introduction

IVF has been widely used since 1978 (Steptoe & Edwards, 1978) to help infertile couples conceive when nature has failed. The IVF field was improved through embryo freezing/thawing, a technique developed by A. Trounson (Trounson & Mohr, 1983). The next major innovation came with ICSI in 1992, through the work of G.P. Palermo (Palermo et al., 1992). C. Chen was the first to publish work on oocyte freezing (Chen, 1986), but results remained too unreliable for this technique to be adopted in routine ART practice. Oocyte freezing became the focus of experimental efforts when in 1999 the first birth using oocyte vitrification was reported by L. Kuleshova (Kuleshova et al., 1999). M. Kuwayama in a 2005 publication confirmed that this new procedure could be useful in clinical practice (Kuwayama et al., 2005). Within a few years his method for freezing the human oocyte has become the standard for the field. Its clinical application has been widely developed and is now routine, even if other variants have since been proposed. There have been efforts to assess the safety of the procedure (Chian et al., 2008, Noyes et al., 2009). Noteworthy is R. Chian's report on the health of 200 babies born after having been conceived with vitrified oocytes. The safety of the procedure is also supported by all the data available on the health of children born from vitrified embryos (Takahashi et al., 2005, Mukaida et al., 2009). Use of the technique has reached France (Boyer et al., 2010) with passage of a new bioethics law in the summer of 2011. From Italy we have a good overview of what takes place on a nation-wide basis once the technique has become standard practice, thanks to the yearly report of ART results published by the country's ministry of health (Relazione del Ministro della Salute, anno 2009).

Some issues are still being debated, e.g. an open versus closed system, infectious risks with Liquid Nitrogen (LN), the safety of cryoprotectants, the health of the unborn children. It is

known that for oocyte vitrification to be most successful an open system is still needed, as shown recently by Paffoni and colleagues (Paffoni et al., 2011). The major concern with regard to an open system is the risk of LN-mediated transmission of infective agents. Researchers testing the safety of the closed system have shown it is possible to introduce contaminants voluntarily into a LN environment. They have suggested taking certain precautions when using an open system. To safeguard samples in the storage container, they recommend sealing the samples after cooling and prior to storage (Bielanski & Vajta, 2009). Parmegiani and colleagues (Parmegiani et al., 2010) suggest sterilizating LN by UV light. However, though safe and easy, UV sterilization is a costly procedure. The debate over the health of children born through oocyte vitrification is one that will not be resolved overnight. However, all the evidence published so far is reassuring. We should remember that when IVF, embryo freezing/thawing and ICSI were first developed, each advance was faced with similar resistance. What has changed is the success of national health authorities in imposing "precaution as a rule" as the only politically correct point of view. Most forget they would have probably banished IVF or ICSI back in the old days. Fortunately, international collaborations have produced quality studies which demonstrate that this new method is almost as safe as the slow-freezing method (Gook & Edgar, 2007, 2009, Cutting et al., 2009; Dessolle et al., 2009; Fadini et al., 2009, Nagy et al., 2009; Smith et al., 2010, Parmegiani et al., 2011). Good results for zygotes (Al-Hasani et al., 2007), day-2 or 3 embryos (Balaban et al., 2008) and embryos in blastocyst phase (Mukaida et al., 2009) have been obtained. The superiority to slow-freezing and the adaptability of fast-freezing to a closed system have also been demonstrated for embryos (VanderZwalmen et al., 2007) but these improvements will have fewer consequences for the future of ART than egg freezing. The new technique will probably render obsolete the cumbersome programmable freezers (Vajta & Nagy, 2009), but that is a mere detail compared to the other gains that can be expected. At this juncture, we must remember our debt to R. Edwards. We continue to be guided by both his brilliant work and his fearlessness and combativity in the cause of innovation, which led to international recognition and eventually the Nobel Prize. The message is clear: we must carry on his footsteps.

Cryobiology has developed steadily over several decades and along with ICSI has provided the conditions for a new clinical approach to ART practice. We are now ready to adopt oocyte vitrification in our biological and clinical procedures. The changes will lead to a more efficient and probably more humane practice.

2. Vitrification: Its importance in IVF

2.1 Increasing the number of fresh embryo transfers

The new approach allows cryopreservation of a part of the oocyte cohort, rather than creating embryos and then freezing any unused ones. Until now, because of the very short lifetime of this particular cell, the procedure required that we fertilize all mature oocytes, verify fertilization, discard all unfertilized oocytes and freeze the non-transferred fertilized eggs (often called supernumeraries or additional embryos) for subsequent attempts. This procedure results in only one fresh embryo transfer, often undertaken in less than optimal conditions because of the ovarian status of hyper stimulation. The frozen embryos may serve in subsequent attempts, programmed close to the initial attempt in the case of implantation failure, or after parturition. In some cases, the frozen embryos are not reused

because the couple has decided against transfer (they may feel they do not want any more children, or divorce or death may have intervened in the meantime). Currently, many frozen embryos are being conserved simply because the couple is undecided whether to proceed with another transfer or most often cannot face the decision to destroy them. In France, the couple legally has three alternatives: end the cryo-conservation of their embryos, allow early adoption by another couple facing a double sterility, or donate their embryos to research. (Paradoxically the law also forbids research on embryos conceived for that purpose.)

In Italy, a 2004 law (Legge N40) forbidding cryopreservation of the embryo forced resumption of work on oocyte freezing, with some encouraging results published by E. Porcu (Porcu et al., 1997). But even with modified slow-freezing in association with ICSI, results were not sufficient for the rest of Europe to follow suit. It was only after the publication of work by M. Kuwayama that IVF centres realized that oocyte freezing could be used in their practice. With rapid improvements in open system freezing, A. Cobo published results from a donor program showing that vitrified oocytes were equal in quality to fresh oocytes (Cobo et al., 2008, 2009). Because of their 2004 law, Italian teams realized that their situation was ideal to evaluate the efficiency of vitrification in an IVF program (Chamayou et al., 2006). Two major publications demonstrate the validity of the new procedure (Rienzi et al., 2010; Ubaldi et al., 2010). The embryo obtained with a vitrified/rewarmed oocyte has the same implantation rate as a fresh one demonstrated by L. Rienzi (Rienzi et al., 2010). Her report in Human Reproduction was the publication's most frequently downloaded work of the year 2011. A few months later F. Ubaldi from the same Roman team demonstrated that cumulative rates of pregnancy from the same cohort are equivalent to the cumulative rates from repeated attempts (Ubaldi et al., 2010). These publications underline the quality of the embryo produced from vitrification and its ability to implant. Oocyte vitrification will soon be widely adopted in IVF centres, as suggested in a recent meta-analysis review (Cobo & Diaz 2011). Each laboratory can decide to propose egg vitrification as an alternative to embryo freezing. In most centres employing micromanipulation, the technique itself is easily learned but its introduction in IVF programs requires some thought. Each centre will need to adopt its own strategy. In our centre we plan to conduct a study in which couples will be asked whether they prefer embryo or egg freezing, thus respecting their choice in the matter. It is expected some patients will still prefer to freeze their embryos, though once informed of the poorer implantation rates, they may decide to choose egg vitrification. (Currently in France, out of 100 births, 85 result from fresh transfer compared to 15 from thawing cycles.) We may of course modify our approach when comparative results for transfer of embryos produced from vitrified eggs are available on a large scale. Our study, to be conducted on the first couples for whom egg vitrification will be available in our centre (Boyer et al., 2009) is complementary to that of L. Rienzi (Rienzi et al., 2010). The benefit will be evaluated in terms of the probability of transfer for one selected oocyte. In a recent brief survey at our centre, half of the couples questioned said they would prefer oocyte freezing. We expect this proportion will increase when oocyte freezing becomes a real option.

In France, because the law prohibits research on human embryos produced for that purpose, research is conducted with embryos which for whatever reason have been rejected for human reproduction. This needs to be made clear to prospective parents (and to lawmakers as well).

2.1.1 The risk/reward balance of ovarian stimulation

Ovarian stimulation can proceed as usual; there will be no changes to the first step of IVF. We learn from the Italian experience that only 50% of the oocytes are of good quality, that is to say, appropriate for ICSI or vitrification. The first examination of the oocytes is crucial for a good result. An attempt at transfer will take place and the selected unused eggs will be vitrified for delayed microinjection. Egg banking schedules must take into account the number of couples who choose embryo freezing and also the number of embryos to be produced and transferred, as determined in our discussions with the couple. For transfers of embryos produced from vitrified oocytes, appointments will be based on the patient's menstrual cycle. In scheduling here we must be careful not to overload laboratory capacity.

An oocyte thawing cycle will be planned if the patient is not pregnant from the first transfer. Thawing of oocytes will be done 2 days after the LH surge or after triggering with hCG. All the cycles used for transfer will be free from ovarian stimulation risk. The natural cycle is preferred as it is the most favourable for embryo implantation.

The true challenge for centres offering an oocyte vitrification option will be scheduling the interventions. Clinicians must have a voice in the process. The laboratory calendar will have to take into account both fresh transfer and thawing cycles. If the natural cycle is most favourable for implantation, as has been reported, we will have to ensure that the laboratory can cope. The best day for the patient may not always be possible for the biologist!

2.1.2 Managing the oocyte cohort after pick-up

Centres must master the new procedure as developed by the leaders in the field, but for experienced biologists with skill in micromanipulation, the learning curve is not particularly steep. Within a very short time our centre was able to obtain oocytes or embryos of as good a quality as before vitrification and which, when transferred, resulted in ongoing pregnancies. (Fig 1, 2 and 3)

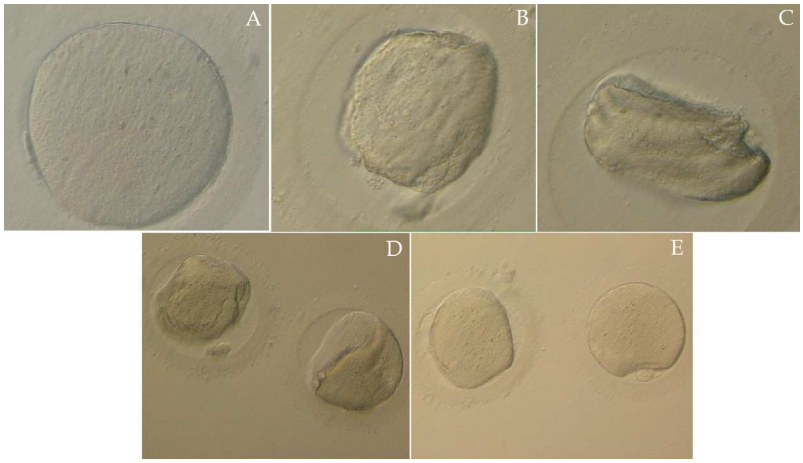

Fig. 1. Oocyte dehydration. In (A) the oocyte before dehydration begins. (B-D) the oocyte during dehydration: shrinkage. (E) the oocyte regaining original form with cryoprotectant filling.

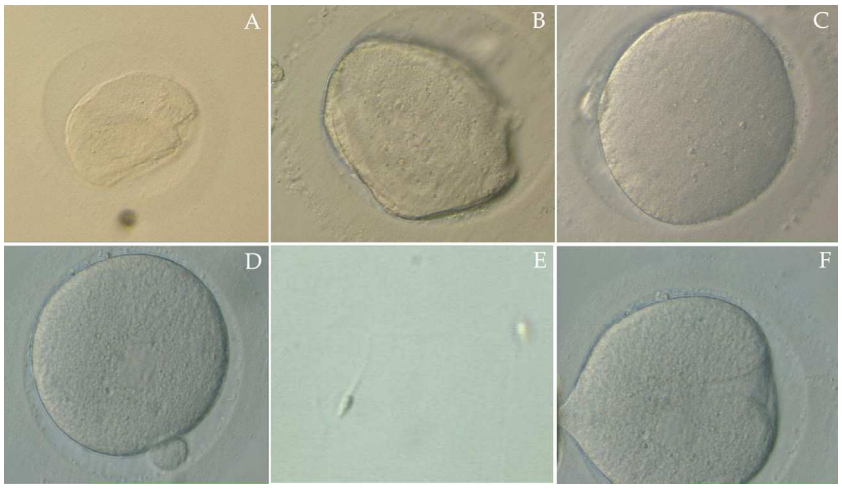

Fig. 2. Oocyte thawing and ICSI. (A-C) Oocyte shrinkage and re-gaining form during thawing. (D) Oocyte post-thawing. (E) Sperm selection for ICSI. (F) Oocyte post-ICSI.

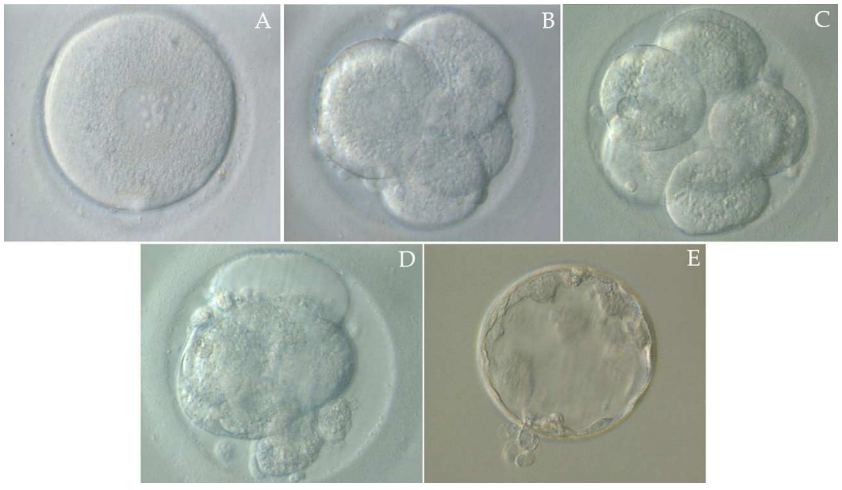

Fig. 3. Fresh oocyte development after vitrification, thawing and ICSI, identical to fresh oocytes development. (A) Zygote, day 1 in culture. (B) 4-cell embryo, day 2. (C) 8-cell embryo, day 3. (D) Morula, day 4. (E) Blastocyst, day 5.

The difficulty today remains deciding how many oocytes should be fertilized and how many vitrified. The daily practice of Italian teams and the results published in their annual report indicate that some 50% of the recovered oocytes are of good quality -- mature, in metaphase II, fertilizable and freezable. Based on their experience, it would be probably reasonable to propose 3 to 6 oocytes for fertilization, and vitrification of the rest. We can adjust these figures after some years of experience, as we did with ICSI. The number can be decided with each couple before starting the procedure. As a result we should be able to limit significantly the number of embryos produced as well as the number of frozen oocytes, without eliminating embryo freezing altogether.

After the denudation step, ICSI can be performed on part of the cohort as usual, followed by a fresh embryo transfer. The single embryo transfer is more frequent nowadays and the over-production of embryos is no longer necessary. The second portion of mature oocytes from the cohort can be vitrified for each patient. They will be used in a future attempt if the first embryo transfer has failed.

2.1.3 Overseeing the thawing of vitrified oocytes

As with single ovarian stimulation, most patients have more oocytes than they need for an embryo transfer. In more than half of all IVF cycles, there will be spare oocytes for at least one other fresh transfer, this time from the vitrified oocyte. For the second fresh transfer, the thawing of the selected oocytes must be scheduled early in the morning to allow for at least 2 hours in the incubator before fertilization. ICSI will be performed in all the surviving oocytes, with more than a 90 percent survival rate expected. Embryos are obtained and the transfer is organized as usual.

2.2 Impact on egg donation

Oocyte vitrification will have a major impact on egg donation by eliminating the complex coordination of donors and recipients. The need for synchronization between donor and recipient teams vanishes, simplifying all the steps necessary in preparing the donation. The creation of frozen egg banks will free us from the restraints which had previously limited our activity. Throughout the world, the mis-match between supply and demand has generated a lucrative business in which some of us have unfortunately become involved. Originally in almost all countries egg donation was developed for the altruistic purpose of providing healthy eggs to young women who for various reasons -- genetic, immunological, viral, and iatrogenic—were unable to produce them on their own. But in practice much of the demand has come from women who have turned to ART because of their age. Egg vitrification has opened the door to a new type of demand. A woman under 30 may now choose to preserve her fertility, even if she has no husband or plan for children at the time. Whether this possibility is a good thing is still matter of debate. However, we may legitimately ask if it is not better for a woman to preserve her own eggs at the age of 25 than to travel to a foreign country at the age of 45 to buy the eggs of young women there. Undoubtedly, we need to reflect on the implications of what has been termed "social egg freezing" (Lockwood, 2011). It is probably best to seek the answer from young women themselves rather than from bioethics committees whose members are often close to

retirement. It is instructive in this regard to be in contact with teenage girls. We have often been surprised by their level of comfort with ART compared to older generations. The high cost of the new procedure poses problems of another order. With the advent of oocyte vitrification, the entire issue of egg donation will have to be re-evaluated, just as sperm donation had to be rethought when ICSI was first introduced.

2.2.1 How egg donation is currently organized

Young healthy women are asked to give their oocytes in return for some form of compensation, usually monetary. In most countries egg donors are first recruited. Synchronized cycles between recipient and donors are organized, eggs are shared with several recipients (depending on the number collected), and excess eggs are attributed to the recipient in case of implantation failure, resulting in super numerous embryos which are frozen even if the number is low (Boyer et al., 2010). One or more freezing/thawing cycles are usually included. With oocyte vitrification there is a risk we will see the explosive development of a market for oocytes especially in poor countries where young women have few opportunities for work and the payment is comparatively high (as with organ donation). We are facing a cross-border organization for egg donation where physicians in foreign countries compete to provide the most medically secure environment for the recipient. It is no secret that demand will come from patients over 40 in wealthier countries who have no other possibility of delivering successfully.

Over a period of ten years egg donation in Europe has increased more than three-fold, from 4500 cycles in 1998 to 15,000 in 2007, with half the donations coming from Spain. Even if the results -- a 60 percent pregnancy rate per cycle -- are good, the development of egg donations is worrisome because most patients are older women beyond the age of child-bearing. It is a flagrant example of the incursion of market practices in the medical profession. This problem is due to the nature of the demand but also to the growth of medical clinics run for financial profit. Biologists working in these clinics must reach goals set by financial directors. Health institutions today are guided more by financial considerations than medical. Today we see franchising of IVF units used as a tool to expand business. The unregulated European market has produced a situation which parallels the market worldwide, where certain practices migrate to countries with lower costs and/or looser regulation. Some centres propose an "all-in-one" stay that includes air tickets, hotel and on-site facilities. The world has seen a similar development in the fields of surrogacy, dentistry and cosmetic surgery.

2.2.2 How egg donation can be organized in a near future

The availability of unpaid oocytes is probably part of the solution. The possibility and quality of egg banking were assessed in a study by A. Cobo from IVI, presented at the ESHRE meeting in 2010 and published under the title, "A randomized, prospective, controlled-clinical trial to test the efficacy of oocyte banking in oocyte donation programs" (Cobo et al., 2010). This work, which demonstrates that ICSI using vitrified eggs produces the same results as with fresh recovered ones, marks a turning point in egg donation.

Oocyte vitrification makes in easier for each country to regulate its activity to fit the needs of its own population. Donations can be organized on a national scale, supervised by the health authorities of each country. Oocyte vitrification also will diversify the origin of donations. Oocytes from donors will be available but also from women who have undergone IVF treatment resulting in a successful pregnancy but who still have oocytes stored in the clinic where ICSI was performed and extra-numerary oocytes cryopreserved. Some of these women may agree to donate their "unused" oocytes to a national bank, and a national network can be organized. After some years of vitrified oocyte use, the number of eggs available for donation will tend to self-sufficiency.

2.3 Preventing loss of fertility

Female fertility is naturally limited to the years between puberty and menopause. The 15 best years are from the ages of 20 to 35. By the age of thirty, half of the oocytes available after birth have vanished (Gougeon et al., 1994, Faddy et al., 1995, Scheffer et al., 1999 Alviggi et al., 2009).

Oocyte freezing offers new possibilities for women who face loss of fertility either through familial risk or during medical treatment of serious pathologies (oncological diseases, etc.)

2.3.1 Identified familial or personal risk

When genetically-induced ovarian failure appears, it is often too late to preserve fertility because of the lack of oocytes, even if the woman is still young. However, the early prevention of fertility loss in patients with a known family history of premature infertility is possible. In some families, premature ovarian failure was identified in a woman's relatives who carried genetic mutations that interfere with the fertility timeline. In some cases a woman, although fertile at the age of 20, discovers she has lost her fertility ten years later, like her mother or aunt. In other cases, the discovery of the rearrangement of sex chromosomes may predict a shortened fertile life. It has been reported that carriers of Turner's syndrome can have their own oocytes in the early stages of the reproductive period (Haseltine et al., 1984).

By performing ovarian stimulation and collecting some oocytes while a woman still has antral follicles, it is possible to perform IVF later when she decides to use her eggs. Collecting them early in a woman's life can protect her from programmed premature menopause. With a few good oocytes, she maintains the possibility of bearing children later on.

2.3.2 Consequences of sterilizing treatments

Treatment for chronic or ontological diseases can affect a woman's fertility, partially or definitively. In almost all such cases, survival and cure rates are high and the woman is young enough to conceive after recovery. In the past ten years, the medical focus for such patients has been the cryopreservation of cortical fragments of the ovaries, and the programming of a graft for a hormonal ovarian cycle recovery. In rare cases an attempted pregnancy has been suggested to restore fertility. Some births have been reported but remain low due to the difficulties of the surgical procedure (Roux et al., 2010). By vitrifying oocytes in some women who are able to undergo ovarian stimulation before treatment,

preservation of fertility will be more efficient (Porcu et al., 2008, Noyes et al., 2011). The new method provides a better alternative for women facing loss of sterility through treatment of certain diseases.

2.3.3 Embryo freezing has new rival for preserving female fertility

However, the most interesting prospect offered by oocyte vitrification is societal -- it is now possible to avoid cryopreservation of the embryo. Many prominent bodies have argued that cryopreservation of the embryo is safe and efficient, thus legitimizing its use for the preservation of woman's fertility. This has been the position of the American Society for Reproductive Medicine (Practice Committee of ASRM 2008), the French College of Gynaecology and Obstetrics (Bringer et al., 2010) and probably that of many other countries. We regard this as a true mistake. Take the case of the Evans couple which was brought before the European Court of Human Rights (European Court of Human Rights 2006). In 2000 the Evans were faced with a difficult situation. Mrs Evans was diagnosed with an ovarian tumour. They were advised by medical staff to proceed with IFV as a couple, and as a result six embryos were obtained and cryopreserved. The couple later divorced and Mrs Evans was denied use of the embryos. Mr Evans would not consent to an embryo transfer. She took her case to the European Court of Human Rights which ruled in her former husband's favour. The position of the European Court was that his right to withdraw consent was stronger than her right to use the embryos without his consent. For Mrs Evans it was a double condemnation -- her ability to have her own children was taken away, first through illness and again by the courts. With oocyte vitrification such an outcome could have been avoided, because she would have been sole owner of her eggs.

It should also be noted that the current debate over the future of unused embryos could become irrelevant. Should they be destroyed? Given to another couple? Is it legitimate to refuse embryo transfer post-mortem? Or to accept post-mortem transfer but within certain time limits? And if so, how long after death? And how many transfers should be allowed? As long as the preservation of embryos is the preferred method, these issues will continue to plague societies.

A few years ago in the French city of Toulouse a woman was refused an embryo transfer because her husband (the "father") was no longer alive. The court ruled that her embryos be proposed for "very early adoption" because of a 2004 law in France prohibiting destruction of human embryos.

Today, we are no longer being forced to choose between the right to motherhood and the right not to become father. Women are now able to preserve their gametes. Oocyte vitrification will resolve a number of ethical issues surrounding the use of the human embryo, without creating new dilemmas. We must be prepared at some point to admit our error in promoting embryo freezing as a means of preserving fertility and recognize that this is not a minor issue.

As underlined by Cobo, the American Society for Reproductive Medicine concludes, ''Oocyte cryopreservation presently should be considered an experimental technique only to

be performed under investigational protocol under the auspices of an Institutional Review Board." The French College of Gynaecologists et Obstetricians reaches a similar conclusion. All such professional organizations have a duty to rectify their position, once proof of error has been reasonably established.

3. Conclusion

The time has come to reorganize our daily practice, integrating oocyte vitrification into our routines. The efficiency of the combined techniques of ICSI and oocyte vitrification has transformed our environment, necessitating the creation of cryobiology units in all our labs where that has not already been done. We may rejoice in this recognition of our speciality in the field of medicine. We must continue to form a new generation of specialists, in a professional world very different from what we once knew. In over 30 years of practice we have been witness every ten years to major changes in the field. We must share our experience with the new generation, while hoping fervently they will have the freedom to follow. In today's often oppressive regulatory environment, practitioners are caught up in concerns over quality control, their CE mark and ISO15189 accreditation. We will lose our souls this way. Regulatory pressures have led not only to modifications of all our procedures, they have transformed the structural functioning of our units. As biologists we must reassert our control in the lab. We cannot delegate the most critical aspects of our procedures to technicians who lack the understanding to take medical decisions.

The advent of oocyte vitrification, like the introduction of ICSI before, poses a real challenge. This challenge will be met with success by all those who have chosen to dedicate their lives to reproductive biology. We are on the threshold of a cryobiological revolution. We can preserve oocytes today. In the future we will be able to preserve entire organs. We are fortunate to be working in this amazing field; may we remain passionate about our true mission -- to help patients. The introduction of oocyte vitrification represents a major step in the field of reproductive biology. It will transform our procedures, curtail the cryopreservation of embryos and resolve many issues surrounding the status of the human embryo. The advances are medical but they will have broad political, social and legal impact as well.

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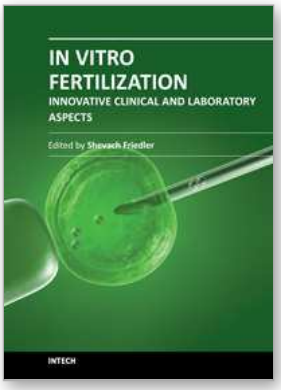

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The field of In Vitro Fertilization is a relatively new field in medicine, constantly on the move. This field is an exquisite example of the vast power in the complementary use of basic research with clinical practice and opened a new route of great basic and clinical research possibilities. The knowledge base that allowed the accomplishment of the idea of in vitro fertilization and embryo transfer has much developed since. The vast body of research pertaining to this field allowed deepening our understanding in the processes related to reproduction. In this book on in vitro fertilization we present new and interesting updated information in various aspects of this field. This work is a result of collaborative work of an international group of professionals dedicated to contribute to the advancement of our knowledge.

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