

## Article

# Vitrification in assisted reproduction: myths, mistakes, disbeliefs and confusion



Gábor Vajta obtained an MD degree (1976), a speciality (1979) and a PhD degree in human pathology (1988) in Hungary; and a Doctor of Veterinary Sciences degree (1999) in Denmark. He is adjunct professor of the University of Copenhagen, Denmark and James Cook University, Australia; and consultant academic director of Cairns Fertility Centre, Cairns, Australia. He is author of more than 100 publications, and member of the Editorial Board of Cloning and Stem Cells.

Dr Gábor Vajta

G Vajta<sup>1,4</sup>, ZP Nagy<sup>2</sup>, A Cobo<sup>3</sup>, J Conceicao<sup>1</sup>, J Yovich<sup>1</sup>

<sup>1</sup>Cairns Fertility Centre, 58 McLeod Street, Cairns, QLD-4870 Australia; <sup>2</sup>Reproductive Biology Associates, Atlanta, GA 30342, USA; <sup>3</sup>IVI Universidad de Valencia, Plaza de la Policía local, 3 46015 Valencia, Spain

<sup>4</sup>Correspondence: e-mail: gabor.vajta@cairnsmfertility.com.au

## Abstract

The purpose of this work is to update embryologists and clinicians on different approaches in human oocyte and embryo cryopreservation, by clarifying some misunderstandings and explaining the underlying reasons for controversial opinions. The work is based on literature review and critical analysis of published papers or conference abstracts during the last 24 years, with special focus on the last 3 years. Due to the latest advancements in techniques, cryopreservation now offers new perspectives along with solutions to many demanding problems, and has developed from a backup procedure to a successful alternative that is an indispensable constituent of assisted reproductive techniques. However, this progress is not free from controversies, at some points is rather serendipitous, and many factors, including human ones, hamper the selection and widespread application of the most efficient technique for the given task. A better understanding of the basic features of the two rival approaches (slow-rate freezing and vitrification), a clarification of terms and technical details, and a balanced, pragmatic evaluation of possible risks and potential, or definite, gains are required to accelerate advancement. Alternatively, the increasing flow of patients to the few assisted reproduction clinics and countries that are highly successful in this field will enforce the required changes in methodology and mentality worldwide.

**Keywords:** cryopreservation, embryo, freezing, human, oocyte, vitrification

## Introduction

During everyday routine in a cryopreservation laboratory it seems to be forgotten that it is the work of the assisted reproduction specialist that allows one of the eternal dreams of humankind to be realized. In fact, this is at present the only possible way to stop and restart the human lifeline, either indirectly (through gamete cryopreservation), or directly, by deep cooling human beings after conception and before implantation. However, the philosophical and moral aspects of this activity are not really of general concern. Specialists simply perform their duties, and use the available techniques that are within the law to assist those who need help.

All cryopreservations in assisted reproduction are performed for a single purpose: to gain time. This may be for a good reason (in the case of postponement of fertility or pregnancy), or it may be used as a tool to meet logistic needs (for example, at gamete or embryo

donation), or to ensure a backup (for repeat embryo transfer).

In regard to spermatozoa, doctors are content to benefit from the convenience of a relatively efficient freezing technique, the principles of which were established long before the start of human assisted reproduction (there is also benefit from the fact that sperm cells are usually abundantly present, thus efficiency of the technique is less critical). As the technique has become a routine and the offered flexibility is evident, its value is now not even appreciated, although for some individuals who have very few spermatozoa, a more efficient storage procedure would certainly be required. On the other hand, the difficulty and inefficiency of oocyte cryopreservation has long challenged researchers to establish an efficient protocol. However, because the difficulty has been emphasized for so long, there appears to be a failure to exploit the potential of emerging techniques.

The third major application, embryo cryopreservation, has been well established (first introduced over two decades ago; Trounson and Mohr, 1983), although it may create the greatest ethical and legal dilemmas. Currently cryopreservation is mostly used as a method for handling supernumerary embryos to avoid transferring and causing multiple gestations, as well as to avoid discarding potential human beings. Earlier concerns regarding potential danger do not seem justified. New data (Pinborg *et al.*, 2008) have demonstrated that babies born after cryopreservation are at least as healthy (maybe even healthier) than their freshly transferred counterparts. Some preliminary (unpublished) data from various respected sources even suggest a possible way to improve pregnancy rates by transferring cryopreserved embryos using a more physiological model, instead of the artificially stimulated environment, especially in the case of single blastocyst transfers.

Accordingly, cryopreservation has become one of the most important constituents of human assisted reproductive techniques [approximately 22,000 cryopreserved embryo transfer cycles a year in the US alone (Society for Assisted Reproductive Technology, 2006) and about four times more worldwide], equally important as ovarian stimulation, oocyte retrieval, IVF, embryo culture and embryo transfer.

## The three camps

As discussed in many recent reviews (Jain and Paulson, 2006; Vajta and Nagy, 2006; Gook and Edgar, 2007; Youssry *et al.*, 2008), two main groups of methods have been established first for embryos, later for oocytes, and very recently also for spermatozoa: slow-rate freezing and vitrification. Not much has been written, however, about the turbulent atmosphere that has arisen around and between these methods. Although the above-mentioned reviews and close to 1000 research papers published in the past 15 years have discussed different approaches for gamete and embryo cryopreservation, the consensus on which method to use seems to be farther than at any other time. In fact, both human and domestic animal embryologists seem to be divided into three more or less distinct groups. The first, rather small, group are devoted fans of vitrification and want to start all cryopreservation work by discarding all traditional freezers. The other, almost equally small group strongly refutes this approach and tries to formulate new ways to improve traditional slow-rate freezing while quietly obstructing the alternative. The largest camp, comprising the vast majority of embryologists, do not understand much of the problem and are just plain confused. They see respected scientists, supposedly professionals in the subject, quarrelling and disagreeing about almost every aspect of cryopreservation. They hear new (and often misused) terms and categories, contradicting statistics, *pro* and *contra* arguments regarding benefits and dangers/risks, cost-efficiency and labour intensiveness, and they simply cannot decide. They are also discouraged by the primitive tools and simple approach in vitrification, which is in sharp contrast to the sophisticated computer-controlled traditional freezers. What they continue to do is what they did before; cryopreserving the way they know works, applying the more-or-less efficient traditional freezing for all purposes.

Unfortunately, this conservative approach is not at all unusual among human embryologists. From one point of view it is

strange, emanating from the representatives of a profession that was established from an extremely brave act (maybe 'audacious' is a more appropriate attribute) performed 30 years ago (Steptoe and Edwards, 1978), and has resulted in approximately 3,500,000 babies and happy families worldwide. On the other hand, the conservatism is quite understandable if one considers the environment in which these people have to work. Few professions are surrounded by such a controversial atmosphere and suspicious public attention; few professions have been hampered by such bureaucratic frames. The absurdity of these frames becomes quite obvious if one considers the extreme variations in assisted reproduction laws in different states belonging to the same federation, including the European Union, USA, or even Australia; and these may also change sharply according to changes in governments. Under these circumstances, just its existence and survival means a considerable risk for an assisted reproduction laboratory; why then face new challenges of questionable value?

Moreover, the conservatism is contagious; it is not restricted to assisted reproduction laboratories, gynaecologists propagate it to general practitioners, and directly or indirectly to patients. Any extensive list of medical and social indications in regards to oocyte cryopreservation published in peer-reviewed journals is of limited value if this information does not reach the patient or is trapped within the filters or suppressed by comments such as: "a new technology just in the experimental phase", "the efficiency is still low", "nobody really knows the potential risks", etc.

This conservatism, either paradoxical or understandable, is a fact that must be taken into account, but cannot change much. The duty of professionals is to minimize or eliminate other factors that hamper advancement. As mentioned above and discussed in detail below, there are still a lot of things to do.

## Terms

The first problem is related to terms and expressions. Although clarification is a seemingly simple task, and it has been professionally performed earlier (Shaw and Jones, 2003), there are still traps, misunderstanding and misuses that need further discussion.

## Vitrification

Vitrification is a physical phenomenon describing solidification of water or water-based solutions without ice crystal formation. It can be partial or total, and may occur in some areas or the whole mass of the cooled sample respectively. In cryobiology, the phenomenon is used for procedures where the whole solution containing and also infiltrating the sample solidifies *entirely* without ice crystals, and also returns to the liquid state without ice formation (although for the latter criterion, the consensus is not entirely sound). However, in contrast to the common belief often described in reviews, neither high cryoprotectant concentration of solution nor increased cooling rates are indispensable conditions of vitrification. It is true that, among other factors, both high level of cryoprotectants and rapid cooling *facilitates* vitrification. There is an inverse correlation between the two factors: the higher the cooling rate, the lower the required cryoprotectant

concentration, and *vice versa*. But pure water can also be vitrified when an extremely high cooling rate ( $10^7$ °C/s, i.e. close to  $10^9$ °C/min; Rall, 1987) is applied, although this possibility is rather theoretical and hard to achieve under common laboratory conditions. On the other hand, with considerable increase in the concentration of cryoprotectants, solutions may also vitrify with moderate cooling rates. In reproductive cryobiology, this second possibility cannot be fully explored, as all cryoprotectants are more or less toxic, and may also have a detrimental osmotic effect on oocytes and embryos. Accordingly, the art of vitrification in human assisted reproduction is to create a practical way to achieve high cooling and warming rates, and to find the lowest level of the least hazardous cryoprotectant combination that ensures a safe vitrified state of both the extra- and intracellular solution under the given cooling and warming conditions.

Another common myth is that cryoprotectants are needed to increase the *viscosity* of the solution. This confusion is created by the fact that vitrification can also be described as an extreme increase in viscosity at low temperatures. However, at ambient temperatures there is no direct correlation between the viscosity of cryoprotectants and their efficiency at supporting vitrification. Ethylene or propylene glycol are not viscous at all, and do not cause a dramatic increase in the viscosity of water either; however, they are among the most efficient and most widely used cryoprotectants in vitrification, in contrast to the highly viscous glycerol that is widely used for traditional freezing, but abandoned several years ago for vitrification purposes.

The phenomenon of vitrification can easily be detected with the naked eye or with a stereomicroscope: the cooled solution should remain transparent, without any traces of white pellets indicating ice formation. However, things are not as simple as they might seem. Although visual observation is practical and easy, and it is the only available possibility under common laboratory circumstances, it cannot be applied in certain situations. For example, the easiest way to achieve the preferred high cooling rates is to decrease the volume of the solution surrounding the sample, and immerse it directly into liquid nitrogen. In some very efficient vitrification procedures only a tiny layer or a film of solution surrounds oocytes or embryos, making optical evaluation of the state of solidification (either crystallized or vitrified) impossible. On the other hand, retrospective analysis based on the successful outcome may lead to false conclusions. It is known that partial or total intracellular vitrification also occurs during traditional slow-rate freezing, and may ensure survival of cryopreserved samples. The same phenomenon may occur with extremely rapidly cooled small samples with low or zero cryoprotectant concentration; even high and consistent survival rates do not prove that vitrification of the whole solution has occurred during the process.

## Freezing and thawing versus cooling and warming

Scrupulous linguistic purism is often regarded as an ‘academic’ (i.e. senseless) problem, and a feature of ageing scientists. However, the terminology for cryopreservation (including vitrification) was established decades ago and has been proven to be useful to define what is being discussed. As stated clearly by Shaw and Jones (2003), freezing and thawing can only be used for procedures where ice crystals are formed or melted.

Cooling and warming only means changing of temperature, and can be used both for traditional freezing and vitrification. The common consensus among cryobiologists is to use freezing, frozen, thawing, thawed, etc. for traditional slow-rate freezing and related procedures, and cooling, warming for vitrification (the term ‘devitrification’ cannot replace warming after vitrification; it describes a rather harmful phenomenon, when ice crystals form in the vitrified sample, usually as the result of inappropriate warming). It would be difficult or misleading to replace terms widely used in assisted reproduction, such as ‘thawed embryos’, ‘thaw cycles’ etc. with ‘warmed embryos’, ‘warm cycles’, but a compromise could be to use ‘cryopreserved embryos’, ‘cryo-cycles’. As a first step in the right direction, all ‘thawing solution’ labels, commonly used by companies offering vitrification kits should be replaced by labels denoting ‘warming solutions’. The professional term would create even more credit for the company and their suggested vitrification procedure.

The use of other terms in human assisted reproduction also requires reconsideration. They are not strictly related to cryopreservation, but may create confusion and misunderstanding in this field, too. The difference in cryobiological features between oocytes and zygotes (although not really understood) is striking in many mammalian species including humans. Talking about *pronuclear oocytes* might be regarded as a vain attempt to escape legal restrictions, but is definitely misleading regarding the chronology, (cryo-) biological behaviour and future potential. Another euphemism is the *pre-embryo*, a widely used slang derived probably from the preimplantation embryo, but in this form (purposefully?) suggesting something preceding the embryo stage, ‘less important’, ‘just something before’, i.e. in sharp contrast to the real situation.

## General statements

It is necessary to deal with some statements that have been published in review articles or made in plenary lectures at international meetings during the past 3 years. There is no intention to make the dispute personal, therefore sources are not cited: however, there is a definite need to enlighten the background and if needed, argue with these statements.

### “Vitrification is a new technology”

Many factors (including subjective ones such as age) contribute to deciding what should be termed ‘new’. However, the first mouse obtained after embryo vitrification was reported by Rall and Fahy in 1985, and accordingly it was born earlier than some of its colleagues in the embryo laboratory! It is also worth mentioning that in 1986, the event was hailed as the ‘rediscovery of vitrification’, and reported in *Human Reproduction* as a potential alternative to slow-rate freezing (Ashwood-Smith, 1986). It must be acknowledged that the overall acceptance and application of vitrification for human oocytes and embryos previously advanced (and still advances) slowly, but it has been widely used in experimental and domestic animal embryology since the early 1990s, and with the introduction of new approaches, it has dominated some fields of reproductive cryobiology before the turn of the millennium.

## “There is no evidence that vitrification is harmless”

In principle, this is true. There is no definitive evidence that vitrification is harmless. The same is applicable, however, to traditional freezing and to all in-vitro procedures in assisted reproduction. Nobody can predict the consequences of human IVF on the second and third generation. However, for vitrification, there is 23 years' experience in animal embryology (accordingly remarks like “... vitrification, where [there] is a lack of basic information on the biological implications” do not seem to be justified), and an exponentially growing database in humans. So far, more than 500 papers and at least another 500 abstracts have been published on vitrification of mammalian oocytes and embryos, and only one, printed 20 years ago, reported an increase in malformations in mouse fetuses (Kola *et al.*, 1988). The technique used by the authors was a very preliminary one and basically different from any recent vitrification procedures. The long exposure times could have contributed to both the low success rate and genetic aberrations. On the other hand, there are hundreds of papers presenting statistically significant, sometimes dramatic improvement regarding in-vitro and in-vivo survival rates when vitrification was compared with traditional freezing.

How long should one wait to be reassured? Cautiously conservative colleagues must be reminded that their traditional freezing was introduced shortly before vitrification, and its widespread application in humans was based on much more fragile data regarding the harmlessness of the technique than those available now for vitrification. The same is applicable to intracellular sperm injection (ICSI), embryo biopsy, assisted hatching, and almost all techniques now routinely used in embryo laboratories worldwide.

## “Long-term stability of tiny vitrified samples remains to be demonstrated”

Again, for how long? Should one wait for several decades? So far, no evidence has been provided that these samples (if appropriately stored at  $-196^{\circ}\text{C}$  or safely below  $-150^{\circ}\text{C}$  in the vapour of liquid nitrogen) suffer more damage than other samples, including traditionally frozen ones. The tiny volume means more susceptibility to accidental warming, but with precautions that are routinely applied in all cryopreservation procedures these vitrified samples don't seem to present any increased risks regarding long-term storage.

## “Can be done in closed container” versus “Containers that can be sealed to exclude liquid nitrogen, and therefore eliminate the risk of infection during cooling and storage are not available for micro-volume samples”

These seemingly technical details have profound consequences on the routine use of vitrification in human assisted reproduction, therefore they are discussed now.

In fact, both of these contradicting statements are true. The main problem with vitrification is the conflicting needs for high cooling/warming rates and hermetically isolated containers. In spite of various approaches, including solid surface vitrification, use of liquid nitrogen slush and extremely thin sealed capillaries, no method can compete with the cooling and warming rates of a sample that is surrounded by a thin film of cryoprotectant and exposed directly to liquid nitrogen. Without the direct contact, the cooling rate is compromised; with the direct contact, there is a risk of contamination.

For many embryos, including mouse, bovine, ovine and caprine blastocysts, the slightly compromised cooling rate provided by a closed system is appropriate. However, in human assisted reproduction, especially for human oocytes, the extremely high ( $>20,000$ , preferably  $>50,000^{\circ}\text{C}/\text{min}$ ) cooling and warming rates seem to be indispensable. In spite of the attractive advertisements of industrial producers, to date no closed system can compete with open minimum volume vitrification methods in human oocyte cryopreservation. An indirect but very convincing piece of evidence for this statement is that all papers reporting high survival and in-vitro–in-vivo development after vitrification of human oocytes were based on the open systems.

This unfortunate contradiction between various requirements offers a considerable opening for attacks (“direct contact with liquid nitrogen may transfer diseases”), and opponents of vitrification never fail to use it. Elimination of any possibilities of iatrogenic infection is one of the most imperative parts of the ‘*nil nocere*’ principle, and the first thing that should be safely excluded when a new method is introduced. Curiously, the approach is much more permissive when old techniques are used, for example face masks in surgery theatres. Even in embryology, traditional freezing is mostly performed in straws that are not completely impermeable to the most dangerous viruses and other infective agents under the extreme temperature, pressure and consequently mechanical conditions to which they are exposed.

However, just like the Gordian knot, this problem can also be resolved by cutting it into two parts, by separating the cooling from storage. The direct contact is needed, but just at the cooling phase, and this step can be performed in factory-derived liquid nitrogen. For extra safety, filtration of liquid nitrogen with commercially available  $0.2\text{-}\mu\text{m}$  filters commonly used at media preparation or exposure to UV light is also suggested. Dewar flasks and stainless steel containers can be easily decontaminated by a simple flush with diluted bleach or other efficient antimicrobial solutions, and separate storage of clean liquid nitrogen should not present a difficult logistical problem in a laboratory. After cooling, the samples and the carrier tool [open pulled straws (OPS), Cryotop, etc.] can be placed into pre-cooled straws, preferably high-security CBS straws, heat-sealed and stored in common containers without any danger of cross-contamination. At warming, one end of the straw can be cut, the carrier tool with the sample can be removed and immersed into the warming medium directly; at this phase there is no need for sterile liquid nitrogen.

This approach was documented as early as 1998 (Vajta *et al.*, 1998), but was disregarded. Recently, however, its

safety has been proven by independent experts, probably the most relevant scientists in this field (Bielanski and Hanniman, 2007); the same group who had demonstrated earlier the danger of liquid nitrogen-mediated transmission of infective agents between open vitrification systems under experimental conditions (Bielanski *et al.*, 2000, 2003). On the basis of this recent proof of safety, the International Embryo Transfer Society's new manual (see [www.iets.org/manual.htm](http://www.iets.org/manual.htm)) includes this approach for safe vitrification. Even if the standards are different, there is good reason to expect that if concerns spread from the animal world to human embryology, the same will happen with the solutions as well.

Alternatively, vapour phase storage could also be a simple strategy providing optimal conditions for storage at low temperatures without direct contact with liquid nitrogen, thus decreasing or preventing the risk of cross contamination during storage (Cobo, personal communication). The safety of this system was tested in dry shippers (Bielanski, 2005a,b), but may require further confirmation for the more sophisticated vapour phase storage tanks (Bielanski, personal communication). Additionally, these containers must guarantee a very stable temperature inside the chamber, safely below the glass transition temperature of water. Fortunately there are commercially available, although rather expensive, systems that fulfil this requirement.

## Technical details

Below are collected some repeatedly published opinions or commonly accepted views that are based on wrong assumptions and may hamper advancement for years.

### “Failed fertilized human oocytes were used to test optimal parameters...”

Considering the extreme scarcity of experimental material to establish the appropriate parameters for cryopreservation of human oocytes, this seems to be an acceptable compromise. One may even assume that these double-handicapped oocytes (seemingly delicate and fragile) will be even more sensitive to any insult than the freshly aspirated counterparts. Accordingly, a cryopreservation method that results in high morphological survival in these samples should be even more efficient when used for fresh oocytes. Surprisingly, however, the opposite is the truth (Stachecki and Cohen, 2004; Vajta and Nagy, 2006). For some unexplained reason *in-vitro* incubation for a longer period (24 h or so) makes human oocytes more resistant to morphologically detectable irreversible damage that occurs immediately or shortly after vitrification (lysis, discolouration of the cytoplasm as the sign of chilling injury, disappearance of the double-refracting cell membrane). As a consequence parameters that seem to work with 100% efficiency in failed fertilized oocytes may result in 0% survival when used for freshly aspirated human eggs.

### “Optimal equilibration and dilution temperature for human oocytes is 37°C”

The statement (expressed repeatedly by different authors) has been made on the basis of a single observation, the occurrence

of damage to the meiotic spindle. It may be correct for this isolated phenomenon. However, pretty fluorescent images may not impress patients who are exclusively interested in the ultimate outcome: birth of healthy babies. Apart from the need to preserve an intact spindle, there are many other factors that influence the cumulative success of human oocyte cryopreservation, including toxic and osmotic effect of cryoprotectants. Both these factors are temperature dependent, and the analysis of their individual and direct effect is very difficult. On the other hand, there is sound experimental evidence illustrating spontaneous recovery after damage of the spindle (Borini *et al.*, 2008). The fact that the overwhelming majority of babies born after oocyte vitrification were the results of a technique where equilibration was performed at slightly elevated room temperature (approximately 25–27°C) supports the view that in this complex procedure, analysis of a single factor may lead to a false conclusion.

### “Vitrification is more demanding in the laboratory than freezing...”

Having taught hundreds of embryologists (many of them with considerable experience in traditional freezing) on practical courses, having seen their amazement regarding the simplicity of the OPS and Cryotop technique and their excellent performance after their initial trials, the authors cannot share this statement. Time frames of exposure to final cryoprotectant solutions may be precisely determined, but any embryologist with average skills required to handle human oocytes and embryos can easily work within them. On the other hand, according to the authors' experience in teaching the method, other steps of the procedures are so easy that even relatively inexperienced technicians can learn them in less than 1 day.

### “One at a time”

In contrast to the earlier appraisals regarding the speed of the entire vitrification procedure compared with traditional freezing, concerns have arisen lately when mass application of the vitrification procedure was implemented. In contrast to traditional freezing, where after equilibration and loading the procedure is performed entirely, or almost entirely, automatically, in the vitrification process the actual level of instrumentation requires manual work during the whole procedure for each sample, or small groups of samples (for example two or three oocytes vitrified together). Recent, highly efficient vitrification protocols use extended initial incubation in the diluted cryoprotectant solution, accordingly the length of the procedure increases significantly. However, this change also offers a possibility for parallel work. As the time-length of the first incubation (note: the first incubation only) is not strictly determined, the usual  $\pm 2.5$  min frame provides 5 min, say  $5 \times 1$  min for five (group of) oocytes or embryos to be vitrified in one run. Moreover, with some practical arrangement and experience, it is also possible to run two first incubations in parallel, overlapping each other. Accordingly, up to 10 (groups of) embryos or oocytes can be cryopreserved in 30 min: this time efficiency is completely comparable with that of traditional freezing.

## Assumptions and comparisons

“Equivalent results with traditional freezing’, ‘The paucity of live births ... alike”; “There is no sound evidence that vitrification is the way forward”

Fans of vitrification could list hundreds of publications that seem to confute these sentences. However, it must be acknowledged that several factors interfere significantly with all comparisons.

Firstly, as described earlier, in cryobiology ‘vitrification’ is a term, not a method. There are numerous different possibilities to achieve ice-free solidification of the solution containing the sample, and embryologists seem to be very creative to apply nearly all of them. There are infinite approaches including different holding media, various types and concentration of cryoprotectants, diverging times and temperatures at dehydration and dilution, many different carrier tools and cooling rates, etc. Quite evidently, the outcomes are also highly variable. Accordingly, if one investigates the efficiency and applicability, one cannot just talk about vitrification, it is necessary to specify the method, including the carrier tools, the applied parameters, cryoprotectants, etc.

Due to these differences, attempts to make systematic comparison of published results in the human field fail to provide enough data and especially a convincing amount of reliable publications. According to a recent meta-analysis regarding vitrification versus slow freezing of human embryos, out of 873 potentially relevant studies only four met the somewhat compromised criteria of scientific soundness (Loutradi *et al.*, 2008). The explanation of this surprising outcome is rather evident. To perform a large-scale, randomized prospective study is generally a demanding task, and an average size human IVF clinic cannot afford it. Most clinics that perform vitrification now have changed the cryopreservation method in 2–6 months, based on their subjective impressions rather than a scientifically significant, objective assessment within their units.

Moreover, in most comparisons the outcome is rather questionable: if performed by scientists devoted to vitrification, the very best, cutting edge vitrification technique is compared with a common traditional freezing method, and *vice versa*.

So far, probably due to the prestigious material and the tense atmosphere between the devotees of the two approaches, a large-scale comparison between the best vitrification and slow-freezing methods, performed by acknowledged experts in both techniques respectively, is scarcely available. The only exception seems to be the very recent publication of Balaban *et al.* (2008), in which in a randomized and controlled study a total of 433 day-3 human embryos were either vitrified or slow frozen, and their survival, metabolism, in-vitro and in-vivo developmental potentials were compared, resulting in favourable results after vitrification. Another study was also published about the same subject, with the same conclusion by Li *et al.* (2007), unfortunately in Chinese and only with the abstract in English, creating some difficulties for a critical analysis.

One may suppose that commercialization of vitrification will offer a solution. Unfortunately, however, the impressive recent activity of companies in this field during the past 2 or 3 years did not help much, in contrast. Poor copies and inappropriate versions of successful and efficient tools have been produced and distributed. Ready-to-use solutions are offered with modifications to meet the requirement of the legal and commercial environment and not the need of oocytes and embryos. Moreover, agents responsible for marketing and distribution of these tools and solutions have no clue about the basic principles of vitrification and details of practical application. Needless to say, the ultimate result is often disappointment and a return to the traditional approaches.

## Concluding remarks

The rather pessimistic atmosphere of this review reflects the slight frustration of authors who make repeated efforts to achieve a more rapid advancement in this important field of human assisted reproduction, and repeatedly face the problems described above. However, in the long term, there is definite hope. Apart from the scientific community, there is another forum that determines the success of a given procedure: the final outcome being the satisfaction of patients. No scientific argument can compete with healthy babies born from a given procedure, and no devotion to one or other procedure can resist the pressure caused by the flow of patients to those successful clinics. Some signs indicate that the process has already started and will certainly lead to the right decision during the next few years.

## References

- Ashwood-Smith MJ 1986 The cryopreservation of human embryos. *Human Reproduction* **5**, 319–332.
- Balaban B, Urman B, Ata B *et al.* 2008 A randomized controlled study of human day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Human Reproduction* **23**, 1976–1982.
- Bielanski A 2005a Experimental microbial contamination and disinfection of dry (vapour) shipper dewars designed for short-term storage and transportation of cryopreserved germplasm and other biological specimens. *Theriogenology* **263**, 1946–1957.
- Bielanski A 2005b Non-transmission of bacterial and viral microbes to embryos and semen stored in the vapour phase of liquid nitrogen in dry shippers. **50**, 206–210.
- Bielanski A, Hanniman A 2007 Non-cross contamination of bovine embryos with microbes using the OPS vitrification system. *Reproduction, Fertility and Development* **29**, 232 (abstract).
- Bielanski A, Bergeron H, Lau PC *et al.* 2003 Microbial contamination of embryos and semen during long-term banking in liquid nitrogen. *Cryobiology* **46**, 146–152.
- Bielanski A, Nadin-Davis S, Sapp T *et al.* 2000 Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology* **40**, 110–116.
- Borini A, Bromfield J, Hutt K *et al.* 2008 Human meiotic spindle dynamics following slow-cool cryopreservation. *Human Reproduction* **23** (Suppl. 1), i32–33 (abstract).
- Gook DA, Edgar DH 2007 Human oocyte cryopreservation. *Human Reproduction Update* **13**, 591–605.
- Jain JK, Paulson RJ 2006 Oocyte cryopreservation. *Fertility and Sterility* **86**, 1037–1046.
- Kola I, Kirby C, Shaw J *et al.* 1988 Vitrification of mouse oocytes results in aneuploid zygotes and malformed fetuses. *Teratology* **38**, 457–474.

- Li Y, Chen ZJ, Yang HJ *et al.* 2007 Comparison of vitrification and slow freezing of human day 3 cleavage state embryos: post-vitrification development and pregnancy outcomes. *Zhonghua Fu Chan Ke Za Zhi* **42**, 753–755 (in Chinese).
- Loutradi KE, Kolibianakis EM, Venetis CA *et al.* 2008 Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertility and Sterility* **90**, 186–193.
- Pinborg A, Loft A, Rasmussen S *et al.* 2008 Danish national controlled cohort study on neonatal outcome of 1267 children born after transfer of cryopreserved IVF and ICSI embryos in 1995 to 2006. *Human Reproduction* **23** (Suppl. 1), i51 (abstract).
- Rall WF 1987 Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* **24**, 387–402.
- Rall WF, Fahy GM 1985 Ice free cryopreservation of mouse embryos at  $-196^{\circ}\text{C}$  by vitrification. *Nature* **313**, 573–575.
- Shaw JM, Jones GM 2003 Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Human Reproduction Update* **9**, 583–605.
- Stachecki JJ, Cohen J 2004 An overview of oocyte cryopreservation. *Reproductive BioMedicine Online* **9**, 152–163.
- Stephoe PC, Edwards RG 1978 Birth after the reimplantation of a human embryo. *Lancet* **12** (8085), 366.
- Trounson A, Mohr L 1983 Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* **305**, 707–709.
- Vajta G, Nagy ZP 2006 Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive BioMedicine Online* **12**, 779–796.
- Youssry M, Ozmen B, Zohni K *et al.* 2008 Current aspects of blastocyst cryopreservation. *Reproductive BioMedicine Online* **16**, 311–320.

*Declaration: The authors report no financial or commercial conflicts of interest.*

*Received 13 March 2009; refereed 16 April 2009; accepted 8 October 2009.*