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REVIEW

Current Data on the Vitrification of Human Embryos: Which One is the Best; Zygote, Cleavage or Blastocyst Stage?

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Abstract

Human gametes and embryos at different developmental stages show various physiological necessities and requirements in order to survive *in vivo*. All laboratory procedures can cause damage to human gametes and embryos. Cryopreservation procedures are such unphysiological circumstances mainly on account of severe temperature and osmotic alterations. Since the early 1980s, two common methods of cryopreservation have been used. Both of these methods have finally depend on the freezing and solidification of cell or tissue. Recently, the one known as vitrification has been claimed as the future of cryopreservation because of increased survival and success rates. However, this method is a non-equilibrium technique of cryopreservation that shows critical requirements of much higher concentration of permeable cryoprotectants and rate of cooling. Thus, it is a more vigorous mean of all possible cell damage except the formation of intracellular ice crystals that is totally prevented by vitrification. Nevertheless, there is no adequate cumulative data on the outcomes of vitrification performed at different stages of human embryos. The aim of this review is to assess the possible differences of outcomes of vitrification performed at different stage of human embryos.

Keywords: vitrification, zygote, embryo, blastocyst

Özet

İnsan Embriyolarının Vitrifikasyon ile Kriyoprezervasyonu: Hangi Gelişimsel Evre Seçilmeli; Zigot, Klivaj veya Blastokist?

İnsan gamet ve embriyoları *in vivo* ortamda değişik gelişimsel hücre evrelerinde iken canlı kalmak için değişik fizyolojik ihtiyaçlar göstermektedirler. *In vitro* ortam olan tüm laboratuvar prosedürleri ise insan gamet ve embriyolarının hasarlanabileceği ortamlardır. Kriyoprezervasyon uygulamaları fizyolojik olmayan ve ciddi ısı ile ozmotik değişikliklere ihtiyaç duyan güncel laboratuvar uygulamalarıdır. 1980'lerin başlarından itibaren kullanılan düşük oranlı dondurma ile vitrifikasyon olarak adlandırılan iki ana kriyoprezervasyon metodu bulunmaktadır. Her iki metot temelde hücre veya dokunun donma ya da katılaşması esasına dayanmaktadır. Yakın zamanda tanımlanan vitrifikasyon artmış çözme sonrası yaşam ve başarı oranları ile insan gamet ve embriyolarının kriyoprezervasyonunda gelecekte tercih edilmesi gereken metot olarak nitelendirilmiştir. Fakat, bu metot bir "non-equilibrium" teknik olup daha yüksek permeabl kriyoprotektant konsantrasyonu ile daha yüksek soğutma oranına ihtiyaç duymaktadır. Bu nedenler ile, vitrifikasyon ile kriyoprezervasyon esnasında intraselüler buz kristallerinin oluşmasının önlenebilmesi haricinde hücre hasarının daha yüksek olacağı düşünülmüştür. Bu derleme ile insan gamet ve embriyolarının değişik hücre evrelerinde yapılan vitrifikasyonun sonuçları ve güncel bilgiler özetlenmiştir.

Anahtar sözcükler: vitrifikasyon, zigot, embriyo, blastokist

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Introduction

Cryopreservation has been widely used since the publication of the first reports about biochemical and successful clinical pregnancies with frozen-thawed human embryos in the early 1980s (1). Subsequently, the first reports of successful deliveries were defined in the mid-1980s (2-4). Since then, cryopreservation of gametes and embryos resulted in thousands of live births in which the slow-cooling cryopreservation has commonly been used. Undeniably, these techniques are enhancing clinical outcomes and cumulative conception rate of couples followed by a single cycle of ovarian stimulation. Results expressed as the augmentation of the delivery rate per oocyte harvest vary in literature between 2% to 24% (5,6).

Human gametes and embryos at different developmental stage show variable physiological necessities and requirements in order to survive *in vivo*. The subzero temperatures and other conditions human gametes and embryos encounter during cryopreservation are not physiological situations (7,8); and these cells are susceptible to damage during all steps of these procedures. The characteristics of cryopreservation methods; such as exposure time of cells to the different cryoprotectant solutions, and to their different concentrations have critical roles in survival and viability of human oocyte and embryos (9,10). The rate of formation of intra-cellular ice crystals and requirements of the cells at different development stages are also concerns of the outcomes of cryopreservation (3,8,11).

A glass-like solidification method, vitrification, was assumed to lead to improved viability and survival rates of cells because of the prevention of intra-cellular ice crystallization (12). Also, only one embryologist without the use of any costly equipment can perform this method within a few minutes (13). More recently, it has been addressed as the future of cryopreservation of human gametes and embryos due to improved outcomes regarding the rates survival and pregnancy (14). However, vitrification has also been defined as a more vigorous in means of all possible cell damages except the formation of intracellular crystals. Thus, vitrification of human gametes and embryos at different stage might yield variable results. The aim of this review is to assess the possible impacts of different development stages of human embryos on outcomes of vitrification.

The methods of reviewing

Targets of this review were; a) to assess whether vitrification at early (cleavage or zygote) and late stage (blastocyst) of human embryo alters the outcomes of vitrification. b) If so, to predict the preferable stage of vitrification of human embryos to attain the highest yields. The literature listed in MEDLINE (January 1990 to October 2006), EMBASE (January 1990 to October 2006) and reference lists of the articles were used as the source of this review. The keywords used in searching of the databases were as follows; Vitrification , Slow rate, Freezing, Cleavage, Pronuclear (PN), Blastocyst, Outcome, survive, pregnancy, cryopreservation, implantation, embryo, and culture. All keywords were used either alone or along with "Vitrification" and/or with additional mentioned keywords in several research steps. Totally 214 articles were found to be related with the topic. Articles were revised by two authors and scored according to their aim, methodology, and type of study, or in reviews, the type of revision. Finally 74 articles, mainly comprising randomized, retrospective, observational studies and reviews as well some case reports, were selected and used in this manuscript.

Main outcomes

The primary outcomes of the study are survival and viability rates after thawing. In addition secondary outcomes such as clinical and live pregnancy rates, as well implantation rates were also investigated. All available data have been evaluated throughout the articles.

The advances in prevention of cell damage in Vitrification method

The history of cryopreservation of human gametes stretches back some 200 years to the first recorded experiments involving cooling followed by a successful rewarming of spermatozoa in snow by Spallanzani et al. in 1776 (15). Since then, more advances have been achieved in cryopreservation of human oocytes and embryos (16). However, the search of a possible cryopreservation method without any potential of cell damage has been the main issue during this era.

Notably, three potential cellular damages during cryopreservation have been defined previously. The first one is the chilling injury that occurs at higher temperatures such as between +15 and -5°C. This injury mainly damages the cytoplasmic lipid droplets and microtubules including the meiotic spindle (17). The next and the most common damage is the formation of intracellular ice crystals, which is the main source of fracture and damage of zona pellucida or cytoplasm and occurs between -50 and -150°C (12). The last one, incurred under –150°C, is the least dangerous one.

Vitrification is a non-equilibrium method of cryopreservation, and may be regarded as a radical approach in which cells are rapidly plunged to liquid nitrogen at -196°C after a very short period of equilibrium procedure (18). Nevertheless, the procedure requires much higher concentrations of cryoprotectants that may also cause possible toxic and osmotic effects when compared to slow rate freezing (14). Thus, an increased probability of all other forms of cell injury caused by cryopreservation except the formation of ice crystals has been claimed by this method.

Physically, there is a close link between the cooling rate and concentration of cryoprotectants; higher cooling rates reduce the required concentration of cryoprotectants or vice versa (19). Therefore, establishment of specific balances between reliably highest cooling (and warming) rates and a safe concentration of cryoprotectant without any toxic effect are critically required for preventing the consequent cell damage in vitrification (20-23). Therefore, those working with vitrification have established their own unique procedures,

by making alterations in concentration of cryoprotectants, cooling rate and/or carriers, and attempted to improve its superiority.

Aims and advantages of cryocarriers

Direct contact with cells for vitrification requires large volume of cryoprotectants and the possibility of transmission of viral pathogens which made the vitrification procedure quite hard for daily use previously (24). Therefore, numerous carrier systems, assumed to prevent both direct contact and requirement of large volume, have been introduced during this era. The open pulled straw, flexipet-denuding pipettes (FDP), microdrops, electron-microscopic (EM) copper grids, traditional straws, hemistraw system, small nylon coils, and the minimum volume cooling by cryotops and recently the closed cryotips are such examples (13,25-27). However, the widest experience has been mostly with the use of cryotop, cryotip and cryoloop (13,25). In 2005, Kuwayama et al. reported improved vitrification success with the use of cryotop in human oocytes (13).

Kuwayama et al. also published a comparison between open system, the CryoTop and a closed vitrification system the CryoTip over 13 000 embryos at different stages. This is the largest study up to today concerning vitrification in which the authors suggested that cryotop is an efficient and reliable way to freeze cleavage embryos, blastocycts and oocytes in daily practice.

The idea of transmission of viral pathogens possibly to embryos vitrified and stored in contaminated nitrogen was raised by the work of Bielanski et al. (24). This agrees with the fact that many viruses and some bacteria, such as Stenotrophomonas maltophilia most commonly, may survive after exposure to liquid nitrogen and potentially cause contamination that significantly suppresses fertilization and embryonic development *in vitro* (28). However, cross contamination of these agents and transmission between samples are still need to be evaluated (28). Thus, a reliable coverage of cell isolation and all rapid cooling devices is required. CryoTip has been recently suggested to eliminate the danger of contamination of cells while maintaining the high efficacy of the procedure.

Economical evaluation of Vitrification

Kuleshova and Lopata highlighted the advantages and disadvantages of slow cooling as compared with the technique of vitrification (5). They could show a satisfactory control of solute penetration or dehydration rate in slow cooling method. Also, vitrification takes a total time of about 10 minutes that is nearly 10 times less than the time required for slow cooling. Furthermore, they also reported that slow cooling is quite an expensive method when compared to vitrification regarding equipment and running costs.

In 2005, Kuwayama et al. also stated that primary disadvantages of slow cooling in cryopreservation of human embryos are the requirement for an expensive programmable freezing machine and its being a time consuming procedure (13,25,29,30). On contrary, vitrification could be performed without the use of costly equipments and could be completed by one specialist within several minutes. Therefore, the introduction of vitrification was assumed to provide significant benefits for any busy IVF lab.

Effect of developmental stage on cryopreservation damage

Clinical lessons from cryopreservation of human oocyte; oocyte vs embryo.

It has been well demonstrated that cryopreservation of human gametes and embryos have resulted in different success rates according to the developmental stage of the cell (11). Mainly the immature cells seem to be more sensitive than those in the latter stages, concerning clinical and laboratory applications or procedures. The methods of cryopreservation, especially vitrification, surely affect cells and lead to damages due to unphysiological situations that cells do not encounter normally. However, today the advances in the cryopreservation methods as well in daily practices of laboratories, such as successful culturing of human embryos to further stages, allow professionals to approach to the physiological reproduction. Therefore, it should not be underestimated that the basis of artificial reproduction is to help patients achieve the successful natural conception.

The ultra-structure of human oocyte is quite sensitive to changes of temperature and extracellular osmotic pressure. Thus, during freezing and thawing, human oocyte can have several types of cellular damage such as cytoskeletal disorganization, chromosome and DNA abnormalities, spindle disintegration, premature cortical granule exocytosis, related hardening of the zona pellucida and plasma membrane disintegration. It is not a surprise that rate of maturation, fertilization, and cleavage were found to be low in cryopreserved human oocytes when compared to fresh oocytes (14). Also, it has been shown that the outcomes of cryopreservation of human oocytes are unfavorable when compared to the results of cryopreservation of human embryos (31,32). This latter data completely supports the higher sensitivity of human oocyte to temperature and osmotic alterations than the human embryos with a significant decrease of survival (67% vs 54%) as well a reduction of pregnancy rates in half (14.2% vs 28.2%) (31,32).

It has been mentioned previously that embryos and gametes of human can be damaged at all stages of cryopreservation. The leading example for this was the initial beliefs concerning the meiotic spindle. Previous experiments presumed that meiotic spindle was lysed and damaged in the cryopreservation procedure (33). However, more recently, it has been shown that meiotic spindle simply disassociates due to the decrease in temperature, and can reform with normal function by the increase in temperature in 4 or 6 hours (34).

Methods have been developed for *in vitro* maturation of immature human oocytes (35). Initially, this was presumed to

overcome the problem of damaging the meiotic spindle in frozen oocytes (36). The presumption was based on the arrestment of meiosis at prophase I, and the protection of chromosomes by the membrane of the germinal vesicle in the immature stage. Also, there are not any microtubule structures yet formed at this stage. Notably cryopreservation of the immature oocyte can be beneficial in IVM cycles especially in patients with premature ovarian failure. Tucker et al reported one birth after cryopreservation of immature oocytes collected in a stimulated cycle by traditional slow rate cooling and rapid thawing protocol (36). Furthermore, Cha et al. used vitrification for cryopreservation of immature oocytes retrieved from unstimulated cycles of patients with polycyctic ovarian syndrome (37). However, they reported a cleavage rate of 90%, absence of successful implantation and dissatisfaction with the vitrification method (36,37).

Recently, vitrification was suggested to be more suitable for cryopreservation of human oocyte than the slow freezing method by the application of higher concentration of cryoprotectants and a rapid cooling speed for preventing the formation of intracellular ice crystals (33,38). Kuleshova et al. reported a pregnancy from vitrificated oocytes while Yoon et al. reported six deliveries (19,39). More recently significantly increased success rates with the vitrification of MII oocytes by the use of cryotop method which was initially described by Kuwayama et al., were reported (25). Nevertheless, it is still too early to support the idea of oocyte vitrification and its routine application in human reproduction.

Vitrification of human zygotes and cleavage embryos: Possibilities and Success of Technique

In 1985, Rall and Fahy were the first to report the efficacy of the vitrification method in embryo cryopreservation (12). However, the application of the method to human embryos was limited initially probably due to fears of toxicity caused by the high concentration of the cryoprotectants. After the initial work of Mukaida et al. who reported successful vitrification of human 4-8 cell embryos by the use of the method developed for mouse embryos (vitrification solution containing ~7 mol/lit ethylene glycol) other groups also confirmed that vitrification is indeed applicable to human embryos (25,40).

Subsequent to these first successful applications, it was shown that the pronuclear stage embryos could survive with high rates after vitrification and warming procedures (25). This might be due to the hardening of the zona pellucida after the cortical reaction that occurs with and after the process of fertilization, which gives the ooplasmic membrane more stability to cope with the low temperature and osmotic changes. In the early part of this decade, different survival rates were reported mainly because of the variation of vitrification technique (26,41,42). However, the majority of studies comprising vitrification of early stage embryos, reported as high survival rates as over 80% (Table 1). The majority of studies reported pregnancy rates in the range of 22-30%, which were completely in the acceptable ranges and much higher than the rates of slow rate freezing procedures (25,26,41-44, Table 1). Also, more recently even pregnancy rates as high as 35% have been reported with vitrification of both cleavage embryos (45) and PN stage zygotes (46). These reported successful pregnancies and recent data suggest that vitrification of human zygotes and early stages embryos are perfect alternatives of slow freezing. Especially in countries where cryopreservation of later-stage human embryos is not allowed by law or due to religious reasons vitrification seems to be quite beneficial (46-48).

El-Danasouri et al. reported that the rate of survival has tended to increase with the increasing number of blastomeres and cell stage of cleavage embryo (42). Higher pregnancy and slightly higher survival rates were commonly attributed to the further stages of human embryos such as 8 cell (45) and blastocyst stage (49). However, other studies showed at least equal (46), or even higher survival rates (25) with vitrification of PN stage zygotes. It should not be underestimated that the differences of pregnancy rates between further stage embryo vitrification might also be due to the later transfer day. However, a recent Cochrane review indicated that there is not any difference between the rates of live birth after embryo transfers in fresh cycle at day 2-3 and day 5-6 (50). The authors also indicated that in the absence of data on cumulative live birth rates resulting from fresh and thawed cycles, it is not possible to determine if this represents an advantage or disadvantage in terms of outcomes after cryopreservation. Thus, survival rates should be taken as a main predictor of success with inadequate data on live births after vitrification of embryos at different development stage. Moreover, the rates of formation of blastocyst stage embryo after different freezing protocols also suggest the idea of early stage vitrification of human embryos (25,51,52).

In a recent study published by our group (53) using the slow freezing method for cryopreservation of human zygotes, the pregnancy rate per embryo transfer was reported to be 10.2%, while with the use of vitrification the pregnancy rate was found more than three times higher (46). For this reason, in our center the use of slow freezing method was stopped completely and has been replaced by a routine vitrification program after a long period of practicing the conventional slow freezing method.

More recently, successful pregnancies after repeated vitrification of human embryos have been reported (54,55). *In vivo* maturation of oocytes were also initially used in both of the mentioned cases. Thus, vitrification of early stage human embryos is an acceptable, viable and a better alternative of slow rate freezing with increased rates of pregnancy and survival. Especially, centers using routine day 3 transfers mostly seem to have the benefit. Possibility of addition of the *in vivo* maturation and of pre-implantation genetic diagnosis procedures widens the range of application of vitrification at early stage of human embryos.

Table 1. The outcomes of selected studies on vtrification of zygote and early stage human embryos

Authors	Year	Stage of embryo at vitrification	Cryoprotectants	Type of cryocarrier	Total number of vitrified embryos	Rate of survival	Rate of pregnancy	Rate of implantation	Special results
Al-Hasani et al. (46)	2007	Zygote	EG/DMSO/S	Cryotop	339	89%	36.8%	15.6%	Rate of abortion 17.24%
Kuwayama et al. (25)	2005	PN stage		Cryotip		100%	-		
		Cleavage stage	EG/DMSO	Cryotop	13 000	98%	27%	NA	Rate of delivery 48-51%
		blastocysts				90%	53%		
Zhu et al. (43)	2005	Embryos	EG based	Open pulled straws	957 (514 thawed)	72.2%	19-22%	NA	NA
Rama Raju et al. (45)	2005	Embryos (8-cell)	EG based	Open pulled straws	40	95%	35%	14.9%	NA
Hredzak et al. (44)	2005	Cleavage	EG/S	100 microl pipetting "tip"	215	69%	27%	NA	NA
Isachenko et al. (67)	2003	PN zygotes	EG based	Open pulled straws	59	71%	NA	NA	NA
Liebermann et al. (26)	2002	Embryos	EG based	FDP	266	83.80%	NA	NA	NA
		Oocytes	EG based	FDP	928	80.60%	NA	NA	NA
El-Danasouri et al. (42)	2001	Embryos (8 cell)	EG based	Open pulled straws	215	49.3% (increases with cellstage of embryo)	30.5%	10.4%	NA
Saito et al. (41)	2000	Embryos (8-16 cell)	EG /Ficoll/HTF	Open pulled straws	98	100%	16.2% (5/31)	NA	NA

DMSO: dimethylsulphoxide
EG: ethylene glycol
EG/S: ethylene glycol and sucrose
FDP: flexipet denuding pipette
HTF: human tubal fluid
NA: not available

Vitrification of blastocyst stage embryo:
Is it the best in embryo selection and clinical outcomes?

Today, great advances of embryo culture systems allow us possible further culturing of human embryos. Especially, prolonged culture of embryos to day 5 gives the chance of much detailed assessment of the embryonic development parameters (56). The possibility of selecting the best embryo for transfer claimed to lead to a favorable reduction of multiple and high order pregnancies by day 5 transfer (57). On the contrary, day 3 transfer along with cryopreservation of embryo at early development stage might be beneficial in conditions where further assessment and possible selection of embryo could not be available due to low number of PN zygotes especially less than three (58). Therefore, possibility of selection and assessment of embryos is also an important issue in selecting the stage of cryopreservation. Nevertheless, the quality of the development of the early embryo determines the quality of the blastocyst and so, the results (56). This underlines the importance of following each embryo day by day to select the blastocyst(s) with the best potential for vitrification. However, it does not make a difference in vitrification of embryos at early versus blastocyst stage. One can assume that selection of best embryo(s) for vitrification is done by cryopreservation at further development stages. However, prolonged and extended culture reduces the number of cryopreserved embryos, which is an important disadvantage of blastocyst vitrification (50).

Blastocyst and further stage of human embryos have different physiological requirements than early stage embryos which affect the survival chance after unphysiological situations such like ultra rapid freezing (11). A major factor that affects the survival rate of blastocyst is its fluid-filled cavity called as “blastocoele”. As expected, the formation of intracellular ice crystals is directly proportional to the volume of this blastocoele. In a study, Vanderzwalmen et al. initially encountered low survival rates after vitrification of blastocyst (22). However, they were able to overcome by reducing the blastocoelic cavity and puncturing it with a special pipette before the procedure. Thereafter improved survival rates have been reported (Table 2).

Mukaida et al. also showed moderate survival and acceptable pregnancy rates by vitrification with the use of cryoloop as a carrier in early of this decade (23). The increased practices of combined use of various cryoprotectants as well application of different cryocarriers, has led to increased success in the outcomes of vitrification after 2002 (13,23,26,25). In this dynamic era, almost all groups studying vitrification tried to improve their technique by introducing various advances to vitrification technique. Thereafter several different techniques, which are almost the same, using different dilution and equilibrium steps as well various kind of cryocarriers have been reported (14). Nonetheless, majority of the studies described the usage of combination of EG and DMSO as common cryoprotectants and cryoloop or cryotop as the leading cryocarriers.

After 2002, the outcomes of vitrification in blastocyst stage were improved and reported to give as high as 100% survival with 53% pregnancy rates (Table 2). Furthermore, most of the recent reported data on survival and pregnancy rates were above 90% and 50%, respectively (Table 2). These recent data mainly suggested that vitrification seems to be the future of cryopreservation with the highest rates of pregnancy and survival outcome ever reported. The most stunning and strong data came from the study of Kuwayama et al. in 2005 (25). This study comprise cryopreservation of 13 000 embryos by both slow rate freezing and vitrification as well containing data about vitrification of human embryos at different development stages. They stated in that study “Vitrification is a simple, efficient and cost-effective way to improve cumulative pregnancy rates per cycle” (13,25). Therefore, vitrification is also a cheap and time saving tool apart from its efficiency and safety proved by many reports of successful pregnancies and deliveries derived from the vitrified embryos, blastocysts and human oocytes (14).

As mentioned in vitrification of early stage embryos, zona pellucida can also be damaged and hardened, which is presumed to cause a reduction in implantation, due to freezing and vitrification procedure (23,59). Therefore in some studies assisted hatching (AH) was added to the freezing and thawing procedure and performed prior to transfer of vitrified embryos (Table 2). Adjunction of AH has been found to be beneficial in vitrification cycles by increasing pregnancy and implantation in a study (59). Furthermore blastocyst with intact zona pellucida were shown to survive and resist much better than their counterparts with partial or total loss of zona (60). However successful cryopreservation of blastocysts which totally hatched and got loss or escaped from their zona with acceptable rates of survival and pregnancy were also shown in that study.

Vitrification was reported to be a beneficial tool for cryopreservation of biopsied blastocysts, and found to be superior to different types of slow rate freezing with or without rapid thawing (61). Therefore, once again, vitrification was indicated to be useful in pre-implantation genetic diagnosis of further stage human embryos. It can also be assumed to be beneficial in synchronization of endometrium especially in donation cycles. On the other hand, two studies indicate that day 5 blastocysts have higher rates of survival (29), as well pregnancy and implantation (49), rather than their day 6 counterparts. Nevertheless, similar rates have also been described in vitrification of morula and early stage blastocyst (62). Therefore, this data needs to be evaluated for the specific requirements and needs of embryos at different developmental stages.

On the other hand, blastocyst stage embryo has also the advantage of possessing many cells, and the loss of few blastomeres during the freezing and thawing might not compromise the integrity of the entire specimen. Also, there have not been reports on any increase in the incidence of chromosomal aneuploidy. An increased rate of DNA frag-

Table 2. The outcomes of selected studies on vitrification of blastocyst stage human embryos

Authors	Year	Stage of embryo at vitrification	Cryoprotectants	Type of cryocarrier	Total number of vitrified embryos	Rate of survive	Rate of pregnancy	Rate of abortion	Rate of implantation or special notes
Li berman et al. (49)	2006	Day 5 blastocysts	EG based	FDP	254	95.9%	48.7%	NA	IR 33%
		Day 6 blastocysts				97.5%	42.8%		IR 25%
Kuwayama et al. (25)	2005	PN stage		Cryotip	13 000	100%	-	NA	Cryotop is superior
		Cleavage stage	EG/DMSO			98%	27%		
		Blastocysts		Cryotop		90%	53%		
Zech et al. (60)	2005	Blastocysts	EG/DMSO	Hemi-straw	177	64-82%	21-35%	NA	SR increases with intact ZP
Takahashi K et al. (64)	2005	Blastocysts	EG/DMSO/S	Cryoloop	1129	85.7%	44%	22%	Congenital defects 1.4%
Huang CC et al. (65)	2005	Blastocysts	EG/DMSO/S/HSA	Cryoloop	249	77.1%	53.8%	NA	Super-cooled liquid nitrogen
Stehlik E et al. (61)	2005	Blastocysts	EG based	Cryotop	41	100%	50%	NA	NA
Hiroaka et al. (66)	2004	Blastocysts	EG/DMSO	Cryotop	49	98%	50%	NA	IR-33%
Cremandes et al.(62)	2004	Morulas	EG/DMSO	Cryoloop	30	73%	Not transferred	NA	NA
		Early Blastocysts			33	82%			
Vanderzwalmen et al. (59)	2003	Blastocysts	EG/DMSO	Hemi-Straw	281	60%	27% (Ongoing)	NA	AH let more favorable implantation rate
Mukaida et al. (29)	2003	Blastocysts	EG based	Cryoloop	444	79%	36%	NA	
Mukaida et al. (30)	2003	Blastocysts	EG based	Cryoloop	725	80.4%	37%	27.6%	Day 5 survival rate is higher (87%)
Cho et al. (68)	2002	Blastocysts	EG based	EM	293	50-82%	34.1%	NA	Six step dilution of cryoprotectant is better
Reed et al. (69)	2002	Blastocysts	EG/DMSO	Cryoloop	15	100%	25%	NA	IR 15.4%
Vanderzwahlen et al (22)	2002	Morula	EG/Ficoll/S	Straws-Direct plunge	167	54.5%	22.7%	NA	Puncturing of blastocoele increases survival and pregnancy
		Blastocysts				20.3-58.5%	4.5-20.5%		
Mukaida et al. (23)	2001	Blastocysts	EG based	Cryoloop	60	63%	31.5%	16%	NA

AH: assisted hatching
DMSO: dimethylsulphoxide
EM: electron microscopic grids,
EG: ethylene glycol
FDP: flexipet denuding pipette
S: sucrose
ZP: zona pellucida
SR: survival rate

mentation was defined in frozen/thawed bovine blastocysts suggesting a possible damage from cryopreservation (63). Thus, a special attention still should be given to this issue; although, Takahashi et al. indicated a normal incidence of congenital defects and anomalies after vitrification of blastocyst (64).

Conclusion

Today, vitrification seems to replace the former slow rate freezing protocols by improved survival and clinical outcomes. Although different stages of human gametes and embryos show different physiologic necessities and features which can affect their survival especially after laboratory procedures, without doubt outcomes of vitrification of human embryos at different development stages are quite encouraging. Therefore, vitrification should be accepted as a real, viable and a more efficient alternative of cryopreservation of human embryos. Recent data also suggest the possible usage of vitrification on human oocytes with similar improved outcomes (38). However, there is still some awareness regarding higher sensitivity of oocytes to rapid temperature alterations that leads to possible chromosomal damage in that stage.

Nevertheless, vitrification at both cleavage and blastocyst stage of embryo seem to be favorable and efficient in view of increased outcomes such as survival and pregnancy rates. On the other hand, more advanced pregnancy rates have been reported by vitrification at blastocyst stage. However, merely pregnancy rates are not a fair comparison parameter due to controversies about the differences of the transfer day. On the contrary, we should have the benefits of great advances in both embryo culturing and vitrification techniques. Acceptably high rates of pregnancy were also reported by vitrification at cleavage and PN stage. However, vitrification of cleavage and PN stage human embryos are important and critical alternatives given similar survival rates, as well high rate of formation of blastocyst. Especially vitrification at this stage will serve quite well in such countries where further culturing is not allowed. The possibility of easy and safe application of early pre-implantation techniques and *in vivo* maturation procedure are also other advantages which professionals have benefited from by vitrification of embryos at all stages. Therefore, in the current practice vitrification, wether at the cleavage and PN or blastocyst stage, should be recommended instead of slow rate freezing which is not only costly and but also necessitates programmable freezers.

There are some unanswered questions; first, should we really expect less chromosomal damage in blastocyst stage due to increased inner cell number? Second, can other early selection assessments improve our embryo scoring and help for better selection at early stage embryo vitrification? Third, is reduced number of vitrified embryos at blastocyst stage critical for success or does this give the chance of the better selection of the vitrified embryos. Nevertheless, there is not a quite reasonable point yet to select blastocyst stage vitrification rather than early stage vitrification, where all mentioned advantages can also be obtained by the vitrification of the early stage embryo along with extended embryo culturing.

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