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# The Present and Future of Embryo Cryopreservation

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## Abstract

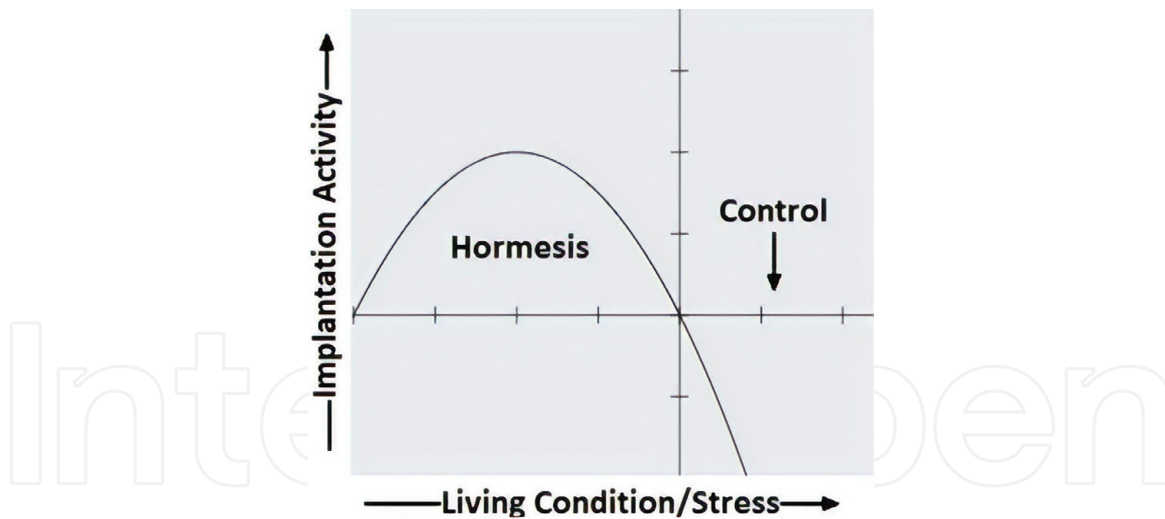
Embryo freezing technologies have widely been used in human IVF practice and in animal industry. In this chapter, we will review the development of embryo freezing technology and the application of the method, which will concentrate on discussion of the arguments in favor of and against freezing, as well as the latest results of success rates, comparing them with the other basic assisted reproductive technologies methods. Then, we will present our viewpoint for the future application of embryo freezing methods and their place in reproductive medicine. The analysis of facts and suggestions should enable researchers to rethink the position of cryobiology in reproductive medicine. It should be considered that the method of cryopreservation is not only a technology for storing embryos but also a method of embryo treatment that can potentially improve the success rates in infertile couples. There is also a theory that describes the “treatment” effect of freezing an embryo, which may explain the higher success rates of frozen embryo transfer (FET) compared to fresh embryo transfer (ET). The authors of the “Theory about the Embryo-Cryo treatment” believe that freezing and thawing could activate endogenous survival and repair mechanisms in preimplantation embryos.

**Keywords:** embryo, cryobiology, embryo treatment, frozen embryo transfer, ART methods

## 1. Introduction

During the last couple of decades, assisted reproductive technologies (ART) have become one of the fastest developing branches of medicine. Since they are the main method of fertility treatment, much research has been done in this area. Enormous amount of work has been done to elucidate the benefits of cryopreservation of stem cells, embryos, gametes, tissues, and organs. The idea of maintaining the viability of living reproductive cells and tissues of various species after long-term storage provides a chance for animal and human reproductive applications [1, 2]. Due to the constantly improving cryopreservation techniques, we are now able to preserve cells and tissues by cooling them to extremely low temperatures, such as  $-195.79^{\circ}\text{C}$  (the boiling point of the liquid nitrogen). Cooling down biological objects to such degrees prevents any biological activity, including all the biochemical reactions involved in cell death.

Among the biggest scientific achievements, cryopreservation of embryos came into prominence more than 45 years ago [3, 4]. In 1972, Whittingham and associates



**Figure 1.**  
*Mechanism of hormesis (License: 4371720640523).*

and Wilmut succeeded in cryopreservation of eight-cell mouse embryos [3, 4]. Since that time, vast number of embryos from various mammalian species have been frozen, thawed, and eventually transferred successfully, thus proving the benefits from this ART procedure. In 1983, Trounson and Mohr achieved the first human pregnancy from frozen embryo with the same procedure used successfully for cryopreservation of mouse and cow embryos [5].

Without doubt, the successful cryopreservation of embryos has greatly improved the chances for a successful outcome after a single cycle of ovarian stimulation and in vitro fertilization (IVF). There is also a theory that describes the “treatment” effect of freezing an embryo, which may explain the higher success rates of frozen embryo transfer (FET) compared to fresh embryo transfer (ET). The authors of the “Theory about the Embryo-Cryo treatment” believe that freezing and thawing could activate endogenous survival and repair mechanisms in pre-implantation embryos [6]. The idea is that the thawing process induces low levels of stress, which leads to hormesis. This controlled stress could lead to the repair of mitochondrial damage and protein misfolding. This theory may explain the higher success rate of FET compared to fresh ET in women of advanced reproductive age, the higher miscarriage rate after thawed blastocyst transfers compared to thawed early cleavage embryos transfers and the higher perinatal parameters of children born after FET (**Figure 1**).

While much has been discovered about embryo cryopreservation, there are still many things that have to be defined more accurately, such as the freezing medium composition or the stage of the embryo during freezing. Embryo freezing methods are constantly being improved, but they, as well as the freezing equipment, require improvements. Scientists are looking for answers to these and many more questions while the final goal remains clear—successful cryopreservation followed by as high as possible pregnancy rates. In this chapter, we will try to present the most important aspects of cryopreservation. In the end, we hope that the potential of cryobiology in reproductive medicine will have been acknowledged.

## 2. Cryobiology and reproductive medicine

Cryobiology represents a branch of biology which studies the effects of low temperatures on organisms, biological systems, or biological materials. Those low temperatures range from hypothermic to cryogenic ( $-150^{\circ}\text{C}$  or lower).

While cryobiology is mainly focused on the “living world,” in the last decades, it has been expanded to involve treatment of nonliving things, as well. With the development of highly sophisticated cryobiological techniques, like cryosurgery, embryo and gamete preservation and others, this biological branch has the potential to affect everyone's lives in the future.

The idea of freezing human gametes for their future use encouraged scientists to incorporate cryobiology in the field of reproductive medicine. Polge et al. in 1949 have been recognized as the first researchers who cryopreserved spermatozoa while using glycerol as a cryoprotectant [7], although Bernstein and Petropavlovski 12 years earlier demonstrated that glycerol has a cryoprotective role in the cryopreservation of spermatozoa [8]. Encouraged by those findings and driven by the potential benefits of freezing human gametes and embryos, scientists soon began to study much more in the biology of cryopreservation. Rapid progress was made, and the first birth from the use of human frozen spermatozoa was achieved in 1954 [9]. In those days, scientists used spermatozoa for their cryopreservation studies, since they have motility, which was useful when assessing the vitality of the frozen/thawed probe. Nowadays, cryopreservation of spermatozoa could be achieved easily and is a routine procedure, performed worldwide.

Freezing oocytes was much harder to achieve, and it took scientists some time. Chen in 1986 reported the first pregnancy, resulting from slow freezing and rapid thawing of human oocytes using DMSO (dimethyl sulfoxide) as a cryoprotectant [10]. However, earlier concerns were raised regarding damage to the meiotic spindle, loss of cortical granules, and the low success rates as compared to the relative success of embryo cryopreservation, which led to little interest in oocyte freezing until 1990s [11]. During those times, Bernard et al. and other researchers demonstrated that reasonable oocyte thaw survival [12] and subsequent fertilization could be achieved [13]. Gradually, interest was raising, and through hard work, oocyte freezing is now also a routine procedure.

One of the very important attainments that cryobiology has achieved is the ability to successfully freeze and thaw human embryos. Scientists first discovered how to successfully freeze an embryo, and only after that, they achieved successful oocyte cryopreservation. Whittingham et al. achieved the first successful embryo cryopreservation when the research group froze mouse embryos in polyvinylpyrrolidone (PVP) [3]. The first baby born after a frozen/thawed blastocyst transfer was reported by Cohen et al. in 1985 [14]. Embryo cryopreservation is now a routine procedure and there is sufficient published data supporting its effectiveness.

### **3. Embryo cryopreservation**

Cryopreservation of embryos is a very delicate procedure, which also hides some risks to the things that are frozen. During the freezing process, embryos are exposed to physical stress, caused either by the direct effects of the low temperature or by physical changes induced by ice formation.

The direct effects of the low temperature may induce damage to cell structure and function. The mitotic spindle is especially sensitive to cold shock injuries. The extent of the damage, caused by the freezing procedure, depends on various factors, like the shape and size of the cells, the membrane composition, and its permeability. All these factors are variable and are species specific. Embryos and oocytes have the ability to repair some damage in order to survive and develop properly.

The formation of ice crystals is detrimental to cells. The damage that the cells suffer is not due to the crystallization of ice but rather due to the high concentration of solutes occurring when water is removed in order to form ice [15].

Cryoprotectants (CPAs) act to reduce cellular damage by increasing the volume of the unfrozen residual phase. When the first cryopreservation experiments took place, two opposing methods had been developed simultaneously—a method of slow freezing of the cells and vitrification. Since these methods were very different, scientists started to compare them, in order to elucidate their benefits and drawbacks. Vitrification offers the possibility of eliminating the formation of ice crystals [16], and over the years, it has gradually replaced slow freezing as the preferred method of cryopreservation in the field of reproductive medicine. The main reason behind this fact is that vitrification achieves better survival rates, and moreover, it is less time consuming to perform and does not require highly specialized and expensive equipment like in the slow freezing technique.

### **3.1 Cryoprotectants**

Cryoprotectants (CPAs) are substances that protect cells/tissues from the damage that may occur during the freezing process. In order to achieve successful cryopreservation of any biological material, the freezing protocols must be optimized, starting with the correct choice of CPA and ending with the thawing process and post-thawing handling of the material. The choice of the most appropriate CPA for a certain freezing procedure is difficult to make, because it must take into consideration the CPA's toxicity, permeability, and also its physicochemical properties. CPAs are widely used to improve the cryosurvival rates, although their mechanism of action is not fully understood. One of their properties is to lower the freezing point of a certain solution, while also protecting the cell membrane during the freezing process. CPAs may also act to stabilize intracellular protein structure. As mentioned earlier, freezing an embryo is a very delicate procedure and embryos may be damaged by chilling, fracturing, ice formation (intra and extracellular), the chemical toxicity of CPAs, osmotic swelling, and osmotic shrinkage [17]. Chilling injuries can lead to changes in lipid-rich membranes and can also result in cytoskeletal disorganization. The mechanical effect of a solidified solution may cause fracture damage, especially to embryos. One of the first documented studies that introduced the concept of cryoprotectants was that of Polge et al. [7, 18], which assessed the use of glycerol in sperm freezing, and it also provided the basis of many future investigations concerning CPAs.

Regarding their structure, CPAs are small molecular weight solutes possessing high aqueous solubility and polar groups that interact weakly with water [19]. CPAs are generally divided into two groups based on their ability to penetrate through the cell membrane—permeating (PM) and nonpermeating (NPM). It is important to point out that PM and NPM cryoprotectants are often used together in order to achieve a successful cryopreservation of cells and tissues. In fact, the core of a cryopreservation solution is made of a mixture of those CPAs, and it also includes various components, like some salts, pH buffers, and others. In the PM group are included some of the most studied CPAs like glycerol (G), ethylene glycol (EG), dimethyl sulfoxide (DMSO), formamide, propylene glycol (PG), and others. PM cryoprotectants are the most important component in the vitrification solution. G and EG are the most commonly used PM CPAs. NPM cryoprotectants include saccharides, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and others. They are large molecules, usually polymers. Sucrose is the most commonly used NPM cryoprotectant. The effect of these NPM agents is the dehydration of the cells by osmosis. They also act to stabilize the cell's membrane [20] and aid the entry of PM cryoprotectants [21]. Moreover, NPM CPAs are added during the thawing process as they act to reduce the osmotic shock.

Nowadays, there are many freezing media produced by biotechnology companies, which are made by mixing various substances to achieve maximum

cryoprotection. The composition of these freezing media varies greatly, since there is no “perfect formula” and therefore the search goes on.

## 3.2 Methods

Practically, two methods for embryo cryopreservation have been used—slow freezing and vitrification. Here, we will briefly review both of them and we will discuss their positives and negatives.

### 3.2.1 Slow freezing

Slow freezing is a conventional cryopreservation method, which is based on a slow cooling rate and use of a low concentration of CPAs. This leads to less toxicity to cells/tissues; however, it does not eliminate ice formation. In 1972, two scientific groups published the first survival of murine embryos after slow freezing [3, 4] and live offspring [3]. Nowadays, after the introduction of the vitrification method, slow freezing is gradually being replaced.

Protocols based on the slow freezing method include an equilibrating step, during which the cells or tissues are placed in an aqueous solution containing PM CPAs in low concentrations (1.0–1.5 M) and sucrose (0.1 M) before which they are placed in ampules or straws. After the exposure to CPAs, initial cellular dehydration is observed followed by a return to isotonic volume with the permeation of CPA and water. After loading the specimen in the straw/ampulla, the temperature is being lowered down slowly with the aid of a controlled rate freezing machine which allows samples to be cooled at different rates, and finally, the frozen objects are placed in liquid nitrogen for storage. The slow cooling is performed to ensure that the cells/tissues are dehydrated before intracellular ice formation occurs. However, the optimal rate of cooling varies greatly among cells and tissue types [22]. A crucial step during the slow freezing protocol is the so-called ice crystal seeding which can be performed either manually or automatically. It takes place after the ampules/straws, preloaded with the embryos, are cooled below the melting point of the solution which is around  $-5$  to  $-7^{\circ}\text{C}$ . At these temperatures, solutions remain unfrozen due to the supercooling (lowering the temperature of a solution below its freezing point without extracellular ice formation). Supercooling leads to improper cell dehydration and to avoid such fate, most commonly manual ice nucleation is performed by touching the ampules/straws with a prechilled with liquid nitrogen cold object like forceps which leads to ice crystal formation. In this way, the remaining water in the cells is driven away due to the osmotic imbalance, caused by the formation of ice crystals. After ice crystal seeding, the process of slow freezing continues at various cooling rates. When the temperature has reached values ranging from  $-30$  to  $-80^{\circ}\text{C}$  depending on the protocol, the ampules/straws are plunged into liquid nitrogen.

In conclusion, we must say that despite the acceptable results achieved by slow freezing, it also has its negatives, for example, it is time consuming, as freezing an embryo usually takes between 2 and 3 hours depending on the cooling rate. Furthermore, it requires expensive controlled-rate freezers.

### 3.2.2 Vitrification

Vitrification is an alternative approach to the slow freezing method for the cryopreservation of embryos/gametes. Vitrification differs from slow freezing in that it avoids the formation of ice crystals both intracellularly and extracellularly [23]. This method is easier to conduct, does not require expensive equipment like

programmable freezers, and is not that time consuming when compared to the conventional slow freezing.

Physically speaking, vitrification is the solidification of a solution at low temperatures by elevation in viscosity during cooling and not by ice crystallization [24, 25]. The first successful vitrification of embryos was published in 1985 by Rall and Fahy, who froze mouse embryos using DMSO, PEG, and acetamide [23]. Commonly used freezing solutions for vitrifications are composed of permeating CPA (EG, DMSO, G, acetamide, PG, with a concentration of over 4 M) and nonpermeating CPA (most commonly sucrose, >0.5 M). After numerous experiments and further improvements of the vitrification technique, like replacement of DMSO with EG and mixture of several CPAs [26], vitrification was applied to human embryos and live births were achieved with both blastocyst and cleavage stage embryos [27, 28]. Assisted hatching (AH) was added to the freezing/thawing procedure and is performed before the transfer of vitrified embryos. It was reported that AH is beneficial in vitrification cycles by increasing pregnancy and implantation rates [29]. Although several methods of AH had been developed—mechanical, piezo, chemical, and laser, the latter turns out to be the most used one with one of its main advantages lies in minimizing the exposure of embryos outside the incubator. A recent meta-analysis conducted in 2016 encompassing 36 randomized controlled trials and 6459 participants reported increased clinical pregnancy rate and multiple pregnancy rate in couples after AH and nonsignificant difference in miscarriage rates between the AH group and the control one [30]. Despite fears about the safety of AH and the increased chance of multiple pregnancies, many IVF facilities apply the procedure on every thawed embryo. The idea behind this is to improve the implantation and clinical pregnancy rates especially in women with history of repeated IVF failure. Embryos with thicker zona pellucida could benefit the most after AH. A large study by Knudtson et al. with more than 150,000 FET cycles reported a slightly decreased live birth rate in the first autologous FET cycle after AH [31]. Therefore, the application of AH should be carefully considered, and prospective studies should be carried out in order to elucidate its benefits and negatives.

The vitrification protocols for embryo freezing consist of several steps. In the first place, embryos are exposed to high concentrations of CPAs, after that, they are loaded into carriers, most commonly straws, and finally those straws are cooled as fast as possible, reaching a cooling rate of thousands of degrees per minute. To achieve vitrification of solutions, there must be an increase in both the cooling rates and the concentration of CPAs. Those two factors are inverse proportionally connected since, the higher the cooling rate, the lower the required CPA. It is important to mention that there are some concerns regarding the use of high concentrations of CPAs, because they could harm the cells during vitrification. That is why a mixture of CPAs is used during vitrification in order to reduce this toxicity.

Vitrification techniques include the so-called “open” and “closed” systems or carriers. The idea behind them is unambiguous, and with the open carriers, the embryos are directly exposed to the liquid nitrogen, which increases the cooling rate, but hides a potential risk of cross contamination of the probe during the storage in liquid nitrogen. On the other hand, the closed systems isolate the sample from the liquid nitrogen which lowers substantially the risk of contamination; however, the cooling rate is inferior compared to the open carriers. There are dozens of different carrier devices available commercially, but there are not many comprehensive studies that compare the carriers and their efficiency. When comparing open and closed systems (VitriSafe carrier, open and closed variation) for blastocyst freezing, a prospective study by Panagiotidis et al. documented no significant difference between the two carrier systems [32], which highlights the importance of the thawing process. Kuwayama et al. compared open (Cryotop) and closed

(CryoTip) systems for the vitrification of blastocysts and also found no significant difference (survival rate 97 and 93% for Cryotop and CryoTip, respectively, deliveries 51 and 48%). These observations support the thesis that closed systems are comparable to open ones, because they also reduce the risk of cross contamination.

We hope that vitrification will be optimized in the near future and questions regarding the composition of the vitrification solution, the most appropriate carrier type, and others would be answered in due time.

### *3.2.3 Method comparison*

In the pool of studies that compare slow freezing of embryos and vitrification, Kuwayama et al. reported that vitrification of 5881 human PN stage embryos resulted in 100% survival, 93% cleavage, and 52% blastocyst rates. In contrast, after slow freezing of 1944 PN stage embryos, the results were 89% survival, 90% cleavage, and 41% blastocyst rates [33]. When freezing cleavage-stage embryos on day 2 with the slow freezing method, the survival rate of 77.0% was reported in a study by Xue et al.; however, when using vitrification, the authors reported 96.6% survival rate when  $P < 0.05$  [34]. A study in 2015 documented the survival rate of 96.95% after vitrification of day 3 embryos, in contrast to the 69.06% of the embryos survived after slow freezing, post-warmed excellent morphology embryos: 94.17 vs. 60.8% [35]. The study included 592 frozen/thawed embryos. Regarding blastocyst cryopreservation, a large retrospective study by Richter et al. in 2016 included 4862 slow frozen blastocysts and 2735 vitrified blastocysts, with no statistical difference between patients BMI and age [36], and reported interesting findings. A survival rate (authors define survival as having >50% of cells intact after thawing) of 95.6% was achieved in the vitrification group versus 91.9% in the slow frozen group, when  $P < .0001$ . They also found that the percentage of intact cells was more after vitrification/warming compared to slow freezing and thawing, 95.3 vs. 88.7%,  $P < .0001$ . It is important to mention that currently, there is much debate as to the developmental stage at which human embryos are best to be cryopreserved [24, 37].

In 2014, a population-based cohort study in Australia and New Zealand included 11,644 slow frozen and thawed blastocysts and 19,978 vitrified and thawed blastocysts. A higher clinical pregnancy rate per embryo transfer cycle was reported for the vitrification group (32.7%) than in the slow frozen one (23.8%). The mean maternal age for the slow frozen group was 33.6, while for the vitrification group—34.2. This is one of the largest known studies in this field; however, a possible drawback could be the lack of information available on clinic-specific cryopreservation protocols and processes for slow freezing-thawing and vitrification-warming of blastocysts and the potential impact on outcomes [38].

All of these results highlight the advantages of vitrification and the drawbacks of slow freezing. Overall, vitrification turns out to be the better method of cryopreservation in the field of ART.

### **3.3 Consequences of freezing an embryo**

There are two major concerns regarding embryo cryopreservation. One of them is about the survival rate after embryo freezing. The second major concern is that the freezing process may induce cryodamage to the embryo. Cryodamage is a collective term which includes various types of injuries that a biological object could experience during the freezing and thawing, like formation of ice crystals, physical stress, and also other types of damage we have mentioned earlier. Assessing survival rate after thawing is the most used technique to evaluate the effects of the



cryopreservation process on the embryos. However, freezing an embryo also does not allow the inspection of other types of damages, which occur at the molecular level—DNA damage, altered gene expression, and protein function. These alterations require specific molecular biology methods in order to be assessed, and their impact on the embryo is not that clear. In contrast, when the survival rate after freezing is being assessed, we must say that this approach is straightforward and yields distinct results.

When talking about the embryo survival rate nowadays, with the constantly improving cryopreservation techniques, a survival rate of more than 90% or even 95% could be observed depending on the vitrification protocol, carrier, embryologist experience, thawing process, and other variables. While this rate is indeed very high, unfortunately there is still a risk that a frozen embryo would not survive after thawing. The survival rate is different for the different stages at which the embryos are frozen. In fact, it is still unknown at which stage of development, the embryos are most suitable for freezing and therefore further research is needed. Moreover, the stage at which the embryo is frozen is connected to different types of cryodamage. At the PN stage, there is evidence that embryos may suffer integrity damage of the pronuclei after cryopreservation [39], and therefore, their developmental potential could be significantly impaired. At the cleavage stage, there is evidence of zona pellucida damage [40] and changes in metabolism [41]. Reduced implantation rates have been observed after the loss of blastomeres in day 2 grade 1 embryos with <10% fragmentation in a study with 363 thawing cycles [42]. Blastocyst cryopreservation represents a demanding task due to its size and the presence of blastocoel. Formation of ice crystals is probably the main factor affecting blastocysts survival rates, since the blastocoel contains large amounts of water. In order to reduce the negative effects of the blastocoel on survival rates, it was proposed that blastocysts should be frozen at the contraction stage or the blastocoel should be collapsed artificially before freezing [43] which can be done, for example, with an ICSI pipette. Despite all these difficulties, blastocyst survival rates seem to be higher compared to early cleavage embryos, as shown in a study by Cobo et al., where 6019 embryos were vitrified using Cryotop as a carrier [44]. In the study, 97.6% day 6 embryos survived compared to 95.7% day 5 embryos, 94.9% day 2, and 94.2% day 3 embryos. As a consequence of the freezing procedure, zona pellucida may become thicker, which could affect the implantation ability of the embryo, and this is why assisted hatching is performed with the idea to overcome this problem.

### **3.4 Artificial shrinkage**

In a well expanded blastocyst, the large blastocoel may interfere with the permeation of CPAs during the vitrification procedure which in turn would decrease the survival rates after thawing. Mukaida et al. back in 2003 stated that blastocyst survival rate after vitrification/warming correlate negatively with the expansion of the blastocoel [45]. Artificial shrinkage (AS) of the blastocoel by different methods—laser pulse, microneedle, micropipetting, and 29-gauge needle was developed with the idea of overcoming this obstacle. A study by Gala et al. in 2014 encompassing 185 warming cycles reported a higher survival rate after AS (99.0%) compared to 91.8% survival rate in the control group without AS [46]. Darwish and Magdi in 2016 assessed clinical pregnancy rates, implantation rates, and blastocyst survival rates in more than 400 patients, divided into two groups—untreated expanded blastocysts and blastocysts undergone AS by laser pulse [47]. The study group found that after AS, there was significantly increased survival rates (97.3 vs. 74.9%), implantation (39.1 vs. 24.5%), and clinical pregnancy rates (67.2 vs. 41.1%).

Despite the promising results of AS, this technique is relatively new and not well studied. More studies must be carried out to validate if the procedure is safe and to assess its impact on the developing embryo. Moreover, a high survival rate can be obtained without AS, and therefore, the use of AS is questionable.

## **4. Embryo cryopreservation and ART**

All of the advances that had been made in the last two decades regarding embryo cryopreservation would be of no significance if the success rates after FET were minimal. So what is the place of embryo cryopreservation among other basic ART methods? What are the positives and negatives of having embryos frozen/thawed and transferred instead of having a fresh transfer? We will try to give answers to these very important questions.

### **4.1 Embryo storage**

The main indication of embryo cryopreservation is for storage purposes. We have reviewed the basic cryopreservation methods. Our interpretation of this thought is: however, no matter how much they have improved recently, they could not be successful unless proper storage and thawing of the frozen objects are carried out. After freezing, the embryos are placed in storage tanks which are filled with liquid nitrogen. There is substantial variety of storage tanks and automated storage systems have been recently introduced, which offer optimal storage conditions and safety. It is not known for how long embryos can be stored in liquid nitrogen without affecting their potential, because embryo freezing was developed during the 1980s, which means that the longest time an embryo has been stored is around 35 years, and there is little chance that patients would come back for them after such a long period. There are some differences in the laws regarding embryo cryopreservation, and therefore, embryo storage limit varies between countries, for example, 3 years in Portugal, 5 years in Denmark, Norway, and many other countries, 10 years in Austria and Australia, 55 years in the UK, while in Venezuela, embryo freezing is prohibited [48]. However, a storage time of 5–10 years is most commonly observed.

Keeping embryos in liquid nitrogen raises some concerns about the safety of the procedure. First of all, liquid nitrogen that is used by the IVF laboratories has chemical standards of purity, not biological. That means that there might be some kind of contamination and we should think if there is any way to sterilize this liquid nitrogen. Bielanski et al. describes the potential for viral transmission from experimentally contaminated liquid nitrogen to vitrified embryos, stored in open freezing containers [49]. From a pool of 83 batches, 21% were positive for viral association. In contrast, vitrified embryos in sealed plastic cryovials and straws were free from viral contamination. These data support that sealing of the freezing container might prevent exposure to contaminants; however, that does not mean 100% safety, as the seal can be damaged. This information leads us back to the idea of sterilizing the liquid nitrogen. However, if this is possible, it should be evaluated if it can be applied practically.

Regarding the thawing process, it is very similar in both vitrification and slow freezing technique. The idea is to submerge the frozen object into a solution prewarmed at 37°C which is the core temperature in human body. Closed systems are usually plunged into water baths, while open systems could be put directly into a prewarmed medium. As mentioned before, CPAs are used for the cryopreservation of embryos and those CPAs must be removed during the thawing process and also

the cells must be rehydrated. This happens by incubating the embryo in decreasing concentrations of the CPAs and increasing concentrations of water.

## **4.2 Embryo freezing in clinical practice**

Increasing the reliability of the embryo freezing/thawing procedure has enabled a wider application of the method in assisted reproduction.

Indications to why we freeze embryos and do not switch to fresh ET are: OHSS risk and significant increase in progesterone on the day of HCG administration during stimulation. Progesterone levels above 1.5 ng/ml in patients in advanced reproductive age are associated with lower success rates with fresh ET [50], low responders, inadequacy of the cervix, requiring hysteroscopy, areas affected with Zika virus, or the analysis of endometrial implantation “window.”

When patients have cryopreserved embryos it provides them with additional embryo transfers thus increasing the chances of achieving a pregnancy from a single stimulated cycle. This also means that further cycles of hormonal stimulation are not required. Having supernumerary embryos frozen also facilitates the so-called single embryo transfer which helps in avoiding multiple pregnancies. Additionally, freezing of embryos enables patients to decide when is the most appropriate time to start their conceiving efforts.

For patients suffering from cancer, embryos can be frozen before the patient starts cancer treatment, which is done because chemotherapy may negatively affect one's reproductive ability, and this may be their only option of having offspring.

Freezing embryos also gives the opportunity for genetic testing (PGD/PGS) which is essential in couples with recurrent pregnancy loss and older women who possess higher risk for chromosomal abnormalities.

The frozen embryos may be donated for scientific purposes or they may be used in a donor program if permitted by law, which is also an advantage.

Embryo cryopreservation has many benefits; however, there are some potential risks, and we hope that they will be eliminated in the near future, as this area of research is further developed.

## **5. Present and future perspectives**

Looking at SART statistics, we can find that the number of frozen embryo transfers has increased more than 2.5 times over the 2004/2013 period. Accordingly, for 2004, we have 15,474 frozen embryo transfers vs. 40,015 FETs for 2013, while the number of fresh embryo transfers remains relatively unchanged, respectively, for 2004—2087,089 fresh ET and 87,045 for 2013. On the other hand, there has been a significant increase in the success rate of FET compared to fresh ET. In 2004, the average success rate of FET was 27.8%, while that of fresh ET was at 33%. In 2013, the average success rate of FET was 40.1% and of fresh ET it was 36.3% [51]. Another interesting fact supporting the increased success rate of FET is data from the Japanese National Registry on the number of ART procedures and their success rate based on births. The most significant is in 2014 when the children born after ART were 47,292, with 77.4% of them being after FET [52].

But when discussing frozen embryos and their clinical practice use, the first question arising is the risk to the offspring, when we are applying that technology. The risk for at least one major congenital abnormality of the children born after FET was not increased compared to the children born after fresh ET [53]. On the contrary, the increase in blastogenesis birth defects appears greater for fresh ET than for FET, and the frequency of Down syndrome was statistically more likely

in the children born after fresh ET than FET [54]. On the other hand, FET has advantages in that it decreases the risk of low birth weight (LBW) (<2500 g), very low birth weight (<1500 g), very preterm birth (VPTB) (<32 gw), placenta previa, small, placental abruption, gestational age, antepartum hemorrhage, ectopic pregnancy, and perinatal mortality [55, 56]. If we look at the weight indicator of the newborn after ART, the results of the studies show that ART-born children have a lower weight, and the risk of LBW in newborns is 2.6 higher than those spontaneously conceived [57]. However, after FET, children are being born 90–190 g heavier than those after fresh ET. This brings them statistically closer to the children born after a spontaneous pregnancy [54, 58].

Here a question arises: is the change in the weight of the newborn due to epigenetic reprogramming?

Human studies have shown that different ART methods, such as ovarian stimulation and supraphysiological levels of sex steroid hormones, culture media, and embryo cryopreservation, may be associated with intrauterine growth change, resulting in altered birth weight profiles, which may be caused by epigenetic modifications [59]. Furthermore, some reports indicate that children conceived by ART have an altered lipid profile, fasting glucose, body fat distribution, and cardiovascular function [60, 61]. ICSI/IVF children showed a significantly decreased DNA methylation at birth. Studies suggest an impact of ICSI on the offspring's epigenome(s), which may contribute to phenotypic variation and disease susceptibility in ART children [62].

Differences in DNA methylation between IVF and non-IVF twins on a genome-wide scale and their results show evidence for epigenetic modifications that may in part reflect parental subfertility [63].

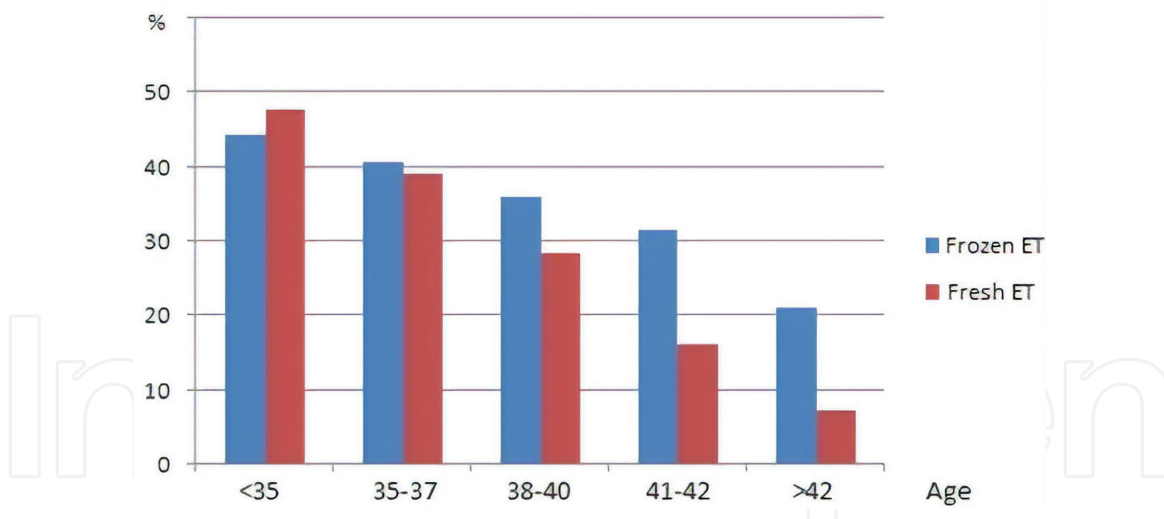
The methylation profiles of ART and IUI newborns were different from those of naturally conceived newborns. But the profiles of ICSI-frozen (FET) and IUI infants were very similar, suggesting that cryopreservation may eliminate some of the epigenetic aberrations induced by IVF or ICSI [64].

In addition to the above mentioned advantages, frozen ET, compared to fresh ET, also has some drawbacks, such as: macrosomia (OR 1.64), large for gestation age (OR 1.54), post-term birth (OR 1.40), and placenta accrete (OR 3.20) [65]. In cases with preeclampsia, the risk after FET in twin pregnancies is 19.6% with risk difference 5.1% in fresh ET, while in singleton pregnancies, the risk is 7.0% after FET with risk difference 1.8% in fresh ET [66]. Probably, the main reason for the increase of these complications is the protocol of the endometrium preparation for FET. Analyzing the Japanese assisted reproductive technology registry in 2014, Saito et al. [67] found that pregnancies following FET after hormone replacement cycle (n = 75,474 cases) have significant higher risks of hypertensive disorders and placenta accrete compared to FET in natural ovulatory cycle (n = 29,760 cases).

Due to the many advantages of FET compared to fresh ET, a number of clinics have begun to implement the “freeze all” policy, so they do not perform ET in the stimulated cycle, but rather freeze all embryos. In a subsequent or later cycle, with or without HRT (hormone replacement therapy), the embryos are thawed, and ET is performed.

The first publications of results show optimism, because the FET success rate is significantly higher than that of the classic ET approach. In support of these results, it was reported by Lopez et al. in a retrospective study with 1697 IVF cycles that the FET versus fresh ET rate for women up to 39 years was 44.5–38% and for women after 39 years, 34.9% and 22.7%, respectively [68].

Similar results were presented from another retrospective study made by Santistevan et al. [69]. It encompassed more than 16,000 IVF cases, with



**Figure 2.**

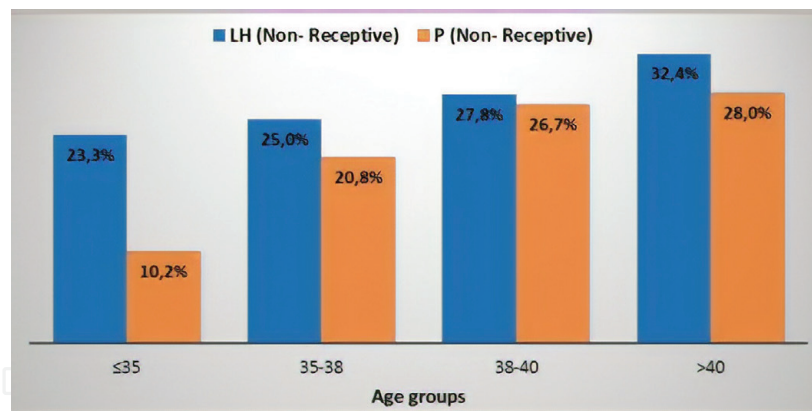
*Age characteristics and fertility in nondonor IVF program after ET of fresh and thawed embryos (SART 2013).*

repeated success rates following FET versus fresh ET in women up to 35 years of age, being 50.240.3%. In women after the age of 35, the ratio was 46–33%, respectively. Another interesting study was published by Zhu et al. [70], which included 20,687 women who started their first IVF cycles using a “freeze-all” strategy. The authors report an average success rate of 50.74% live birth rates, establishing different success rates depending on collected oocytes and the age of the woman.

However, despite these preliminary positive results, it must be emphasized that these are retrospective studies. We cannot find enough proof to convince us to change our treatment strategy toward a “freeze all” policy, as the studies cannot give us an answer when it is better to freeze the embryos and when to apply fresh ET.

For this reason, a number of prospective studies are currently being carried out which are intended to answer those questions. What is the place of “freeze all” policy in treatment of infertility? Some of these studies have already published their results, such as Coates et al., who for the first time compared the success rate of FET to that of fresh ET of euploid embryos. In a study encompassing 179 cases, the authors found a significant increase in the success rate of frozen ET, based on developing pregnancy and delivery [71].

In another prospective study, however, Vuong et al. [72] and Shi et al. [73] did not establish a statistically significant difference between FET and fresh ET. The results in the first study were 36.3 and 34.5% in 782 IVF/ICSI cases of non-PCOS patients, while in the second study, the results were 48.7 and 50.2%, respectively, in 2157 women who were undergoing their first in vitro fertilization cycle. The main conclusions of those articles are that freezing embryos do not decrease pregnancy rates, and the “freeze all” policy only raises expenses. But there were some limitations to those studies. The methodology is based on the embryo cultivation to an early cleavage stage, and if the effect of the freezing procedure is sought, the embryos should be cultured and frozen during the blastocyst phase. In support of that opinion is the analysis of the success rates of 236,191 cases after FET. Holden et al. [74] found that there is a 49% increase of live births after blastocyst-stage FET, compared to cleavage-stage FET. The authors examined only a group of patients up to 35 years of age. However, if we were to analyze the success rate after in vitro procedures in different age groups, we would find that in females more than 37 years of age, the results after frozen transfer are significantly higher than in fresh transfer [51] (**Figure 2**).



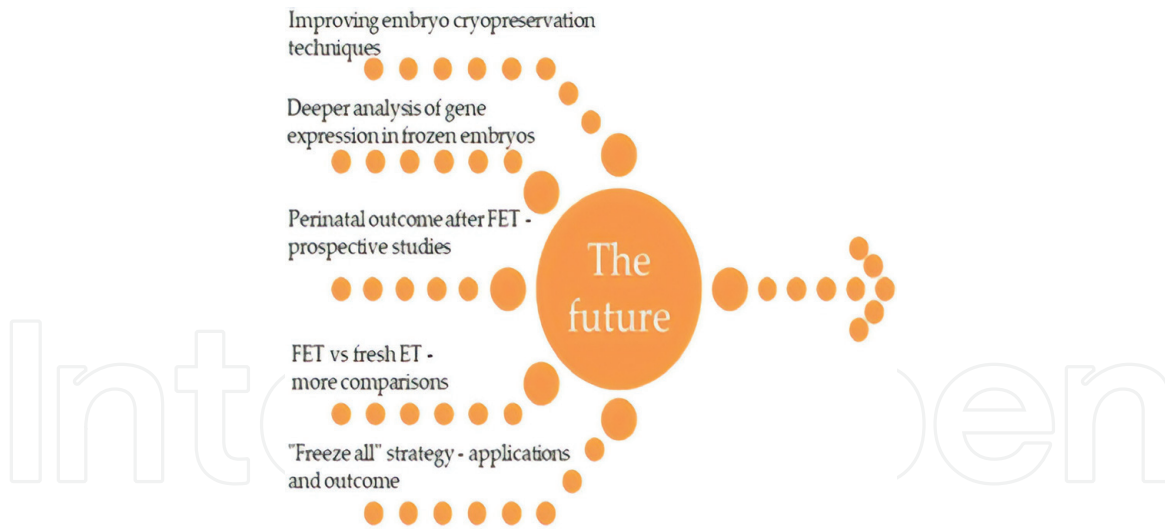
**Figure 3.** Age distribution of cases with endometrial non-receptivity (endometrial receptivity analysis) in LH/HRT protocols.

In spite of controversial results, FET has its own place in treatment of couples with infertility. To date, the main explanation for the high FET success rate is the so-called “Hormonal Theory.” According to it, high levels of estrogen during stimulation have a detrimental effect on embryos and placentation, a negative effect on the preparation of the endometrium for implantation of the embryo. If we accept this hypothesis, we would not be able to explain why women in advanced reproductive age achieve FETs with higher success rates than fresh ET. The results of the studies show that, as women age, there is a higher possibility of a displacement of the implantation “window.” In this situation, it is only logical that the FETs have a lower success rate. Continuing the discussion on this issue, Vladimirov et al. [75] researched the age distribution of cases with a displacement of the implantation “window.” This is a study with 402 women to whom we have applied the endometrial receptivity analysis (ERA) test due to different indications. The results show that, with the increase of the woman’s age, there are increased number of cases of displacement of the implantation “window” with a statistically reliable difference between the groups of up to 35 years and over 42 years (**Figure 3**).

## 6. Conclusion

According to the “Theory about the Embryo-Cryo treatment,” the procedure of freezing/thawing of the embryo probably has a positive “therapeutic” effect on embryos [6]. In fact, results show that FET has a high delivery rate, and the resulting offspring has a better perinatal outcome, compared to children born after fresh ET. The frequency of obstetric complications during pregnancy and children born with congenital abnormalities is lower in FET. However, we still do not have a clear answer to the question, what are the effects of hormonal stimulation and laboratory conditions of cultivation on the normal development and embryo implantation, as well as on pregnancy, birth and development of the individual. These scientific questions and many more still await their answers (**Figure 4**). We believe that applying this freezing approach can reduce the negative effects of the in vitro procedures on embryo development and implantation.

We would like to close this chapter with Mahatma Gandhi’s thought: “A nation’s greatness is measured by how it treats its weakest members.” Our interpretation of this thought is: “The level of human societal development is measured based on how we take care about our unborn children, i.e., the embryos.”



**Figure 4.**

*Possible future directions aiming to improve our understanding of embryo cryopreservation and its place in the field of ART.*

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## Conflict of interest

None.

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