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New Trends in Assisted Reproduction Techniques: Cryopreservation, *In vitro* Fertilization, Intracytoplasmic Sperm Injection and Physiological Intracytoplasmic Sperm Injection

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Abstract

Since that historically the first human birth was registered through *in vitro* fertilization in 1978, the improvement and creation of new assisted reproduction techniques such as intracytoplasmic sperm injection and physiological intracytoplasmic sperm injection have become of great importance nowadays. Louise Brown was the first baby conceived through *in vitro* fertilization in England. Since then, several worldwide studies have reported human and domestic animals outcomes. This fact has solved human fertility problems creating new ways of conceiving. Before the success with *in vitro* fertilization, other assisted reproduction techniques such as cryopreservation were developed. Spermatozoa were the first mammalian cells to be cryopreserved. These events revolutionized all the research in the field of reproductive biology. However, in terms of cryopreservation, intense work needs to be intended since low recovery rates are still reported. The improvement of vitrification-warming procedures is highly important in order to increase embryo development and outcomes. In domestic species, porcine oocytes are the most sensitive cells during vitrification. The future of vitrification success is promising; therefore cryobiologists have a commitment in the field of assisted reproduction. The aim of this review was the acknowledgement of the current status of assisted reproduction techniques, its application, progress and future prospects.

Keywords: Vitrification; Immature oocytes; Porcine; Cryolock; IVF; ICSI; PICSI; Embryo development

Abbreviations

ART: Assisted Reproduction Techniques; CPA: Cryoprotectant Agents; DMSO: Dimethylsulfoxide; EG: Ethylene Glycol; FAO: Food and Agriculture Organization of the United Nations; GV: Germinal Vesicle; HA: Hyaluronic Acid; ICSI: Intracytoplasmic Sperm Injection; IVF: *In vitro* Fertilization; PICSI: Physiological Intracytoplasmic Sperm Injection; PROH: Propanediol; PVP: Polyvinylpirrolydone; PZM5: Porcine Zygote Medium 5; ZP: Zona Pellucida

Introduction

In recent years natural fertilization conditions have declined in some species. In humans, it is known that these difficulties are mainly due to endometriosis. It has been reported worldwide high incidence of polycystic ovarian syndrome, and obstruction of the fallopian tubes, cancer, obesity, azoospermia and oligozoospermia, so the creation of assisted reproduction techniques (ART) has enabled a solution.

In regard to domestic animals, the fertilization rate is generally high. If the male with a normal sperm quality serves a uniparous female during the corresponding oestrus period like bovine, pregnancy rates can reach 60-70%. In the case of multiparous species such as pigs, the pregnancy rate reaches more than 90%. Nevertheless, fertility problems have been reported. The main causes of subfertility are: 1) insufficient sperm quality due to excessive male ejaculations in a short time interval, 2) nutrient deficit, 3) venereal infections, 4) male and female malfunctioning genital system: cystic ovarian follicles, endometritis and anatomic abnormalities [1] and 5) wildlife species conditions: reduced food resources and incestuous mating that homogenize the genome, causing the expression of deleterious alleles [2]. On the other hand, ART have allowed the preservation of endangered species. It is known that its reproduction becomes difficult to achieve due to their dangerous behaviors when collecting biological samples, for example bighorn sheep [3], and the mexican hairless pig [4].

ART refers to all treatments solving natural fertility problems but also for animal production. It is known that at the present time the increase in population and food supply is a worldwide problem. Although, apparently pigs do not have reproductive problems, their overproduction in a small population represents heterozygosity loss, resulting in less genetic variability, inbreeding and extinction. For this reason, the creation of ART has enabled a possible solution.

Among ART, there are four main techniques described: 1) Cryopreservation: slow freezing and vitrification, 2) *In vitro* fertilization (IVF), 3) Intracytoplasmic sperm injection (ICSI) and 4) Physiological intracytoplasmic sperm injection (PICSI).

Cryopreservation

Principles of cryopreservation

Cryobiology, is a term derived from the Greek words cryo= cold, bio= life, logos= science, which studies the effects of low temperatures in the cells and tissues in order to achieve their cryopreservation.

Cryopreservation processes allow maintaining cells, tissues, organs and even organisms generally at low temperatures between -80°C and -196°C, keeping their metabolism completely inactivated. Biochemical reactions are stopped for long periods preserving their development potential and feasibility. Nowadays cryopreservation has been used for gametes conservation, so that both cryobiology and ART have moved in parallel over the last 50 years.

Oocyte cryopreservation is of great importance in various aspects. In humans it can help people undergoing treatments for diseases such as cancer and patients with subfertility problems to achieve pregnancy through the ART. Currently the decision to delay motherhood and the creation of donation banks is something that can be achieved by techniques such as vitrification.

In animal production, vitrification may contribute to the genetic improvement of livestock. At present in production establishments, there has been a decline in genetic diversity of swine and other domestic species, because the commercial demands have caused an uncontrolled overproduction. In addition, cryopreservation is related to the preservation of species in danger of extinction, as regards, the pig is known that some species, especially in European countries are in danger. The Food and Agriculture Organization of the United Nations (FAO) has stated that there is a 20% loss of genetic species of pigs and horses where using vitrification this fact could be solved [5]. Besides, the loss of heterozygosity causes severe problems in species adaptation to the environment from climate changes and disease resistance. The importance of conservation of specific genes is one of the goals of cryopreservation. For example, the gene Booroola FecB fecundity of sheep increases ovulation rate and the number of pups per ewe increasing live offspring [5].

Even that important advance in human gamete cryopreservation have been made, most of oocyte retrieval consists in an *in vivo* matured oocyte or embryos for cryopreservation. But few studies are conducted with immature oocytes or *in vitro* produced embryos for cryopreservation. However, the possibility to cryopreserve gametes is already included in several fertility centers over the world.

Freezing methods (slow freezing and vitrification)

The slow freezing cryopreservation technique is achieved using low cryoprotectant concentrations and the temperature is lowered gradually. The cooling rates are slow producing in some kinds of biological samples ice crystal formation. This technique has been widely used for sperm, mature oocytes and embryos cryopreservation [6-8]. However, slow freezing has several limitations. First of all this procedure is highly expensive because it requires programmable freezing equipment. The whole method is extremely time-consuming, taking long periods to cryopreserve. But the most important finding is that successful outcomes have been overcome by vitrification in humans [9].

The use of cryoprotectant agents

Cryoprotectants (CPA) are substances used to protect the cell against damage that occurs during vitrification and warming, mainly due to the ice crystals formation and inadequate dehydration. CPA alters the physicochemical properties of the solutions; they are water soluble and low toxic molecules, which act by decreasing the maximum temperature. Furthermore, they lower the temperature at which the transition from water to solid state occurs, interacting with water molecules reducing its ability to form bonds between them. Also they act establishing hydrogen bonds with other biological molecules avoiding the fact to lose their original structure and therefore cell viability [10].

CPA are classified according permeability into two groups: permeable CPA (intracellular) and non-permeable (extracellular).

Intracellular CPA are low molecular weight, which allows high diffusion capacity. The commonly employed in cryopreservation of gametes, tissues and other cell types are dimethylsulfoxide (DMSO), glycols such as ethylene glycol (EG) and alcohols such as propanediol (PROH) and glycerol. They act primarily by the following mechanisms: decreasing the freezing point and interacting with the membrane retaining its structure and preventing the increase of high concentrations of electrolytes, since they are capable of binding to them [11]. This fact also allows the osmotic flow of intracellular water to the extracellular place, generating dehydration. The use of high concentrations of CPA at low volumes and setting combinations among these is essential for optimizing cryopreservation processes, specifically during vitrification.

Extracellular CPA is high molecular weight compounds commonly used associated with intracellular CPA. They exert their cell effect promoting dehydration and increasing the osmotic gradient helping incorporation of the permeable agent by the cells, during the vitrification process. The most used are sugars such as sucrose and trehalose, but they have also used other macromolecules such as polyvinylpyrrolidone (PVP), ficoll and the high molecular weight proteins [10]. Although these CPA are employed during the first stage of vitrification, its main effect occurs during the warming process. They act by exerting a high osmotic pressure generated by the output of the intracellular CPA and water re-entry to return the cell to its original state.

Several methods have been used in terms of cryoprotectant exposure. During the "one step" method, cells are directly placed in a hyperosmotic solution with respect to the permeating cryoprotectant. In the removing process, cells with a high intracellular concentration of cryoprotectant are exposed to an isotonic salt solution [12,13]. However, osmotic injuries are not avoided. Many efforts have been made in order to reduce the negative effect of vitrification. Basically, the use of "multi-step" methods instead of the "one-step". In this method, a solution with high CPA concentration is added into a cell suspension step by step and the CPA concentration in the cell increases gradually. However, this method is considered more expensive and difficult practically [14].

Vitrification

Vitrification is the process of converting a liquid state into a vitreous solid state. It has been known an alternative method to slow freezing. This technique uses high cryoprotectant concentrations and rapid cooling rates to convert liquid intracellular water into a vitreous state.

In nature, the lowest temperature recorded was -89.3 in Antarctica, suggesting the relationship between vitrification and life. The first register of vitrification was the idea by Brayley in 1860. He introduced the possibility to vitrify water or any liquid through the use of very high cooling rates. Over history, vitrification is an important method of cryopreservation. From 1937 to 1958, was the first attempt of vitrification of living cells [15]. Luyet stated two intrinsic factors involved in the vitreous state. These include: velocity of crystallization and the size of the zone of crystallization temperatures. Other factor, which is extrinsic, depends on the method employed: the cooling velocity. The main objective for an adequate vitrification process consists of obtaining an enough cooling velocity to prevent ice crystal formation. The way to increase the cooling velocity is by the dehydration of the biological samples prior to cooling. In 1962, all the works performed by Luyet et al. of vitrification ended with unsuccessful results, leading to other cryopreservation methods as freezing. Although, it was not until 1969 that he was able to vitrify human red blood cells [15].

The most important development of vitrification began in 1970. Pierre Boutron introduced the idea that all cells should be protected before vitrification. It was important to consider the terms cooling and warming rates, since the use of CPA is absolutely necessary during vitrification. These CPA should be protective solutions with low toxicity. Boutron proposed three main vitrification problems: 1) low cryoprotectant concentration is necessary to avoid toxicity, 2) high cooling-warming rates are required for complete vitrification and, 3) during warming high concentration is needed but toxic for most cells. He studied the behavior of propylene glycol. However, by this time, there was still no demonstration that nucleated cells could be vitrified and rewarmed successfully [15].

In 1985 [16] the fundamental principles of vitrification and the vitrification of 8-cell mouse embryos were performed, this success remains all the previous works [17], where significant survival of cryopreserved cells became reality only after the discovery and use of permeable cryoprotective agents. Before cryopreservation, these agents should be added to prevent the cells from the cryoinjury.

Since 1985 approximately 2,100 publications can be found with the topic of vitrification underlying that this technique is one of the best developments in ART. Successful pregnancies were reported with 9.4% of implantation rate and 42.9% of deliveries after vitrified-warmed human oocytes [18,19]. In regard to domestic species, bovine oocytes and embryo cleavage stages were vitrified-warmed successfully [20]. In the case of swine, reports in the literature describe attempts to vitrify mature oocytes. The first report was by Nagashima et al. in 1994; they vitrified porcine embryos after depleting cytoplasmic lipid vesicles by centrifugation [21]. The CPA used were EG and sucrose. Only 6% of the oocytes were able to develop to the eight-cell to morula stage. In 2004 [22], it was reported that only 14% of oocytes were able to undergo cleavage after attempted fertilization. Recently other study indicates that vitrified oocytes are able to develop to the blastocyst stage 16% by IVF, increasing the possibility to obtain live offspring [23].

Vitrification devices

Nowadays reports appeared describing the use of different vitrification devices in order to increase cooling-warming rates. In seeking to optimize the cryopreservation procedures some devices have been performed with different freezing speeds and vitrification minimum volume solutions. For example, the Open Pulled Straws, Super Open Pulled Straws [24], solid surfaces [25], Cryoloop, Cryotop, and Cryolock, which among others, runs a minimum volume of solution <0.1 μL, which benefits the vitrification process. In 2005 it was reported the use of the Cryotop device. In this study, the survival rates were high 80-85%, but the development rates were considerably low 1.2-7.4% [26]. In contrast, the Cryolock device is currently used as a tool in ART, which has been designed and developed in order to facilitate the management of the existing techniques. Its use has been especially for the conservation of oocytes and embryos whose characteristics allow adequate insurance and maintenance, as it has a lid that protects the sample from contamination by contact with liquid nitrogen for storage. The survival rates obtained with this device was 96% and maturation up to 49% [27]. The main differences between both devices: Cryotop and Cryolock is that the cooling rates are different. The Cryolock has 23000°C/min and Cryotop 25000°C/min, although it has been indicated that high cooling rates improve vitrification conditions. It is also reported that if the rate is too high >25000°C/min it may cause severe damage to the structure of the cell. Furthermore, the Cryotop can store only one or two oocytes, which represents one of the most expensive devices for experimental purposes instead of the Cryolock allowing the storage of more than eight oocytes.

Impact of the nuclear stage during vitrification

For oocyte cryopreservation, it is known that in several mammalian species such as humans and pigs, oocytes at the germinal vesicle stage (GV) are more sensitive to cell damage compared to those at a mature stage or embryos (Table 1). In swine, this fact has been attributed to their high lipid content, which decreases in the further developmental stages [21]. But also by the structural changes in the membrane of the oocytes during maturation, affecting the lipid-phase transition temperature, which is a determining cryo-tolerance factor [28].

Species	Developmental stage high resistance	Developmental stage less resistance
Bovine	Morulae, early blastocyst and blastocyst	Immature oocytes
Ovine	Morulae, early blastocyst and blastocyst	Immature oocytes
Porcine	Blastocyst	Immature oocytes, matured oocytes, morulae and hatched blastocysts
Equine	Blastocyst	Immature oocytes and hatched blastocysts
Human	Blastocyst	Immature oocytes and matured oocytes

Table 1: Cryopreservation resistance among species and developmental stages.

Immature oocytes have lower permeability to CPA and higher to water. This contributes to their low ability to survive vitrification. Despite this, it has been reported the first generation of live piglets from cryopreserved immature oocytes [29]. Even that mature oocytes show high viability rates and recovery, their developmental competence is decreased. In regarded to embryo vitrification, blastocysts have been successfully cryopreserved with high survival and re-expansion rates [30,31]. However, *in vitro* produced blastocysts

have higher DNA fragmentation and a lower total cell number than *in vivo* blastocysts [32].

An efficient protocol for oocytes vitrification in GV is not been established; improvements to vitrification technique can provide greater opportunities to increase the rate of oocyte retrieval. However, the damage caused by vitrification, remain the main obstacle to the success of the preservation of mammalian oocytes by altering fundamental processes such as fertilization, due to the low recovery of oocytes after cryopreservation [14].

Cryoinjuries

Cryoinjuries mostly depend on cell size, cytoplasm contents, and permeability of the membranes, developmental stage and oocyte or sperm quality. It is known that these characteristics vary between species. The plasma membrane of the oocytes differs from that in the embryo regarding lipid content. This facilitates the permeation of water and CPA promoting dehydration and reducing ice crystal formation. If an adequate cryoprotectant intake does not occur, osmotic injury, and toxic effects can be able to produce alterations in the intracellular organelles, cytoskeleton and DNA [5].

It has been widely reported that DNA is damaged during cryopreservation. The oocytes have also altered distribution of the cortical granules increasing polyspermy and zona pellucida (ZP) hardening. It has also been reported that *in vitro* produced blastocysts present a higher percentage of DNA fragmentation [33], which is related to the lower total cell number. This can be due to higher levels of oxidative stress during vitrification but also during *in vitro* culture. It is known that the response of the cell against oxidative stress can differ depending on stress intensity and length, this response varies from developmental arrest or cell death by apoptosis or necrosis [34]. In some domestic species, including pigs, a higher level of apoptosis has been observed in vitrified blastocysts [35].

In vitro fertilization (IVF)

Nowadays the ART have become more important because of their continued practices in both, human reproduction and animal production. Several IVF unsuccessful attempts have been reported on different species in the 1950s and 1960s. Edwards discovered important principles for human fertilization [36-38]. The most important findings were that spermatozoa had to spend many hours in the female reproductive tract before acquiring the potential to bind to the ZP and reach fertilization. In 1967, Austin and Chang identified the need for sperm capacitation [39,40]. To complete this process, spermatozoa had to be exposed previously to secretions of the female tract and achieve fertilization. These findings allowed future advances in IVF. The most important success was the first human birth registered through IVF in 1978. Interestingly it was not until 2010 that the Nobel Prize in Physiology or Medicine was awarded to Dr. Edwards, for the development of IVF. His achievements have made it possible to treat subfertility, a medical condition afflicting worldwide.

The IVF technique has been achieved with high success rates in different mammalian species. In a porcine study established percentages up to 81% of fertilization efficiency but with a high percentage of polyspermy (42%) [41]. Polyspermy consists in the penetration of more than one sperm into the egg during fertilization and is known that in swine this event naturally occurs. This event is caused by a failure of the cortical reaction, which is a modification of the ZP for blocking polyspermy. Cortical granules release enzymes into the ZP2 and ZP3 protein causing the blocking entry of more than one sperm. These granules are located below the oocyte plasma membrane and have a secretory function. When sperm is in contact with the oocyte membrane, calcium release occurs by generating an intracellular calcium increase that triggers membrane fusion of cortical granules with the oocyte membrane, releasing the contents of the granules into the extracellular space.

As mentioned above, porcine IVF presents several procedure problems such as incomplete maturation of the oocyte and polyspermy. In this way several studies have focused on studying the nuclear and cytoplasmic maturation response during IVF, which can be improved by adjustments during *in vitro* maturation (IVM), these adjustments include: the use of a granulosa cells co-culture system during maturation after the vitrification of immature oocytes [27], cAMP levels during IVM [42] and the use of better culture media such as porcine zygote medium 5 (PZM5) for embryo development [43].

An important IVF success is the birth of *in vitro* produced porcine embryos. Several studies point out that pigs are an appropriate model for creating transgenic animals intended for production of biological products and organs for xenotransplants. Although progress has been made in the IVF field, there are still few reports of pig births [44]. *In vitro* culture during embryo development might increase oxidative stress generating DNA damage. But also this process can alter the distribution of the cortical granules increasing polyspermy. Therefore, porcine IVF faces several procedure problems such as incomplete maturation of the oocyte, polyspermy and deficient *in vitro* culture conditions. However, among the ART, IVF is the most successful because this procedure resembles the natural way to conceive.

Intracytoplasmic Sperm Injection (ICSI) and Physiological Intracytoplasmic Sperm Injection (PICSI)

A high number of studies have been performed in humans and other mammalian species in order to achieve fertilization. Nowadays the most widely used in humans is ICSI. In this technique the sperm is injected directly into the oocyte cytoplasm. The use of this technique goes back to the year of 1962 by the microinjection of a sea-urchin sperm [45]. However, several procedure issues have been reported, this technique bypasses the normal sperm-oocyte recognition and selection during fertilization, leading to sperm decondensation failures [46]. Failure of male pronuclear formation is one of the main reasons for the embryo development after ICSI. Despite this, live births (10%) have been obtained after ICSI. It was also reported the first baby born by ICSI [47]. Since this important fact, several outcome successes have been reported. Up to now, ICSI has been an important find in human assisted reproduction. Its application and results have been very successful compared to other species.

Live births have been obtained in the cow (21%) [48], mouse (8-10%) [49], and sheep (one pregnancy) [50]. However in pigs, the embryonic developmental ability after ICSI is inferior to that resulting from the IVF. In pigs, ICSI is considered to be a useful technique to produce live offspring from non- motile sperm and for preventing polyspermy, which in this species, occurs naturally and during IVF [51].

It has also been reported that lower embryo development rates by ICSI can be mainly due to oocyte activation failures, sex chromosome abnormalities, sperm ejection, and oolema rupture with the introduction to the cell of the polyvinylpirrolydone (PVP) medium,

which is known to be toxic. Therefore, ICSI studies performed in domestic species indicate that by this procedure, fertilization, embryo development and outcome success still remains low up to 6% [23].

Since low success by the ICSI procedure in domestic species has been reported, the new approach to ICSI is including hyaluronic acid (HA) for sperm selection. The PICSI method is based on the ability of sperm to bind to a HA hydrogel, mimicking the natural binding of mature sperm to the oocytes. In nature, sperm encounters HA in the cumulus cells allowing the fertilization process. This is an important event where sperm quality interferes in the subsequent embryo development and implantation. Spermatozoa that is able to bind to HA, display increased viability, cellular maturity [52], unreacted acrosome status and reduced aneuploidy or DNA fragmentation [53]. For this reason, sperm quality plays an important role in determining embryo development.

The PICSI dishes are conventional plastic culture dishes prepared with 3 microdots of HA. The drops need to be re-hydrated by adding 5 µL droplets of culture media such as tissue buffered medium to each microdots during 5 min. Furthermore, a 1-2 µL droplet with treated sperm is loaded in each drop and incubated during 10 min so that sperm could be attached by their head to the HA surface. Sperm are bound by the head to the bottom of the dish with vigorous motility; in contrast, HA-unbound sperm swim free around the droplet.

Sperm selection by HA prior ICSI helps to optimize the procedure highlighting several advantages: 1) In practical terms: HA-bound sperm can be easily recovered by the injection pipette, 2) the culture media in the PICSI dish has no negative effects on the embryo development like the PVP which is known to be toxic, and 3) because of its natural origin, HA can be metabolized by the oocyte.

As well as IVF, PICSI represents a more natural alternative for assisted reproduction, increasing the embryo production rates. Positive trends in fertilization and pregnancy rates by PICSI have been reported [48], demonstrating that the injection of HA-bound sperm improves embryo development.

Conclusions

For reproduction the creation of ART has been important in order to improve fertilization and embryo development. The development of cryopreservation techniques, IVF, ICSI and PICSI has revolutionized the field of human and animal reproduction. Since the first idea of cryopreserved cells at low temperatures, the first baby born by IVF technique, the beginnings of ICSI and its success in 1992 have made further improvements. For example, the PICSI procedure is so far one of the best techniques for *in vitro* porcine embryo development. Although survival and development percentages are still higher by IVF than ICSI or PICSI, this procedure does not exclude polyspermy, which is important to avoid for embryo development.

Among cryopreservation hard work needs to be intended since low recovery rates are still reported. The improvement of vitrification-warming procedures is highly important in order to increase embryo development and outcomes. In regard domestic species, porcine oocytes are the most sensitive cells during vitrification. The future of vitrification success is promising; therefore cryobiologists have a commitment in the field of assisted reproduction. Otherwise, it is fascinating for embryologists and reproductive biologists studying the mechanisms by which *in vivo* fertilization takes place through *in vitro* models. These studies are the starting point for the development of new technologies.

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