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Cryopreservation of female Animal Reproductive organ

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ABSTRACT

Nowadays the strategies have been developed which allow the long-term storage of gametes and embryos. Since mature oocytes are rare and very valuable cells, so the way that they will produce a higher number of oocytes would be useful for the reproductive processes of animals and clinical activities. Low success of the methods such as oocyte cryopreservation led to the focus of research on the freezing of primary follicles containing oocytes in the ovarian cortex. In this article, we describe the ovarian tissue cryopreservation processes.

Keywords: Cryopreservation, Ovary tissue, Oocyte, Veterinary.

INTRODUCTION

Nowadays, researchers follow the proper procedures for storing gametes and embryos of animals. More than two centuries ago, in 1776, Spallanzani was the first to report that human sperm motility was maintained after exposure to low temperatures. one of the important historical events in the cryonics was the discovery of cryoprotective property of glycerol for poultry sperm [1]. In 1958, Sherman and Lin reported that the unfertilized oocytes of mice survived after cryopreservation and thawing again. They used the temperature of -10˚C in a medium containing 5% glycerol; however, none of oocytes live in the temperature of -20˚C. In 1972, Whittingham *et al*., applied mathematical-based methods of the freezing on embryos of mouse, which eventually led to the birth (after the mouse embryo was kept in -196˚C in liquid nitrogen [2]. Freezing of the metaphase II oocytes have had disappointing results because of the difficulties in fertilization and embryo development. Incomplete exocytosis of cortical granules and hardening of zona pellucid, prevent sperm penetration into the oocyte; while there is the possibility of unequal division of chromosomes during the first division of meiosis [3, 4].

These techniques have also other disadvantages, including: disorder in the cell structure during freezing as the result of changes in the organization of molecules and organelles, low success rate of live births from transferred frozen embryos [5], high cost of egg and embryo freezing processes [6], and the low number of antral follicles to obtain oocytes [7]. Low success of the methods mentioned led to the focus of research on the freezing of primary follicles containing oocytes in the ovarian cortex [5, 8]. There are 235000 oocytes in the ovaries of newborn calves and about 130000 oocytes in the ovarian follicles in heifers [9]. The first report on cryopreservation of ovarian tissue was performed in 1950s, during which the mouse frozen ovarian strips were prepared using glycerol as a preservative at a temperature of -79 °C. In that study, the survival rate after freezing of follicle was 5 percent, because the temperature drop was not exactly controlled [5].

The cryopreservation of ovarian and it's replacement, indeed, was strongly supported by showing the possibility of spontaneous pregnancy and birth of live newborn in sheep [10]. The cryopreservation of ovarian tissue in sheep was first published by Gosden in 1994.

First, reports on the possibility of maintaining follicular activity was given for humans [11], and later the live birth by transferring tissue to donor was reported [12-13]. The cryopreservation of ovarian tissue is intended to maintain primary follicles in ovarian cortex. Ovarian tissue cryopreservation and its transplantation again is yet one of the practical methods to preserve the reproductive capability of women and female animals exposed to reproductive damage [14].

• Advantages and disadvantages of cryopreservation of ovarian tissue

Facility of obtaining samples without delay, high number of primary follicles population containing oocytes in each ovary, ability of preserving collected samples without fertilization, restarting of hormonal secretion after transplantation, maintaining and restoring reproductive potential (oocyte of primary follicles, contain small amounts of lipids, and have relatively inactive metabolism. They have no division spindle, zona pellucida and cortical granules, and are highly resistant to freezing) and the ability of obtaining samples from different ages (adult, immature and embryos) are some of advantages of this method. Difficulties of surgery, transplantation of thawed ovary, possibility of transferring malignancy, low success rate, difficulties of performing this process in farms and the presence of differences between species are some of disadvantages of this method [15-20].

• Principles of freezing

Temperatures lower than the freezing point will lead to the formation of ice crystals in the fresh tissues. Since biological membranes prevent the formation of ice nuclei in the intracellular components, ice formation occurs in the extracellular space. To maintaining long-term vitality, living cells must remain suspended for an indefinite time and then return to the normal metabolism. Extracellular ice phase will be increased with prolonged cold, thus the concentration of salts in the extracellular unfrozen part will be increased. In the freezing processes, these changes are reversed with the thermodynamic equilibrium between the intra- and extracellular environments [1]. For long-term preservation of simple or complex cell structures, liquid nitrogen (-196 °C) is used which the intracellular chemical reactions stop at this temperature [5, 18].

The only danger which may occur for frozen cells is DNA damage [21]. Adding cryoprotective compounds to the environment of tissue maintenance, controlling freezing and thawing rates can reduce cellular damage [22]. The greatest risk can occur when temperature decreases to -196˚C or increases to 37˚C. Seeding is essential to reduce the temperature changes at the time that the ice cores are formed. These changes are due to exothermic reactions (resulted by the formation of ice crystals [5].

As there is lower density in ice crystals than water, they occupy a larger volume than water, thus it damages the intracellular organs. At the freezing period, since the salts go out from the frozen part of water, the concentration of electrolytes and other salts can be increased to very high levels, so that it can be toxic to intracellular proteins. Thus avoiding from the formation of ice crystals and the toxic effects of the solution at the freezing period are among the fundamental objectives of a successful cryopreservation [21]. During thawing, the cells are placed in the washing solution, so that cryoprotectants can be washed and removed. It can lead to the conditions opposite to what was at the time of adding this material. The cells are swollen due to the entry of water, and are slowly shrunken as a result of the removal of cryoprotectants. When cryoprotectants and water flow reach equilibrium, cell volume gets its maximum size [23]. When the thawing is rapid, sudden drop in extracellular osmotic pressure may lead to a rapid transfer of free water into the cell, and then can lead to swelling and cell damage; the phenomenon which is called osmotic shock. When the thawin is done slowly, there is the risk of thawed free water and re-crystalization, leading to further damage. Therefore, all methods of freezing have used chemical additives, which are known as cryoprotectants, to prevent cellular damage [21].

• Methods of cryopreservation of ovarian tissue

Important points in tissue freezing include: freezing rate, type and concentration of cryoprotectant, pH of the frozen buffers, inhibitors of apoptosis, thawing rate, storage temperature, and conditions of adding/clearing cryoprotectants [24]. There are three methods of ovarian cryopreservation: slow freezing, glassy freezing (vitrification), and ultra-vitrification. Therefore, it is necessary to set the rate of freezing so that sufficient opportunity is provided for cells and tissues to go out intra cellular water.

Slow freezing:
During freezing, cells are faced with the many physical stresses (such as changes in membrane permeability and cell volume as well as the formation of ice crystals) [19]. In this method, this process can be started with a low concentration of cryoprotectant, and permeable cryoprotectants are used with a concentration of about 1.5 mole (such as ethylene glycol, dimethyl sulfoxide and propanediol) [19, 25]. Cryoprotectants are usually added at room temperature; then, temperature is gradually lowered until -7˚C with a speed of -2°C per minute. During this time, the solution remains liquid. However, there is a possibility of formation of small ice crystals at this temperature.

The solutions usually remain at this temperature about 10-30 minutes so as to be balanced [26]. After this period, the temperature decreases rapidly with a rate of -0.3°C per minute, and reaches to the temperature of -40˚C, because the growth of ice crystals causes a gradual increase in the concentration of salts in the remaining solution and then mechanical damage and osmotic stress. Subsequently, the temperature is lowered to -100˚C, and finally, it is immersed in liquid nitrogen; and the remaining solution is converted into solid and glass states. The whole process takes about 3 hours [21, 27].

The main problem with this approach is the duration of tissue exposure to cryoprotectant, which is a very time consuming. The cooling rate, indeed, should be slow enough to allow sufficient dehydration and prevent intracellular freezing; and in addition, it should be fast enough to avoid the toxic effects of cryoprotectant. Slow freezing and fast thawing provide the least amount of intracellular ice and causes less damage [28].

• Rapid freezing
Alternative method of slow freezing is very fast freezing or vitrification. Penetrating cryoprotectants with high concentrations (1 to 1.6 mole of ethylene glycol or DMSO) is used in this method. Since high concentrations of cryoprotectants have significant toxicity, cells cannot be stored for long periods. The freezing rate is very high (-1500°C per minute), which causes (glass) freezing without formation of ice crystals [29]. On the other hand, the rapid decrease in temperature during the transition period is likely to reduce the thermal stress to the cells, and reduces the amount of damage caused by cooling [30].

Today, new freezing containers are used to allow direct contact between liquid nitrogen and the solution containing the oocytes; and they usually have very high surface to volume ratio. This very fast freezing method should been have also very fast thawing method, so that the formation of ice crystals can be avoided [31, 32]. High concentrations of cryoprotectants such as DMSO and ethylene glycol, have higher levels of toxicity and osmotic damage, which the following ways are used to reduce the damage: a combination of several chemical compounds, the use of less toxic materials, and step-by-step addition of the cells to the pre-cooled solutions [33-35].

Vitrification has two important points: the selection of cryoprotectants, and the appropriate concentration. Vitrification solutions often contain permeable cryoprotectants (such as glycerol, ethylene glycol, 1, 2 propanediol), small disaccharides (such as sucrose, trehalose, glucose) and macromolecules (e.g., propylene glycol, follicle 70, bovine serum albumin) [36].

• Thawing
Conditions of samples thawing are the same significance as the freezing. Inhibition of osmotic swelling during thawing for prevention of irreversible damage and re-formation of the ice crystals are important goals in thawing the samples [26]. Following the movement of water and cryoprotectants from the cell membrane, intracellular ice formation may be increased. Rapid thawing is usually better than the slow thawing. The rate of ice thawing between -196˚c to -10˚c is low; but most of the ice crystals are converted into the water between -10˚C and the thawing point. If the sample is exposed to laboratory condition, the temperature will increased to -50 ˚C in 40 seconds. The washing solutions containing decreasing concentrations of sucrose are used to prevent cell swelling and clearing the cells from cryoprotectants [2].

• Cryoprotectant compositions:
Cryoprotectants are the chemicals that are dissolved in water and lower the freezing point. In all cases they are called “anti-freeze”, except for the freezing of tissues. These substances may be biochemically alcoholic compounds such as propylene glycol. dimethyl sulfoxide (DMSO), glycerol, methanol and ethanol or carbohydrates such as glucose, lactose and sucrose [37].

• Properties of cryoprotectants

Solubility in water, the effect of reducing the freezing point of water, lack of precipitation and lack of hydration are among the properties of a suitable cryoprotectant. All cryoprotectants make hydrogen bonds with water [38].

• Permeating cryoprotectants:

The best protective materials are permeatable cryoprotectants, which are small molecules and pass easily through cell membranes. The molecular weight of most materials is less than 100 Daltons (39). The role of these substances are reducing the formation of ice crystals, reducing dehydration of the cells, and decrease the freezing point of the mixture in low concentrations; but they can prevent the formation of ice crystals in high concentrations. This can lead to glass solid state, a state in which water is solid, but has no increase in volume. The most part of vitrification solutions are these substances that can prevent fully formation of ice crystals. This group of cryoprotectants can also provide second objective of freezing (i.e., preventing the effects of solution), that include ethylene glycol, dimethyl sulfoxide, propylene glycol (propanediol) and glycerol [21, 39].

Table 1: Physical and chemical properties of permeating cryoprotectants

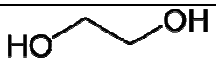
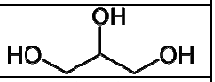
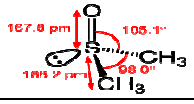
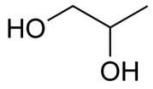
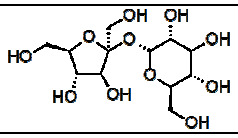
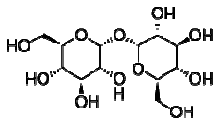
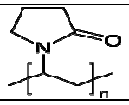
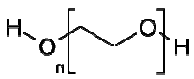
Ethylene glycol		**Glycerol**	
Chemical structure		Chemical structure	
Chemical formula	$C_2H_6O_2$	Chemical formula	$C_3H_8O_3$
Melting temperature	−12.9 °C	Melting temperature	17.8 °C
Molar mass	62.07 g mol^{-1}	Molar mass	92.09 g mol^{-1}
Density	1.1132 g/cm³	Density	1.261 g/cm^3
Dimethyl sulfoxide		**Propylene glycol**	
Chemical structure		Chemical structure	
Chemical formula	C_2H_6OS	Chemical formula	$C_3H_8O_2$
Melting temperature	19 °C	Melting temperature	−59 °C
Molar mass	78.13 g mol^{-1}	Molar mass	76.09 g/mol
Density	1.1004 g cm^{-3}	Density	1.036 g/cm³

Table 2: Physical and chemical properties of non-permeating cryoprotectants

Sucrose		**Trehalose**	
Chemical structure		Chemical structure	
Chemical formula	$C_{12}H_{22}O_{11}$	Chemical formula	$C_{12}H_{22}O_{11}$
Melting temperature	186 °C	Melting temperature	17.8 °C
Molar mass	342.30 g/mol	Molar mass	342.296 g/mol
Density	1.587 g/cm^3	Density	1.58 g/cm^3
Polyvinylpyrrolidone (PVP)		**Propylene glycol (PEG)**	
Chemical structure		Chemical structure	
Chemical formula	$(C_6H_9NO)_n$	Chemical formula	$C_{2n}H_{4n+2}O_{n+1}$
Melting temperature	150 - 180 °C	Melting temperature	----
Molar mass	2.500 - 2.5000.000 g·mol^{-1}	Molar mass	300 g/mol to 10,000,000 g/mol

• Non- permeating cryoprotectants:

They are large molecules that consist mainly of polymers and are added to protective solutions. These substances with the removal of free water inside the cells cause intracellular dehydration. These substances like polyvinyl pyrrolidone and polyethylene glycols, are less toxic than the penetrating cryoprotectants. They can also prevent

damage caused by the cooling due to increase in tonicity of vitrification solutions. There are also other compounds that directly inhibit the production of ice by binding with ice or materials that accelerate the formation of ice (ice nucleators); among this group, the polyglycerol (Z-1000) and polyvinyl alcohol (X-1000) with low molecular weight can be cited [39].

These substances are only used in the vitrification solutions. Non-permeating cryoprotectants play an important role at the time of thawing. During thawing, the water produced by thawing of ice causes a rapid decrease in extracellular osmotic pressure. If intracellular cryoprotectants cannot to prevent the influx of free water quickly enough, osmotic shock, swelling and even rupture of the cell may occur. Thus, the high concentrations of non-permeating cryoprotectants are used in the stage of thawing. Although sucrose is the most common material, other disaccharides may also be used; trehalose is being used for this purpose, recently [21, 40].

CONCLUSION

In recent years, there have been significant advances in the field of cryopreservation and utilization of mammalian ovarian tissue. Ovarian tissue cryopreservation and its transplantation appear to provide effective and reliable methods to preserve the reproductive capability of female animals and laboratory applications.

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