Analytical evaluation of eight-cell cow embryos structure, outwards water and inwards cryo-protectants to ensure successful cryopreservation*

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Abstract

Modern reproductive techniques use cow embryos at different stages of development. Eight-cell embryos feature a layer comprising seven cells (blastomeres), surrounding the inner cell, making a total of eight. This article analyses outwards water paths and inwards cryo-protectants, within the eight-cell cow embryos and effectiveness of their cryopreservation. Reproductive cycles and superovulation in cows were triggered through reproductive hormones injection. After slaughter, oocytes were harvested and eight-cell embryos were produced *in vitro* fertilization and through embryo culture. The study included morphological observations to visualize embryo structure, distribution of blastomeres, cell-layers and analytical evaluation as well, to assess distribution of penetrating cryo-protectants within the inner-cell mass. Findings based on the eight-cell embryo structure suggest that the quantity of outwards water and inwards cryo-protectants is symmetrically distributed. Although, the characteristic of water and cryo-protectants movement is expected to be much better in eight-cell embryos, cryo-protectants properties and the modality of cryo-protectants should differ from procedures applied in other class of embryos. Survival rate of cryopreserved eight cell embryos was lower (6.3%) as compared to the results for 7–8 days embryos. Obviously, a successful cryopreservation of eight-cell cow embryos can only be achieved through a combination of both technical and biological parameters.

Keywords: Water and cryo-protectants; eight-cell embryos and penetrability.

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1 Introduction

Experience in cells and embryo manipulation has shown that successful results might only be achieved, when enough reserve of biological material is available. For instance, preservation of biological material under low temperatures of liquid nitrogen (-196°C) applies different regimens, enabling successful removal of partial water from blastomeres. Biological objects, however, like embryos at different stages of development, react differently, when they are subject to liquid nitrogen preservation techniques. This fact can be attributed to effect of different factors leading to cell destruction: hyper-concentration of salt solutions within the cells, alteration of pH, ionic force produced by the salt solutions, ice formation, excessive dehydration, phasic transformations of bio-polymers and cellular structures (Bishop 1959; Smith 1963; Whittingham 1972 and Willadsen 1977). Moreover, the effectiveness of embryo cryopreservation is also dependent on embryo development stages.

The use of chemical components in cryopreservation procedures in particular, fatty acids serving as protective agents against the effect of low temperatures, has been a common practice. The fatty acids used for embryo cryopreservation comprise penetrating and non-penetrating agents. Penetrating agents such as Dimetil-sulfoxide (DMSO) (Smith 1963) and (Whittingham 1975), Ethylene Glycol defend the biological structures against negative effects of low temperatures from inside the cells. Non-penetrating agents like Propandiol and Polyvinyl-pyrolidone (PVP) are rarely applied due to their inefficiency, although they might be beneficial in protecting eight-cell cow embryos given the membrane properties.

Traditional cryopreservation methods using DMSO at concentration of 1.5 moles established by Wittingham 1972 and Willadesn 1977 are not effective for cryopreservation eight-cell cow embryos, despite better distribution of blastomeres, better removal of partial cellular water and penetration of cryo-protectants in exchange as it is expected according to structural organization of blastomeres. It appears that the membrane structure of this class of embryos is not as easily penetrable as compared to cow embryos membrane structures at subsequent development stages. Consequently, the penetration speed of cryo-protectants in the membrane structure of such embryos will obviously be different. Possibly the penetrability of cryo-protectants in the membrane is related to the stages of embryo development, which increases with embryo's growth. As a result, an analytical study of this class of embryo was carried out to review the structural traits affecting the movement of penetrating cryo-protectants and water removal outwards the cell structure and cell distribution within the eight-cell cow embryos, with parallel cryopreservation using liquid nitrogen, at -196 °C.

2 Methodology

2.1 Superovulation and embryo production

Cows were administered gonadotropins as indicated in the Tables 1 and 2 for triggering synchronization of reproductive cycles and superovulation. Table 1 is with the scheme for stimulation of reproductive cycles using 32mgr of FSH and estradiol, while Table 2 shows the scheme for stimulation of reproductive cycles using 50mgr of FSH and estradiol.

Table 1: Cow scheme and heifers processing using 32 Mgr of FSH and estradiol.

Day	Hormonal administration of 32mgr of FSH and LH‡		
	Morning	Afternoon	Type
1	FSH	FSH	FSH
2	FSH	FSH	FSH
3	FSH + Estradiol	FSH + Estradiol	FSH+LH
4	Administration of Estradiol	Administration of Estradiol and disinfection of uterine cavity	
Evaluation of females response to hormonal administration			

‡FSH=follicle stimulating hormone; LH=luteinazing hormone.

Table 2: Cow processing scheme using 50 Mgr of FSH and Estradiol.

Day	Hormonal administration of 50mgr of FSH and LH‡		
	Morning	Afternoon	Type
1	FSH	FSH	FSH
2	FSH	FSH	FSH
3	FSH + Estradiol	FSH + Estradiol	FSH+LH
4	Estradiol	Estradiol and disinfection of uterine cavity	
Evaluation of female response to hormonal administration compounds			-

‡FSH=follicle stimulating hormone; LH=luteinazing hormone.

Females were slaughtered at abattoir, total hysterectomy was performed, a complete reproductive tract including ovaries were preserved in a solution of warm distilled water mixed with antibiotic (streptomycin) to ensure aseptic environment and kept in thermos maintaining 38 °C, to keeping physiological conditions and was taken to laboratory. Figure 1 represents the hysterectomized cow reproductive tract (see the ovaries and follicles) with different sizes resulting from administration of hormonal reproductive compounds.

Ovaries were punctured and oocytes released up and collected into a Petri dish. Oocytes were double washed with TCM-199 culture medium, and then transferred into a four-well BD

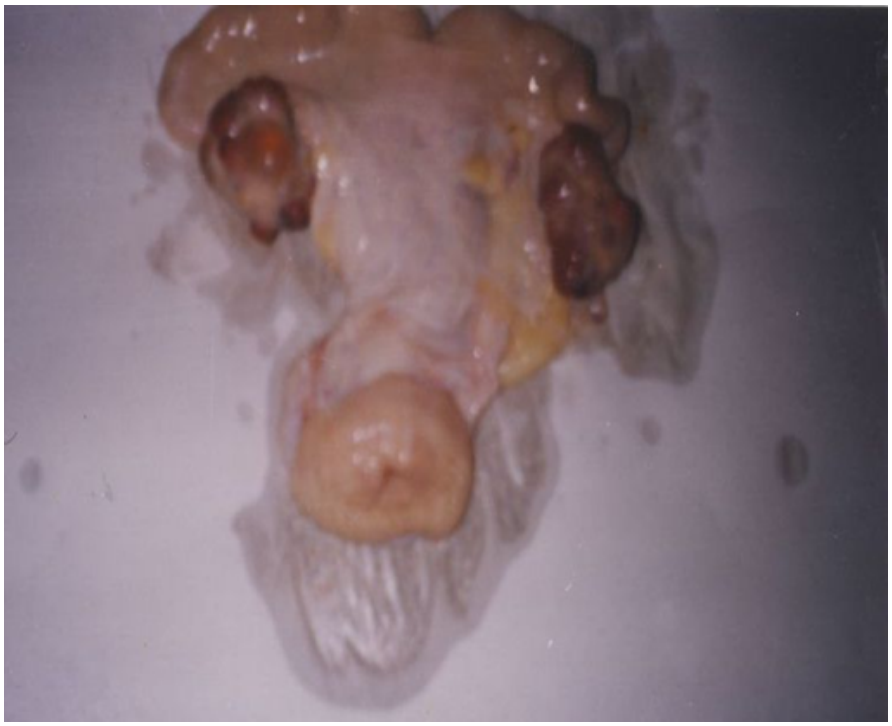

Figure 1: Hysterectomized cow reproductive tract (Mausse 1997).

Flacon, followed by insemination using 50 μl of semen from the Lebediskii breed at 80 million of sperm concentration. Three oocytes were placed into each semen droplet under mineral oil, with subsequent placement into CO2 chamber for in vitro fertilization. The fertilization rate was evaluated 24 hours after insemination of oocytes and the fertilized eggs were rewashed using the TCM-199 culture medium. New TCM-199 medium was used for embryo culture after each 24 hours. Three to four days after insemination, 52 embryos at eight-cell stage of development were harvested and cryopreserved in liquid nitrogen using 1.5M DMSO, at cooling rate of 3° C/min. Four days after cryopreservation, embryos were thawed and brought into ambient temperature followed by evaluation of survival rate through microscopic observation and embryo culture. The average embryo survival rate was too low (6. 3%) as compared to standard survival rates for cow embryos at blastocyst stage of development cryopreserved using the same procedures.

These results led to conduction of subsequent studies to identify the reasons for such failure in terms of dynamics of water and cryo-protectants movement outwards and inwards eight-cell embryos respectively. Morphological observations have enabled a detailed visualization of eight-cell cow embryos, the distribution of blastomeres and respective cell-layer, in one hand. On the other hand, based on structural distribution of blastomeres, an analytical evaluation of movement of water and cryo-protectants was carried out, using Glycerol (1.5 mol). Microscope (KB-45) was used to analyse paths followed by the penetrating cryo-protectants inwards the embryos, in exchange of the outwards water from the inner-cell mass.

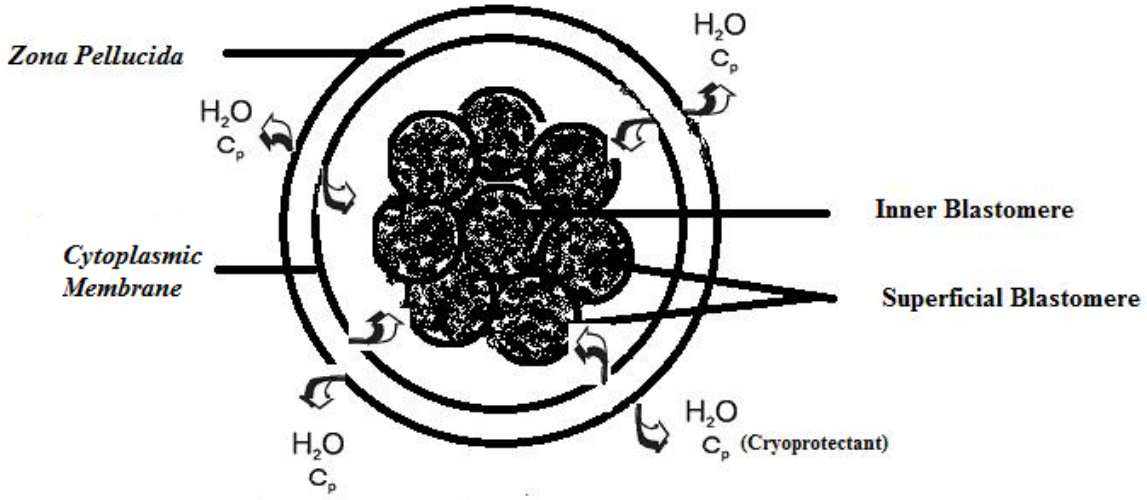

Figure 2: Eight-cell cow embryo schematic layout. C_p:cryo-protectant, H_2O: water.

During observations of water and cryo-protectant transport, it was clear that eight-cell cow embryos are made of blastomeres distributed in a single layer, surrounding one in the middle, a fact favouring symmetric flow of both liquids within the embryonic environment. For better understanding of eight-cell embryo structure and conceptualization of water and cryo-protectant flow inside and outside the said embryo, see the schematic illustration in Figure 2.

Figure 2 illustrates all blastomeres distributed at the same level and the exchange and transport of cryo-protectants inwards and outwards the eight-cell cow embryo. In other words, based on the illustrated eight-cell embryo structure, the exchange and transport of substances is expected to occur symmetrically in all blastomeres. Therefore, the amount of outwards water from the eight-cell cow embryo is expected to be equal to inwards cryo-protectants, a fact which could enable better protection of this class of embryos and ensure high survival rates after their cryopreservation, which is not the case in this class of embryos.

Findings of this analytical study can be supported by Wilmut and Rowson et al. (1973); Pollard and Leibo (1994) and Polge (1994) through independent studies. These researchers have established that the survival rate of eight-cell cow embryos after cryopreservation is not commensurate. This fact is likely associated with low penetrability of cryo-protectants in the eight-cell cow embryo membrane structure leading to lesser application of this class of embryos in cryopreservation activities using traditional method. The cryopreservation techniques were developed taking into account the embryo membrane cryo-protectants penetrability, water release as well as cryo-protectants movement inwards embryos at compact morulae and early blastocysts stages excluding the specificity of eight-cell cow embryos. The reason for such exclusion might have been that the eight-cell cow embryos were not in use in reproductive biotechnology, when the traditional methods for cryopreservation of cells and embryos

were formulated.

Earlier studies by Gardienko et al. (1974) and Ostashko et al. (1982) have established that the penetrability of embryonic membranes during embryogenesis increases in each successive cell division. General perception on these findings make one understand that when divisions take place within an embryo, its membrane structure becomes more penetrable for water and other substances according to earlier studies by Ivanov (1976). Further studies by Bezugly (1984a,b) underscore that in most cases, the division of blastomeres occurs in synchronized way and thus the task on calculating the number of blastomeres can be solved through the following formula:

$$N_k = 2^k$$

where N_k is the number of total blastomeres; k- the numbers of cellular divisions and 2- the total number of blastomeres resulted from each division. With an increase of the number of blastomeres as result of subsequent embryonic divisions, embryo gets another structure with more organized distribution of cells into different layers. The number of cells at j-layer can be defined through:

$$M_j \approx \frac{8\pi}{\sqrt{3}}(j-1)^2 \approx 14^{(j-1)} \quad \text{(Bezugly 1984a)}$$

and the total number of embryonic cells can be determined through the following formula:

$$N_k = \frac{7}{3} j_{max}(j-1)2(j_{max}-1)+1 \quad \text{(Bezugly 1984a, b)}$$

where j stands for the total number of blastomeres in a given layer J, while j_{max} is the total number of layers with blastomeres.

According to these calculations embryos up to 8-cell stage of development represent a structure of isolated cells. After the fourth divisions (16 cells) they get two layers and at later stage of development they get high number of layers. The analysed concept on the dynamic of water and substance as well as the respective formulae, applies for embryos at different stages of development composed of several layers. In this class of embryos, the movement of water and penetration of cryo-protectant is different from that observed in eight-cell embryos and will constantly change as the number of blastomeres increase grow and consequently, the time needed for partial water removal will be much longer.

3 Discussion and Conclusions

The use of traditional methods for cryopreservation of cells and embryos is not successfully applicable for cryopreservation of eight-cell cow embryos. Structural distribution of blastomeres

within the eight-cell embryos enables symmetric distribution and favourable movement of water and cryo-protectants in this class of embryos. Based on the previous scientific findings, it can be suggested that the eight-cell stage cow embryos divisions have not yet reached such a level, enabling cryo-protectants penetration through the embryonic membranes. The findings in Gardienko et al. (1974) and Ostashko et al. (1982) are very supportive to the outcomes from the present analytical study, revealing a weak penetration of cryo-protectants through eight-cell embryonic membranes. Possibly, failure to preserve this class of embryos under low temperature of liquid nitrogen using traditional methods can be attributed to the characteristics of this specific stage of embryo development.

Still based on the concept given by Gardienko et al. (1974) and Ostasko et al. (1982), it becomes obvious that although the characteristic of water and cryo-protectants movement is expected to be much better in eight-cell embryos, cryo-protectants properties and the modality of imbibing this class of embryos with cryo-protectants should be changed. It is suggested that attempts to cryopreserve this class of embryos should be more focussed on cryo-protectants penetration ability through the eight-cell cow embryonic membranes, enabling cell protection from inside the embryo. Alternatively, other studies should be carried out on the use of non-penetrating cryo-protectants given their properties as compared to cryo-protectants applied for cryopreservation of embryos at other stages of development, example morulae and blastocysts. Findings from this analytical study suggest more innovative trials towards successful cryopreservation of eight-cell cow embryos, through a combination of both technical and biological parameters.

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