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**The Role of Membranes Thermobehavior in Chilling
Injury of Bovine Oocyte as an Important Step toward
Cryobanking of Female Genome**

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DCI, LPT, FTIR, GV, MII, LC, PUFA, PC, DPPC, Tm, BHT, RBC

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The Role Of Membranes Thermobehavior In Chilling Injury Of Bovine Oocytes As An Important Step Toward Cryobanking Of Female Genome

Amir Arav and John Crowe

October 2001

ABSTRACT

Objectives: Elucidating the precise events involved in direct chilling injury (DCI) is essential for development of successful procedures for cryopreservation of oocytes. The objective of this proposal was to study the mechanism of DCI in bovine oolema and to prevent the injury by using membrane stabilization factors. This was achieved by exposing bovine oocytes at different stages of development to various treatments and cooling procedures and then comparing the effect of membrane phase transitions on their subsequent DCI. Elucidation of the precise mechanism and kinetics of lipid phase transitions (LPT) and evaluating different strategies to prevent chilling injury will facilitate development of efficient cryopreservation procedures for the short and long term storage of bovine oocytes.

Background: Successful cryopreservation of semen has contributed tremendously to genetic breeding, reproduction technology and biotechnology. Similarly, female genome cryobanking will advance these fields. However, despite two decades of intensive research, successful freezing of the eggs of domesticated animal and human eggs has been limited. Upon exposure to low (above freezing) temperatures, mammalian oocytes and early stage embryos of different animals (bovine, ovine, swine) exhibit a substantial DCI which defined as irreversible damage that occurs to cells during cooling to low, but non-freezing, temperatures.

Major conclusions, solutions, achievements: We evaluated the phase transition temperature in oocytes by using FTIR microscopy, and we found that the phase transition of the membrane lipids of oocytes at the immature stage occurred between 20°C and 12°C, while very broad phase transition, which centered around 10°C, was observed for the mature stage. We also found that holding immature oocytes at the phase transition temperature is more damaging to their membranes than exposure to lower temperatures. In contrast, no significant differences in membrane integrity were observed when oocytes matured in vitro were cooled to the same temperatures. The high rate of polyspermy indicates that a site(s) other than the plasma membrane is affected during cooling of bovine oocytes. These data confirm different authors' observations that mature bovine oocytes are more adequate for cryopreservation than immature oocytes (reported in Arav 1996). We evaluated the effect of the antioxidant BHT on immature oocytes during cooling and found that BHT has a membrane protective property (Zeron et al., 1996). In addition, when oocytes are electrofused with liposomes (Zeron et al., submitted), they become less susceptible to chilling. These observations suggest that an alteration in the lipid composition changes the sensitivity of the oocyte membranes to chilling.

Implications: Scientific- we found that chilling injury is associated with lipid phase transition and it occurs between 12 to 20°C. The LPT is related to the membrane lipid composition. We found that the chilling injury itself is attributed to the transition, and the damage is maximized at LPT temperature. This suggests that chilling injury is a dynamic process which could be avoided by several strategies, including rapid cooling, modification of the LPT by using additives such as BHT, or electrofusion with liposomes or nutritional additives. In agriculture- creation of stable cryobanks of female genome will improve biotechnology and genetic breeding programs. In the following areas of animal reproduction it will facilitate the in-vitro production of embryos, production of transgenic animals, transportation (export/import) of gametes and cryostorage of the germ-cells of domesticated and non-domesticated animals, including endangered species.

Achievements

Following we summarize the main scientific achievement of the present study:

1. Chilling injury is associated with lipid phase transition and occurs at temperatures between 20 and 12°C; (Arav 1996, Pearl 2000).
2. Chilling injury is maximized at the center LPT temperatures (16°C); (Zeron 1999).
3. Chilling injury is related to the lipid composition of the membranes; (Arav 2000)
4. Rapid cooling to temperatures below LPT will delay chilling damages (Zeron 1999).
5. Vitrification with very rapid cooling rate using a new device (Vitmaster, IMT, Israel) allows oocyte survival (Arav 2001 in press).
6. Addition of antioxidants to oocytes improve chilling resistance.
7. Changing the lipid phase transition by electroporation of liposomes with oocytes improve their chilling resistance. (Zeron submitted)
8. Changing LPT by nutritional additives improved their chilling resistance (Zeron , in press)

Lipid phase transitions in bovine oocytes

(1) Assignment of the Phase Transition to Membranes. In the initial studies, we have used Fourier transform infrared (FTIR) microscopy, to study these phase transitions in oocytes. The transition is detected by following the change in vibrational frequency of the CH₂ band with temperature. This band shows a shift of about four wavenumbers when the lipid undergoes a phase transition (from 2853cm⁻¹ to 2849 cm⁻¹). With an FTIR microscope, the method is sufficiently sensitive to permit recording the transition in a single oocyte. Since any hydrocarbon will absorb in the CH₂ stretching region, it is necessary to establish that the signal is from the membrane, and we have taken care to show that this is the case. For example, it is possible to exclude the other major hydrocarbon-containing material, lipid droplets, from the beam by centrifuging the cell. Using this procedure, we found a phase transition in bovine oocytes at the GV stage between 20 and 12°C, with T_m at 16°C, during which chilling injury is maximal (Arav et al., 1996, 2000). After in vitro fertilization bovine embryos and early developmental stages show the same T_m and chilling sensitivity as oocytes at the GV stage. However, with in vitro fertilized embryos T_m shifts to 8°C, and the embryos become cold resistant. We confirmed with other physical techniques that this phase transition is due to a lipid phase transition (Tsvetkova et al., 1999; Crowe et al., 1999). Interestingly, we found that other cells from cattle, notably blood platelets, resist chilling damage and have a low phase transition (Tablin et al., submitted), in sharp contrast with the well known chilling sensitivity of human blood platelets, which have a phase transition similar to that seen in bovine oocytes (Tablin et al., 1996; Crowe et al., 1999).

(2) Lipid Composition is consistent with the observed phase transitions. Lipid profiles of bovine oocytes at the GV stage revealed a low ratio of polyunsaturated fatty acids (PUFA) to non-PUFA in their membranes (0.2:1). However, we found a three fold higher concentration of PUFA in the MII stage, when T_m is depressed (Arav 2000, Zeron 2001). We confirmed by theoretical analysis of the known transition temperatures for this lipid composition that the phase transition should be found where we observed it experimentally (Crowe et al., 1999). We have extended these findings to other species and found a correlation between PUFA ratio and cold sensitivity in bovine and Zebrafish oocytes, in ram, bee, and fowl sperm (Arav 2000).

(3) Rapid cooling through the phase transition. Rapid cooling ($>1000^{\circ}\text{C}/\text{min}$) of GV oocytes to below the phase transition delays chilling damage, but damage nevertheless accumulates rapidly during the cold storage, in the sense that recovery following warming is severely depressed. In separate studies on human platelets we have found that storage below T_m leads to aggregation of proteins in the membrane and lateral phase separation of membrane lipids (Tablin et al., 1999). Thus, we suggest that a similar phenomenon may be found in bovine oocytes.

(4) Vitrification: Vitrification of bovine oocytes at the MII stage, using a new device that permits cooling at a rate of $100,000^{\circ}\text{C}/\text{min}$, showed a cleavage rate after in vitro fertilization of 50% (Arav et al IETS 1996). In subsequent experiments we found that centrifuging the oocytes, which drives the lipid bodies to one side of the cell, improves the cleavage rate, significantly, and 25% reached the blastocyst stage.

(5) Changing the phase transition with additives. The anti-oxidant, butylated hydroxytoluene (BHT), is reported to stabilize sperm membranes and reduce DCI. We have evaluated the effect of BHT on the survival of bovine immature oocytes exposed to low temperatures (4°C). Several variables were examined: cooling rate (rapid, $>100^{\circ}\text{C}/\text{min}$ vs. slow, $<1^{\circ}\text{C}/\text{min}$) and exposure time (0, 4 and 18h). Survival was assessed by continued meiotic development and fertilization shows that both BHT and slow cooling is necessary to avoid chilling injury to bovine oocytes.

(6) Changing the phase transition by electrofusion of sensitive and resistant cells. Since immature oocytes and mature oocytes differ in their chilling sensitivity, we used this phenomenon to explore whether the phase transition can be altered by changing composition. When plasma membranes from mature oocytes were electrofused with intact immature oocytes, very promising results were obtained; 90% of the electrofused immature/mature oocytes ($n=20$) had intact membranes after cooling to 4°C (Arav et al., 1996). These results indicate that chilling tolerance of electrofused oocytes is similar to that shown by the in vitro matured oocytes. We also obtained a higher chilling resistance of electrofused oocytes when the immature oocytes were fused with enucleated matured oocytes ($n=20$).

(7) Changing the phase transition by electrofusion of sensitive cells with liposomes. In view of the above results, we prepared liposomes of defined composition and studied effects of electrofusion of such liposomes with chilling sensitive oocytes. Electrofusion of liposomes prepared from egg phosphatidylcholine (PC) ($T_m = \text{ca. } 0^{\circ}\text{C}$) or with dipalmitoyl-PC ($T_m = 41^{\circ}\text{C}$) respectively decreased and increased T_m of bovine GV stage oocytes. Chilling sensitivity of the oocytes treated with egg PC was reduced drastically, while chilling sensitivity of those treated with DPPC was increased (Zeron submitted). The studies with DPPC require a little more explanation. When the oocytes were fused with this lipid, they showed extreme damage when they were stored at room temperature, but less damage when they were chilled. The reason this is so goes as follows. T_m in these cells is around room temperature (T_m in the untreated cells is 16°C , and T_m of DPPC is 41°C ; the mixture gives a T_m around 25°C). Thus, the seeming paradox is easily explained. At 4°C , the cells are well below T_m , but at room temperature they are in the middle of T_m thus are damaged. This very promising procedure, which has already led to $> 75\%$ recovery of the embryos to blastocyst stage (compared with the unchilled control) of the oocytes treated with egg PC.

(8) Changing the phase transition by nutritional additives: we tried to change the lipids composition in the oocytes by nutrition. We have shown that supplementing 4% of rumen protected fish oil (Ca-fish oil) to the commercial diet did not effect the growth rates of the experimental ewe. However, the Ca- fish-oil diet alter significantly the chilling sensitivity of the oocytes. We found that ewe oocytes are also chilling sensitive in a similar way to cow oocytes. In addition, we found that ewe oocytes have a similar LPT to bovine oocytes which is centered at 14°C . Chilling these oocytes for 15 min, resulted in a decrease of membrane integrity by 35%. However, changes of their phospholipid fatty acids composition by supplementation of Ca-fish oil to the commercial diet affected the chilling sensitivity and resulted in decreasing of 10%.

These differences might be due to the alterations in the proportions of long chain polyunsaturated fatty acids (LC-PUFA) in the membrane. Lipid composition of immature oocytes in bovine (Arav 2000) shows a small amount of LC-PUFA in their membranes. In the present work we have shown that despite increasing concentration of LC-PUFA in the brain, RBC and cumulus cells after supplementation of Ca-fish oil in the diet, the oleic fatty acid (18:1) was the only one which increased after the diet. It was shown in our previous paper (Arav 2000) that a high concentration of Oleic fatty acid in bee's sperm was attributed to their chilling resistance. We propose that the addition of one double bond in the center of the chain, will increase their membranes fluidity and low temperature resistance of membranes.

Potential for application:

Immediate: In order to evaluate the temperatures in which bovine oocytes, sperm or embryos are sensitive we can measure lipid phase transition by microscopic FTIR. In addition the kinetic by which the damages occur should be evaluated by exposure of the cells to the LPT temperature for various period of time.

Medium term: In order to reduce chilling injury in bovine gametes the different strategies which were described above: 1. cooling very rapid through the LPT temperature using vitrification and the VitMaster apparatus. 2. Using different additives such as BHT or trehalose to stabilize membranes during chilling. 3. Changing lipid composition of membranes by electroporation specific liposomes or by nutritions.

Economic benefits:

In Agriculture oocytes cryo-banking will have tremendous effect on genetic breeding and the in vitro production of embryos industry. In Israel, there are over 100,000 cows with a conception rate dropping to 10% in the summer (compared to 50% in the winter), if oocytes cryopreservation will be possible, than oocytes will be cryopreserved in the winter and embryos will be produced and transfer in the summer. Increasing the summer conception rate from 10% to 50% (increasing of 40%) could save 40 million dollars (40,000 cows * 4000 liter milk/4 month * ¼ USA \$). In biotechnology, cryo-banking will facilitate the availability of oocytes and somatic cells for nuclear transfer and transgenesis. Because of the low efficiency in these techniques the cost of cloned or transgenic animal is estimated to be over \$100-500K. We estimate that the possibility of using cryopreserved oocytes and somatic cells will cut the cost of these procedures by one order of magnitude.

Cooperation:

The cooperation between UC Davis and the Volcani Center was as follows:

Assessment of oocytes LPT was done in both laboratories.

Chilling kinetics was performed in Israel with the supervision of Prof. Crowe from UC Davis.

Liposomes were prepared in Davis and sent to Israel for testing.

Students exchanges were done between the centers (Y. Zeron was in Davis and M. Tomchack was in Israel)

Analysis of results and preparation of publications were done in full cooperation.

List of publications:

- ✓ 1. Arav, A., Zeron, Y., Leslie, S. B., Behboodi, E., Anderson, G.B., and Crowe J. H. (1996) Phase transition temperature and chilling sensitivity of bovine oocytes. *Cryobiology* 33, 589-599
- ✓ 2. Zeron, Y., Pearl, M., Borochoy, A., and Arav, A. (1999) Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. *Cryobiology*, 38, 35-42
- ✓ 3. Pearl, M. and Arav, Amir. (2000). Chilling sensitivity in zebrafish (*Brachydanio Rerio*) oocytes is related to lipid phase transition. *Cryoletters* 21, 171-178
- ✓ 4. Arav, A., Pearl, M., Zeron, Y. (2000) Does lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes? *CryoLetters* 21, 179-186
- ✓ 5. Zeron, Y., Ocheretny A., Kedar, O., Borochoy A., Sklan D., Arav, A. (2001) Seasonal changes in bovine fertility: relation to oocyte and granulosa membrane properties follicular fluid. *Reproduction* 121, 447-454
- NA ✓ 6. Zeron Y., Sklan D., Arav, A. (2001) Dietary supplementation with long chain polyunsaturated fatty acid improves follicle quality in the ewe and semen parameters in the bull. *Mol. Reprod. Develop.* (In Press)
- ✓ 7. Zeron Y. Tomczak M., Crowe J., Sklan D. and Arav A. (2001) Changes in oocyte membrane fatty acid composition by either nutrition or fusion with liposomes affects biophysical parameters and chilling sensitivity. *Cryobiology* (In press)
8. Tsvetkova, N. M., N. J. Walker, A.E. Oliver, L. M. Crowe, J.H. Crowe, and F. Tablin. 1999. Lipid composition and thermal phase transition of human platelet plasma and dense tubular system membranes separated by immunoaffinity. *Mol. Mem. Biol.* 16: 265-27
9. Hinch, D.K., A. E. Oliver, and J. H. Crowe. 1999. Lipid composition determines the effects of arbutin on the stability of membranes. *Biophys. J.* 77: 2024-2034.
10. Crowe, J.H., F. Tablin, N. Tsvetkova, A.E. Oliver, N. Walker, and L.M. Crowe. 1999. Are lipid phase transitions responsible for chilling damage in human platelets? *Cryobiology* 38: 180-191.
11. Crowe, J. H. and L.M. Crowe. 2000. Preservation of mammalian cells-learning nature's tricks. *Nature Biotechnology* 18:145-146.
12. Tablin, F., Walker, N.J., Klein, S.D., Field, C.L. and J.H. Crowe. 2000. Animal models for studies on cold-induced platelet activation in human beings. *J. Laboratory and Clinical Medicine* 135:339-46.
13. Martin, M.C., N.M. Tsvetkova, J.H. Crowe, and W.R. McKinney. 2001. Negligible heating from synchrotron infrared beam. *Applied Spectroscopy* 55: 111-113.
14. Oliver, A.E, D. K. Hinch, N. M. Tsvetkova, L. Vigh, and J.H. Crowe. 2001. The effect of arbutin on membrane integrity during drying is mediated by stabilization of the lamellar phase in the presence of nonbilayer-forming lipids. *Chem. Phys. Lipids* 111: 37-57.
15. L.M. Hays, J.H. Crowe, W. Wolters, and S. Rudenko. 2001. Factors affecting leakage of trapped solutes from phospholipid vesicles during thermotropic phase transitions. *Cryobiology* 42: 88-102.
16. Wolters, W.F., L.M. Crowe, N. M. Tsvetkova, F. Tablin, and J.H. Crowe. 2001. In situ assessment of erythrocyte membrane properties during cold storage. *Mol. Mem. Biol.* (in press).