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RESEARCH ARTICLE

VITRIFICATION OF SHEEP OOCYTES USING PROPYLENE GLYCOL AND ITS EFFECTS ON THE VIABILITY AND FERTILIZABILITY AND CHROMOSOME PATTERNS

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ABSTRACT

Analysis of the effect of vitrification using propylene glycol on the post-thaw survival, fertilizability and chromosomal arrangement was conducted. The survival rate and the fertilization rate were assessed in frozen-thawed sheep oocytes after various storage durations employing propylene glycol as the cryoprotectant. The frequencies of chromosomal aberrations in the fertilized embryos were also assessed at various storage durations. Sheep oocytes collected from the local abattoir were vitrified in propylene glycol (5M PROH+1.5M Sucrose) using the standard technique. The oocytes were frozen for the following storage durations 24 hours, 1week and 2 weeks and were thawed and studied for their viability and fertilizability. After fertilization the intact embryos were transferred to fresh medium containing colcemid and were fixed after 48 hours. The embryos were then studied for chromosome abnormalities using Tarkowsky's drying technique. From the results it was evident that vitrification affects the viability, fertilizability and chromosome structure of the oocytes, when compared with the control group. Furthermore increasing the storage duration reduces the viability and increases the chromosome aberrations in the cells.

INTRODUCTION

When conventional therapies fail assisted reproductive technology (ART) comes into view. Assisted reproductive technology has played a major role in assisting individuals with infertility issues. Though this is the case, the assisted reproduction using freshly collected sperm and ova has shown a higher rate of success than that of the cryopreserved gametes. Thus there is a necessity to investigate this in organisms of economic interest. Various studies have been conducted on various organisms like mice, cattle, rabbits and even human beings. Though some success has been achieved in freezing oocytes from cattle by adapting embryo cryopreservation protocols (Aman and Parks .1994; Agca *et al.,* 1998; Asada and Fukur .2000; Baka *et al.,* 1995 and Cooper *et al.,* 1996), the effectiveness of existing procedures based on viable embryos per oocyte frozen remains low compared to use of fresh oocytes in cattle and other domestic animal species, as well as humans. The various steps required for cryopreservation i.e. cryoprotective agent (CPA) loading, cooling below 0°C, seeding, cooling to a low subzero temperature, freezing/storage, thawing, and CPA removal, may contribute individually or cumulatively to oocyte damage that in turn decreases fertilization and development rates (Cohen 1992). Alterations in the cytological components of mammalian oocytes due to procedures required for cryopreservation have been reported for the mouse (Cooper *et al.,* 1996; Johnson and Pickering. 1987), human (Baka *et al.,* 1995; Pickering *et al.,* 1990; Sathananthan *et al.,* 1988; Selick *et al.,* 1995), rabbit and cow (Agca *et al.,*1998; Asada and Fukur .2000;Cohen 1992; Dike .2009**;** Fuku *et al.,* 1995; Fry *et al* ., 1997; Hamano *et al.,* 1992; Hu *et al.,* 1999, Lim *et al.,*1999). The developmental stages at which the oocytes are frozen greatly influence the post thaw survival rate. In vitro matured oocytes have been showed to be more permeable to cryoprotectants that immature freshly collected oocytes. Thus the reports reveal that the fertilizability of mature oocytes was found to be superior to the immature oocytes (Le Gal *et al.,* 1993).

Types and concentration of cryoprotectants are also important factors for the post thaw survival rates of cryopreserved oocytes. Glycerol, Dimethyl Sulfoxide (DMSO), propylene glycol (PG/ PROH) and ethylene glycol (EG) have been conventionally used for the cryopreservation

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of oocytes and they are reported to have different biochemical natures as cryoprotectants. The procedures for successful cryopreservation of oocytes are available for numerous laboratory and domestic animals including mouse and cow (Lim *et al.,* 1992). Le Gal *et al.,* cryopreserved goat embryos by the three step equilibrium procedure using ethylene glycol and obtained 25 per cent developmental rate *in vitro* (Le Gal *et al.,* 1993).Martinez and Matkovic equilibrated sheep embryos with ten minutes interval and obtained 80 per cent developmental rate (Martinez and Matkovic. 1998) In vitro matured bovine oocytes were vitrified using 2M DMSO, 1M acetamide and 3M propylene glycol and only 10 percent post thaw development was obtained (10). However there is paucity of information regarding sheep oocyte cryopreservation procedures (Martinez and Matkovic. 1998, Nowshau *et al.,* 1994).

Successful vitrification requires the use of a highly concentrated, effectively non-toxic solution of cryoprotectants (Rall, Fahy.1985). Thus, to achieve good survival after vitrification, the oocytes or embryos must be able to tolerate exposure and dehydration in these highly concentrated solutions. For this study propylene glycol was selected as the choice cryoprotectant because from comparative studies, propylene glycol has proven to be more effective with minimal morphological abnormalties than other cryoprotectants (Dike. 2009). Hence this study was programmed to analyse the survivability of the post thaw cryopreserved oocytes based on morphological appearance, further maturation and its *in vitro* fertilization after vitrification. The study focused on determining the survival rate and frequency of chromosomal aberrations at various storage durations employing propylene glycol as the cryoprotectant for the vitrification process.

MATERIALS AND METHODS

Sources of Oocytes

Ovaries of mature ewes from India were collected immediately after slaughter from the regional (India) abattoir in normal saline supplemented with 10µL gentamycin per 1ml and transported at 37ºC to the laboratory in vacuum flask within one hour. Upon arrival at the laboratory, the ovaries were removed of extra ovarian tissues and washed 2-3 times with tap water and 7-8 times with 0.9% normal saline at 37-38ºC. The ovaries were then kept in a sterile beaker containing normal saline (37-38ºC) supplemented with 10µL gentamycin per 1ml. The ovaries were sliced as per the standard slicing technique described by Dutta *et al.,* (1993) and the follicular fluid collected was then transferred into 50 ml sterile conical tube and kept at 37ºC in water bath (10 minutes) for the oocytes to settle at the bottom due to gravity. The supernatant was then discarded and the pellet was diluted with HEPES buffered Tyrode's Lactate (TL HEPES) and transferred to 2 – 3, 90 mm petridishes for oocyte screening and graded under the stereo zoom microscope. Oocyte grading was done according to Fry *et al.,* (1997) (9).

In Vitro Culture and Maturation of Oocytes

A & B grade oocytes (18-20 oocytes/droplets) were then transferred to pre-incubated maturation droplets. The oocytes were allowed to mature *in vitro* for 24-26 hours in CO_2 incubator under 5% CO_2, 95% relative humidity and 38.5ºC temperature. After 24 hours of *in vitro* culture of oocytes, the oocytes were examined under the zoom stereomicroscope and assessed for cumulus layer expansion.

Cryopreservation of Oocytes

Cryopreservation of oocytes was performed using propylene glycol (5M PROH+1.5M Sucrose) (7,19) by the vitrification method. Based on the storage duration 4 study groups were maintained: 1 month, 2 weeks, 1 week and 24 hours. The oocytes were partially dehydrated at room temperature at 5 minutes interval. The oocytes were then placed in drops of freezing media containing the cryoprotectant (propylene glycol) and left 45 seconds for equilibration (Nowshau *et al.,* 1994).

Loading of the Straws

After equilibration the oocytes were immediately loaded into the middle of a 0.25µl French mini straw. First 60 µl of 0.5M sucrose was aspirated into the straw followed by 5 mm air space, then 40 µl of freezing media containing cryoprotectant and 20-25 oocytes, followed by 5 mm air space and finally 60µl 0.5M sucrose. The open end of the straw was sealed. The entire operations were carried out at ambient temperature. A total of 350 oocytes were frozen and studied for each group.

Freezing of Straws by Vitrification

After filling, the sealed straws were dipped slowly into liquid nitrogen within 45 seconds and transferred to LN_2 goblets and stored at 196ºC in LN_2 storage containers for three weeks.

Post Thaw Morphological Evaluation of Frozen Thawed Oocytes

After specified storage duration (1 month, 2 weeks, 1 week and 24 hours), the oocytes were thawed After removal of cryoprotectants, the cumulus oocytes complexes were washed four times in Fertilization Tyrode's lactate solution (TALP) medium and freed from cumulus cells by repeated pipetting. Morphological evaluation was done under zoom stereomicroscope.

Morphologically damaged oocytes which were identified as oocytes with dark granulated or fragmented cytoplasm, indistinct vitelline membrane, widened perivitelline space or fragmented zona pellucida were removed and 10-12 morphologically normal oocytes were selected to be placed in the fertilization droplets.

Evaluation of In vitro Fertilization of Frozen Thawed Oocytes

Testes from adult rams collected from the slaughterhouse were washed thoroughly, after removing tunica albugenia, with tap water and 0.9% Saline supplemented with gentamycin. The cauda epididymis was sterilized with 70% alcohol and incised deeply using a surgical blade.

Then using a syringe the sperm TALP medium was used to collect the seminal fluid. The semen collected were assessed

for motility of the sperms and then processed for sperm separation by Percoll method (Papis *et al.,* 1995). The sperms were layered on the top of the percoll gradient. The tube was then centrifuged at 800rpm for 30 minutes. The hazy layer was recovered immediately after centrifugation with a sterile pipette. Sperms were again washed with 10ml of sperm TALP at 400rpm for 10 minutes and the supernatant was removed until 100µl was left in the tube. The motility of the sperms was assessed under lower (10x) magnification of the inverted phase contrast microscope. The concentration of sperms was adjusted to 2x10 sperms/ml using haemocytometer.

The oocytes deemed morphologically normal were then used in the investigation of fertilizability of the oocytes. The pre incubated fertilization droplets with oocytes were inseminated with sperms at a concentration of 2-10 sperms/ml. Now two sets of oocytes were incubated, i.e. freshly harvested oocytes and the thawed oocytes. These oocytes were then incubated with the sperms for 18-20 hrs at 38.5ºC, 5% CO_2 and 95% relative humidity. After 24 hours of fertilization, the presumptive zygotes were observed under the zoom stereomicroscope or phase contrast microscope for evidence of cleavage.

Chromosome analysis of Embryos

Chromosomal analysis was carried out on the obtained embryos after fertilization. The obtained morphological normal embryos were incubated in the T6 medium 4-6 hour and then plunged in acid Tyrode's solution for 7 seconds in 3 steps to slenderize zona pellucida, then the embryos were exposed to the T6 medium containing colcemid for 48 hours; after that embryos were placed in hypotonic solution (1% sodium citrate in water) until swollen (3-15 min). The swollen embryos were individually placed on clean glass microscope slide in a minimal amount of fluid and then spread using the fixation solution described previously by Tarkowski (1966). The slides were stained with giemsa (3%), and examined under a light microscope (Nikon E800, Japan) at x 1000 magnification for numerical chromosome analysis.

Statistical Analysis

Survival rates of the 4 groups were analyzed using the student's *t* test using the INSTAT software and P<0.05 was said to be significant.

RESULTS

All the good quality oocytes (A and B) recovered through the slicing technique were utilized for in vitro maturation. The maturation rate obtained was 76 percent. Assessment of maturation of oocytes was carried out after 24 hours of in vitro culture of oocytes. The oocytes were examined under the microscope and assessed for cumulus cell layer expansion.

Viability Rate

The viability rates for frozen oocytes in the 4 study groups were 90.1% for 24 hours, 83.9% for 1 week, 82.5 % for 2 weeks and 79.2 for 1 month. The control showed a viability rate of 98.3%. (Table 1) the viability rate of the post thaw cry preserved oocytes based on morphological appearance and further maturation of these oocytes *invitro.* The difference between the week 1 and 2 study groups and the control was not a significant difference when compared to control. But as the storage duration was increased to 1 month, the difference was found to be significant (P<0.01). Thus it was noted that by increasing the storage duration, the viability percentage decreased. (Figure 1)

Table 1. Post Thaw Viability of Sheep oocytes Vitrified at Different Time Intervals

Study Groups	Total Oocytes Retrieved	Total Normal Oocytes	Viability Rate %
24 Hours	354	319	[a] 90.1
1 Week	360	302	[a] 83.9
2 Week	360	297	[a] 82.5
1 Month	360	285	[b] 79.2
Control	60	59	[a] 98.3

Difference between [a] and [b] was found to be significant

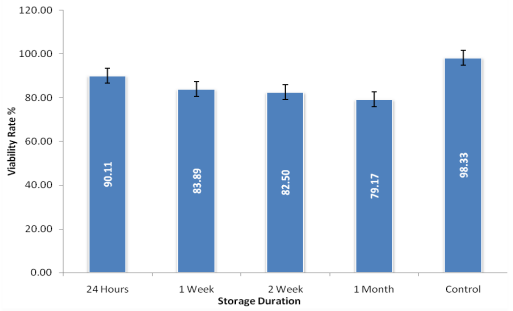

Figure 1. Percentage of Viability after Vitrification at Different Time Intervals

Fertilization Rate

Oocytes obtained after the vitrification process using propylene glycol, upon fertilization, showed that the cleavage rate and the percentage of oocytes developed to the two-cell, four-cell and blastocyst stages were found to be significantly higher in the control and the oocytes frozen for 24 hours (39.10%), 1 week (34.05) and 2 weeks (32.54%) when compared to the cleavage rate obtained for the oocytes frozen for 1 month (21.33%). Thus it was evident that there was a significant difference (p<0.01) between the oocytes fertilized after 1 month of freezing when compared to the control and the other study groups. This is clearly displayed in Table 2.

Table 2. Post Thaw Fertilizability of Sheep oocytes Vitrified Using Propylene glycol at Different Time Intervals

Study Groups	No. of oocytes used for IVF	No. Normal Oocytes showing cleavage	Fertilizability Rate %
24 Hours	154	60.21	[a] 39.10
1 Week	160	54.48	[a] 34.05
2 Week	160	52.06	[a] 32.54
1 Month	160	34.13	[b] 21.33
Control	50	28.15	[a] 56.30

Difference between a and b was found to be significant (P<0.01)

Table 3. Total of Chromosomal Aberrations at Different Time Intervals

Duration	24 Hours	1 Week	2 Week	1 Month	Control
No. of Metaphase Plates Analysed	31	34	39	38	60
*No. Of Aneuploid embryos	10	10	13	13	5
#No. Of Polyploid embryos	1	4	3	8	1
Normal embryos	20	20	22	16	56

*embryos showing chromosomal count between37-43; #embryos showing chromosomal count between77-83.

Chromosome Abnormalities

The number of embryos showing chromosome abnormalities (aneuploidy and polyploidy), were noted in the various study groups (Table 3). As indicated, increasing storage duration, increases the incidence of aneuploidy and polyploidy. In Figure 2, there is an increase in chromosomal abnormalities from the control group to 1 month group attaining an almost stable state in the 1 and 2 week study group. The incidence of abnormality in 1 month study group was the highest showing a significant difference when compared to the control group ($P<0.01$). There are significant differences of aneuploidy between the study groups and the control group. From Figure 2, it was evident that there was a significant difference in total abnormalities rate of 24 hour, 1 week, 2 weeks and 1 month study groups (P-values <0.05) when compared to control group.

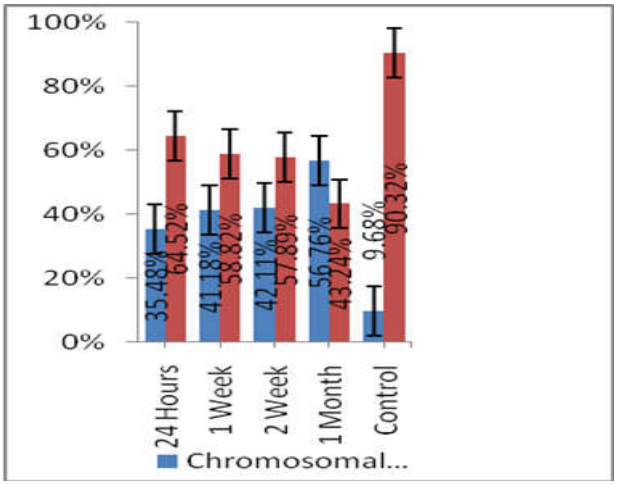

Fig. 2. Percentage of Total Chromosomal Aberrations

DISCUSSION

Availability of viable, cryopreserved sheep oocytes would allow greater flexibility in the use of *in vitro* fertilization and related technologies by providing developmentally competent oocytes when needed. The effectiveness of existing procedures based on viable embryos per oocyte frozen remains low compared to use of fresh oocytes. Thus the study analyses the viability, fertilizability and the rate of chromosomal abnormalities of the frozen oocytes post fertilization. Multiple factors, such as stimulation protocols, freezing procedure and oocyte quality at time of freezing may influence the success of an embryo freezing program (Hu *et al.,* 1999; Niemann,1991; Park *et al.,* 1997). Yet storage duration was found to be one of the most important factors in the vitrification of sheep oocytes. It was noted that by increasing the storage duration, the viability percentage decreased. (Figure 1) This may be due to the fact that freezing and warming of cells imposes a concatenation of stresses including equilibrating with a cryoprotectant, cooling, warming, dilution and rehydration (Aman and Parks, 1994). Other studies have indicated similar findings, indicating that freezing and thawing significantly reduces viability (6, 27). The adverse effects of cryopreservation may also lead to the formation of cracks in the zona pellucida, or injuries to the cell membranes and intracellular components. Ideally the freeze-thaw procedure should not cause any loss of viability, or lead to an increased incidence of genetic aberrations, foetal malformations or losses. Several lines of evidence suggest that the damage may be more closely linked to the freezing properties of cryoprotectant solution (14). Intracellular ice is more likely to be formed when the permeation of the cryoprotectant and its concentration in the cytoplasm are insufficient.

Chromosome abnormalities

From the results it is clear that vitrification using propylene glycol may cause chromosomal aberrations and reduced viability *in vitro.* The incidence and severity of the damage is influenced by the storage duration (Table 2-3, Fig 2). The highest incidence of chromosomal aberrations occurred in the "1 month" study group. These damages appear more closely linked to factors such as ice crystal formation during storage duration in vitrification. Fertilization of oocytes with disorganized spindles could lead to chromosomal aneuploid and arrest of cleavage. It is known that the cytoskeleton of mammalian oocytes and embryos is thermo- and chemo-sensitive and sensitive to stresses connected with cryopreservation (Park *et al.,* 1997; Pickering and Johnson. 1987, Sathananthan *et al.,* 1988; Tarkowski . 1966).

The unequal distribution of chromosomes, between daughter cells results in aneuploidy at multi-polar mitosis or in a polyploid unipolar mitosis (Van Derelst *et al.,* 1988). It is known that aneuploidy is one of the causes of low implantation rate and early embryonic mortality in mammalian species. From this study it is evident that vitrification of oocytes using propylene glycol as cryoprotectant can lead to induction of chromosomal aberrations and thus cannot be suggested as a efficient cryopreservant when employed singly. In addition, rapid cooling, warming and dilution solutions can cause the problem of injury to the zona pellucida which is very sensitive to cryoprotectants and low temperature. However, the morphological survival rate of oocytes was reduced by increasing storage duration. The reason for this is not clear, but may be attributed to the ice crystal formation during freezing and sudden extremes of cooling. Propylene glycol as cryoprotectant showed low potential when employed singly, however, studies involving prolonged duration higher than one month and analysis of the exact type of abnormalities at after each storage stage will help provide an insight into this

reduction in survival rate as the storage duration increases. Further research in this field will improve the long term survival and preservation of the chromosomes in embryo vitrification using propylene glycol as cryoprotectant.

Abbreviations

1. IVM – *In vitro* Maturation
2. IVF - *In vitro* Fertilization
3. CPA – Cryo-protective Agents
4. PG/ PROH - Propylene glycol
5. EG - Ethylene glycol
6. DMSO - Dimethyl Sulfoxide
7. TL HEPES – HEPES buffered Tyrode's Lactate
8. TALP - Tyrode's lactate solution (TALP)

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