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Effect of warming on the survivability and fertilizability of vitrified matured bovine oocytes

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Vitrification has proven to be a very useful tool for oocyte cryopreservation. Success of cryopreservation by vitrification could however be affected during thawing, hence, experiments in this study were conducted to determine the effect of different warming conditions on the survivability and fertilizability of matured bovine oocytes after vitrification. Matured oocytes were vitrified at 10 min equilibration time using MDS, warmed and assessed morphologically. Oocytes deemed survived were subjected to *in vitro* fertilization (IVF). Step- wise, direct and different step- wise combinations were used in determining the survivability and fertilizability of vitrified- warmed matured oocytes. Results were analyzed using ANOVA, DMRT and GLM using SAS ver. 9.0.Results obtained a survivability rate of 88%, 91% and 83%- 93% following direct, step-wise and different step- wise combinations, respectively. The penetration, MPN and cleavage rate obtained after direct and step-wise were 6 %, 3%, 13% and 11%, 7%, 16% respectively. While a penetration rate of 4% was identified at 3:3 step- wise combination alone. Relationship analysis however, revealed that the penetration results were affected by the warming at 13.8% degree of relatedness as confirmed by the relationship analysis.

Keywords: warming, vitrification, survivability, fertilizabilty, bovine oocyte

Introduction

The cryopreservation of mammalian embryos and oocytes has become an integral part of methods to control animal reproduction and development of reproductive technologies (Leibo *et al*., 1996). Several cryopreservation methods have been used to preserve embryos and oocytes of many animal species resulting in the birth of live off spring. One cryopreservation method known of its advantages is the vitirification method. Vitrification has been successfully applied for cryopreservation of bovine oocytes and embryos at various developmental stages (Le Gal and Massip, 1999).

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Although achievements have made early stages of cryopreservation a promising method of conserving bloodlines using various vitrification protocols, optimum conditions for vitrification have yet to be determined. Many factors are known to influence vitirification of oocytes among them are the type of concentration of the cryoprotectant, type of cryoprotective device, number of equilibration steps, time of exposure and number of dilution steps at warming. It is widely believe that each of these factors can affect the results (*Martins et al*., 2005). Many other factors are cell itself, time, temperature, added macromolecules, disssacharides and cryoprotectants. Most common of which is the exposure time of oocytes to cryoprotectant solutions (Otoi *et al.*, 1998). Inappropriate removal of CP during warming is also a critical factor for vitrified oocyte survival. Therefore, the study was performed to evaluate whether modifications of warming procedure could improve the efficiency of the MDS vitrification protocol for in vitro matured bovine oocytes.

Materials and methods

Bovine Oocyte Samples: Immature oocyte samples normally at the Germinal Vesicle (GV) stage were collected immediately and subjected to 22h maturation prior vitrification and freezing processes. After few days of freezing the vitrified IVM oocytes were thawed and all adjudged survivors were subjected to IVF and IVC.

Reagents and Media: Medium for oocyte handling, IVM, IVF, IVC and VS were adapted from the established formulations of the PCC staff biotechnology department. For oocyte handling, TCM- 199 + 10% FCS buffered. Fertilization medium was BO medium. The culture medium was a modified synthetic oviduct fluid (mSOF) supplemented antibiotics.

For the vitrification solution of both methods, the base medium (BM) was TCM-199 medium and 20% FCS. Vitrification solution A (VS_A) consisted of BM + 10% EG and vitrification solution B (VS_B) consisted of BM + 40% EG. The warming solution was BM supplemented with different levels of sucrose.

General Procedure

Oocyte Source and In Vitro Maturation: COCs were recovered from bovine ovaries by aspiration of three to five mm in diameter follicles using a sterile five ml plastic syringe with an 18-G needle. The COCs with a compact, non-atretic cumulus and a homogenous cytoplasm were selected, washed twice in holding medium before allocated in 50 µl drops of maturation medium (5-10 COCs/drop) under mineral oil. COCs were cultured for 22 hr

under controlled gas atmosphere consisting of five % CO_2 in humidified air at 39^0C.

Vitrification using MDS Technique: The *in vitro* matured bovine oocytes were washed twice in the holding medium before it was suspended in 10 minute equilibration time under VS_A at 37^0C before it was transferred to VS_B (45 sec), then aspirated by using a micropipette and dropped in pellet form directly into LN_2. The droplets containing the vitrified oocytes were then transferred into cryotubes and into a LN_2 tank for storage. The pellets were warmed by directly dropping them in the different warming solutions at 37^0 C.

In Vitro Fertilization and Culture: The oocytes in the treatment group that had the highest MII rate were fertilized in vitro. Locally processed beef cattle semen from the Nueva Ecija Stock Farm (NESF) was used for fertilization. For each experiment, two straws containing 0.5 ml semen was thawed in a water bath at 37^0C for 15 sec. The semen suspension was washed twice with the culture medium by centrifugation at 2,000 rpm for 10 min. A 50 µl aliquot of the sperm suspension was introduced in droplets of 50 µl fertilization medium containing the pre- washed oocytes to co- incubate. The suspension was then incubated for 12 hr in an atmosphere of 5% CO_2.

Analysis of the Nuclear and Cytoplasmic Status: Following sperm-oocyte co-culture for 30 hr, representative "presumptive" zygotes were mounted on glass slides and subjected to staining procedures. Presence of polar bodies and a 2^{nd} metaphase plates were regarded as matured while the presence of two polar bodies, a detached tail and a swollen or decondensing sperm head in the ooplasm was regarded as fertilized. Fertilized oocytes with male pronucleus/pronuclei were considered to have undergone cytoplasmic maturation.

Experimentation: Direct, Step-wise and Step-wise Combinations

Warming Methods: Two warming methods using direct and step-wise technique were examined in study I below, while effects of different warming combinations were further investigated in study II.

Study I. Effect of Using Direct Over Step-wise Warming Methods on Oocyte Survivability and Fertilizability

Group of oocytes vitrified from 10 min: 45 sec equilibration time interval was subjected to two warming methods namely; the step- wise method (A) and direct method (B). Method A allowed a three min exposure time to 0.3 M sucrose and a subsequent exposure to 0.1 M sucrose for 1min. While method B

allowed a single exposure for 3 min in 0.3 M sucrose only. Effect of different step-wise combinations was further examined in Study II.

Study II. Effect of Different Step-wise Warming Combinations on the Survivability and Fertilizability of IVM Oocytes

Another set of oocytes were vitrified and warmed under WSA containing 0.3M suc and WSB containing 0.1M suc. The following treatment combinations were used: Treatment 1- 0; Treatment 2- 1 min: 1min; Treatment 3- 5 min:5min; Treatment 4- 3min:3min and Treatment 5-5min:3min.

All survival rates from the exposure to the different warming times were recorded and were further examined for cleavage formation after IVF. For the purpose of examining penetration and MPN formation rates, separate batch of oocytes after subjecting to IVF was subsequently stained and fixed.

All control in study I and II consisted of fresh non-vitrified oocytes matured *in vitro* and directly fertilized.

Statistical Analysis: The parameters used in this study included the percentage inseminated; penetrated, male pronuclei (MPN) formation and cleaved oocytes. All percentages were compared statistically using the Analysis of Variance (ANOVA), Duncans Multiple Range Test (DMRT) and Generalized Linear Model (GLM). All results were analyzed statistically using the Statistical Analysis System (SAS) program ver 9.0.

Results and discussions

Study I. Effect of Using Direct Over Step-wise Warming Methods on Oocyte Survivability and Fertilizability

Changing the warming conditions greatly affected vitrified oocytes post-warming survivability and fertilizability. Table 1 presents the effect of warming on in vitro fertilization of in vitro matured bovine oocytes using the single step and step- wise method of warming.

Table 1. Effect of using direct over step- wise warming methods on oocyte survivability

WARMING METHOD	NO. OF OOCYTES VITRIFIED- WARMED	SURVIVAL RATE (%)
Control	57	100^{a}
Direct	74	88^{b}
Step- wise	66	91^{ab}

Means having the same letter script within a column are not significantly different at the 5% probability level by DMRT; Control-Non-vitrified

Survivability rate results for the two procedures are almost equal at 88% for step- wise and 91% for direct. This shows that warming using direct or step-wise could provide the same degree of providing adequate conditions necessary for maintaining survival after series of vitrification and cryopreservation procedures. Results further indicated that single or series steps of warming time combinations do not cause obvious effects on the survivability of the oocytes as observed by its normal morphological structure post-warming.

Penetration and MPN formation after IVF were higher at 11% and seven % rates respectively, for the step- wise and seven % and three % for direct method. Slightly better result was observed for the step-wise possibly because the oocytes were exposed twice to two warming conditions, hence, could have contributed to the partial if not complete removal of the residual amounts of cryoprotectants left from vitrification procedures. Statistical results, however, showed no differences between the two procedures which implies that whatever method is used, the same effect could be acquired by the oocytes. This also indicates that elimination of toxic component is immediate whatever the procedure is used.

Table 2. Effect of using direct over step- wise warming methods on penetration and MPN formation after IVF of IVM bovine oocytes

WARMING METHOD	NO. OF OOCYTES VITRIFIED-WARMED	SURVIVED-INSEMINATED (%)	PENETRATED (%)	MPN FORMATION (%)
Control	26	100	77^{a}	65^{a}
Direct	30	90	11^{b}	7^{b}
Step- wise	33	88	6^{b}	3^{b}

Means having the same letter script within a column are not significantly different at the 5% probability level by DMRT

Appropriate condition in the study was provided by the temperature (37^{0}C) with solutions of 0.3M sucrose for direct and 0.3M: 0.1M for step- wise. This further indicates that exposure to 0.3M sucrose alone could provide the same level of protection with exposure to high (0.3M) and subsequently transferred to low (0.1M) sucrose solution. Furthermore, results show that the non- vitrified groups remains to be significantly better to cause penetration and MPN formation. The significantly ($p<0.05$) low results for penetration and MPN formation, however, could be attributed to the effects of cryoprotectants upon vitrification or to freezing and to some extent to the warming solution. Although high survival rates were observed, the warming solution(s) and time

combinations themselves were not enough to protect the cells from the toxic effect of the cryoprotectant or to the effect of the freezing to continue their development.

Table 3. Effect of using direct over step- wise warming methods on cleavage formation after IVF of IVM bovine oocytes

WARMING METHOD	OOCYTES VITRIFIED-WARMED	SURVIVED-INSEMINATED (%)	CLEAVAGE RATE (%)	REMARKS (BLASTOCYST)
Control	31	100 [a]	39	8 blastocyst
Direct	44	86[b]	16	Degenerated after 6 days
Step- wise	33	94[a]	13	Degenerated after 6 days

Means having the same letter script within a column are not significantly different at the 5% probability level by DMRT; Control-Non-vitrified

Cleavage rate was observed to be higher (16%) in two warming exposures or the step-wise method than direct method (13%) of warming. Cleaved embryos for the two treatments, however, showed degeneration after six days. Such result indicates that the warming time used regardless of the procedure was insufficient to protect IVM oocytes from cryodamage post-warming, thereby, preventing their subsequent growth and development following IVF.

Study II. Effect of Different Step-wise Warming Combinations on the Survivability and Fertilizability of IVM Oocytes

Results upon exposure to the different warming conditions are reflected below

Table 4. Effect of different step- wise warming combinations on the survivability of IVM oocytes vitrified under 20% bovine follicular fluid (BFF) supplemented vitrification medium

TIME (min)	NO. OF OOCYTES VITRIFIED- WARMED	SURVIVAL RATE (%)
Control	92	97
1:1	60	83
5:5	51	86
3:3	73	92
5:3	45	93

Means having the same letter script within a column are not significantly different at the 5% probability level by DMRT; Control-Non-vitrified

Survival rates using four different step- wise warming combinations provided the highest survival rate at 5: 3 min (93%) warming time combinations followed by 3:3 (92%), 5:5 (86%) and 1:1 (83%). The survivability rates for the different step-wise combinations were close enough to the results of the non-vitrified (97%) groups. Such result indicates that the different step- wise regardless of the time and sucrose combinations provided enough protection for the survival of the vitrified IVM oocytes.

Table 5. Effect of different step- wise warming combinations on the penetration and MPN formation after IVF of IVM oocytes

TIME (min)	NO. OF OOCYTES VIT-WARMED	SURVIVED-INSEMINATED (%)	PENETRATED (%)	MPN FORMATION (%)
Control	30	90[a]	74[a]	0
1:1	30	73[b]	0	0
5:5	24	96[a]	0	0
3:3	28	93[a]	4[b]	0
5:3	19	95[a]	0	0

Means having the same letter script within a column are not significantly different at the 5% probability level by DMRT; Control-Non-vitrified

Penetration was apparent only at 4 % in the 3:3 min time combination. This shows that 3:3, although low could be enough to protect few oocytes from the effects of cryoprotectant and cryodamage.

Statistical analysis shows that the non-vitrified groups revealed a significantly higher ($p<0.05$) result for penetration. Relationships revealed a linear and quadratic relationship for penetrability. This signifies that penetrability is influenced by changing warming conditions and may follow a linear and quadratic pattern of relatedness.

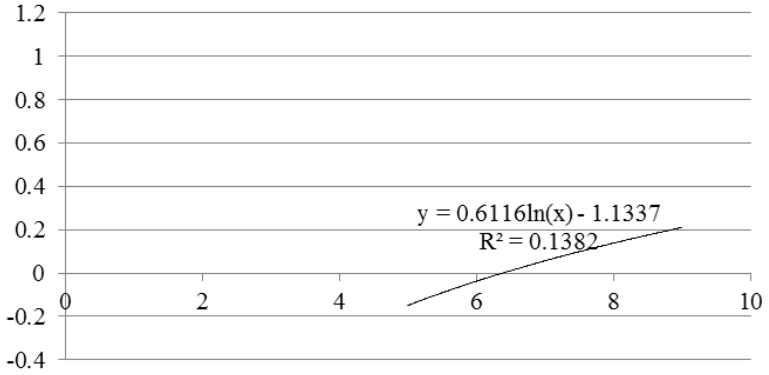

Fig. 1. Relationship of the effect of warming times and penetrability of vitrified oocytes

Based on the result (Fig. 1), statistical analysis proved that 13.8% of the causes of low penetration rates were due to changes in warming condition. Such indicates that low results could not only be attributed to the warming effects alone as the oocytes could have been adversely affected during vitrification and freezing processes prior to warming. At any rate, warming could be a contributory factor (13.8% relatedness) as few oocytes were penetrated post-warming indicating insufficiency in protection of oocytes after vitrification. Again, exact mechanism is unknown.

Although, morphologically normal oocytes were already identified in 1:1 min (7%), 5:5 min (14%) and 3:3 min (68%) 24 hrs post- incubation, none of the IVF oocytes continue to develop. While 31% of the inseminated controls developed into blastocyst and were vitrified for future use. Apparently, cleavage rate was greatly affected for none of the developing embryo continued to develop. This implies that the warming conditions and possibly effects of vitrification and cooling could have caused damage to the oocytes affecting their subsequent growth and development.

Table 6. Effect of different step- wise warming combinations on the cleavage formation after IVF of IVM oocytes vitirified

TIME (min)	NO. OF OOCYTES VITRIFIED-WARMED	SURVIVED-INSEMINATED(%)	CLEAVAGE RATE(%)	REMARKS BLASTOCYST (%)
Control	62	100	45	19 (31%) (Vitrified)
1:1	30	93	0	2 (7%) (MN 24 hr. p.i.)
5:5	27	78	0	3 (14%) (MN 24 hr. p.i.)
3:3	45	91	0	28 (68%) (MN 24 hr. p.i.)
5:3	26	92	0	Degenerated

Means having the same letter script within a column are not significantly different at the 5% probability level by DMRT; Control-Non-vitrified

Survival rates obtained in the study (88% and 91 % for step- wise and direct method, respectively, and 83- 93% for different step-wise combinations) agree with the result of Men *et al*. (2003) obtaining a more than 90% of the vitrified oocytes viable after warming using step-wise procedure. Nevertheless, the detrimental effect of cryopreservation was expressed gradually during *in vitro* culture. Under conditions used in the study of Saunders and Parks (1999), 85% of the oocytes were classified as morphologically normal post- thaw but 50% were osmotically responsive to 1.0 M sucrose after warming, suggesting that the oolema was compromised even in oocytes with normal morphological appearance. Attanasio *et al*. (2007) also found out that warming carried out in a 2-step manner, starting from a lower concentration of sucrose (i.e. 0.25M sucrose for 1 min: 0.15M sucrose for five min), had deleterious effects on

survival rates of the oocytes and subsequently resulting in impairment of post-fertilization embryo.

Such various effects could be attributed to the biological responses of the oocytes to warming conditions. Main biological response to warming conditions is dependent on the ability of the spindle fibers to be restored post-warming. Saunder and Parks (1999) demonstrated that when oocytes were cooled and rewarmed, abnormal spindles formed frequently. Results reported by Aman and Parks (1994) in their study indicates that meiotic spindles in bovine oocytes did not fully reform after rewarming which is similar to human oocytes. In mature bovine oocytes, microtubules appear to be restricted largely to the meiotic spindle, with little evidence for foci of pericentriolar material. More chromosomes were misaligned from the metaphase plate in cooled oocytes rewarmed directly than in those oocytes rewarmed in steps. This difference could be caused by extended exposure to 39^0 C in the absence of a normal spindle, allowing more time for chromosomes to move away from the metaphase plate. ***However***, question still remains on how disruption of the meiotic spindle and dispersal of chromosomes as a consequence of cooling bovine oocytes affect fertilization and early development of the resulting zygote.

Conclusion

Majority of the results revealed that survivability of matured bovine oocytes were not likely affected by various warming times, however, developmental competence was greatly compromised. Hence, factors causing poor growth and development and subsequent fertilization after post- warming needs to be investigated further.

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