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VITRIFICATION OF BOVINE OOCYTE USING DIFFERENT INTRACELLULAIR CRYOPROTECTANTS

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ABSTRACT

The aim of this research was to find out the effect of intracellulair cryoprotectant Ethylene Glycol (EG),Glycerol (Gly)and DMSO (Dimethylsulfoxide) on normal oocyte's morphology, viability and the rate of oocyte's fertilisation after vitrification. Medium of vitrification tested was : 40% EG + 0.5 M Sucrosa (VS_1), 40% GLY + 0.5 M Sucrosa (VS_2), 40% DMSO + 0.5 M Sucrosa (VS_3). The percentage of oocyte with normal morphology after that had been vitrificated in VS_1 solution was obviously higher ($p < 0,05$) than if it was in VS_2 and VS_3 solution. The vitrification of oocyte within a solution with 40% of etylen-glicol (VS1) showed higher viability ($p < 0.05$) than another of two solution of vitrification. The vitrification process of MT II oocyte in VS_1 was showing different significantly ($P<0,05$) by control oocyte to viability and fertilization rate. After vitrification process into VS_1, VS_2, or VS_3, GV-oocyte showed a lower viability ($P<0.05$) than GVBD, metaphase I(Mt I) and metaphase II(Mt II). The *in vitro* fertilization rate of MT-II oocyte after vitrification in VS_1(81.25%) was higher than VS_2 (68.73%) and VS_3 (66.25%). It was concluded that normal oocyte's morphology, viability and the rate of oocyte's fertilisation after vitrification in VS_1 was better than those were in VS_2 and VS_3. GV-oocyte was more sensitive to vitrification solution than it was at GVBD, MT I and MT II.The rate of viability of Mt-I and Mt-II Oocyte after vitrification were higher than GV and GVBD's ones.

Key word : Bovine oocytes, Vitrification,Morphology,Viability,Fertilization rate.

INTRODUCTION

Based on physical phenomena, there are two methods of cryopreservation, those are conventional and vitrification methods. The more obvious different significantly of both methods consists of; in conventional methods was happened liquids freezing process throughout ice crystallization (Rall and Fahy, 1985). However, an over vitrification of this method is simple, cheep enough and unnecessary to special equipment in decreasing the temperature step by step, so that it could be easily applied in placed where it has a liquid-nitrogen container's facility.

The purpose of the research was to develop method for long-term cryopreservation bovine oocyte through improving the new method of cryopreservation using vitrification method that will be simplier and cheaper. We attempted to develop a protocols for bovine oocyte cryopreservation that may be applied especially in under limited facilities in developing country. Therefore, the main objective of this investigations has been the development of an effective method for bovine oocyte cryopreservation. So, it is important to test the viability of frozen oocyte resulted from vitrification method with in vitro models. The data about in vitro maturation rate, fertilization rate of post thawed oocyte then be very important as a basic information. Success of bovine oocyte cryopreservation application will give advantage for further

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research such as IVF, embryo manipulation, cloning, and nuclear transfer. Therefore research on gamet cryopreservation, especially oocyte cryopreservation was a very interesting and important thing to carry out.

MATERIAL AND METHODS

Oocyte preparation

Ovaries were collected from cows at local abattoir and were brought to the laboratory in physiological saline 0.9 % (w/v) at 25 - 30 o C within 3h. Oocytes were aspirated from 2-5 mm follicles with an 18-G needle attached to a 5 ml syringe containing PBS (Phosphate Buffered Saline) supplemented with 5 % (v/v) Fetal Calf Serum (FCS) and antibiotics 100 ug/ml streptomycin and 100 IU/ml penicillin G. The oocytes were put into the maturation medium TCM 199 + PMSG 10 IU + hCG 10 IU, cultured for 24 h in 5% CO2 in air at 38.5 °C.

Vitrification and thawing oocyte

Oocyte were dehydrated conformity with the room temperature in the 30 mm of petrydish filled by 0.25 M and 0,50 M of sucrose, each of them are 5 minutes, then they are being exposured into vitrification solution for 60 second, then be injected into 0,25 ml of straw (mini straw). After it had being exposured in nitrogen stem for 10 seconds, straw being put into liquid of nitrogen at -196^0 C and saved for 4 weeks for further analysis. Medium of vitrification tested is : (1) 40% EG (Ethylene Glycol) + 0.5 M Sucrosa, (2) 40% GLY (glycerol) + 0.5 M Sucrosa, (3) 40% DMSO (Dimethylsulfoxide) + 0.5 M Sucrosa

The Oocyte that it had after been vitrificated by thawing on air for 10 seconds, it had been put into water bath at 37^0 C for 1 minutes. The contains of Straw were filled to the petrydish containing 0.5 M and 0,25 M of sucrose for 5 minutes.

Morphological Evaluation

Oocyte classified had a normal morphology if it showed circular pattern with complete plasmic membrane and homogenize compact cytoplasm. Abnormal Oocyte: the pattern was not circular, fracture of pelucide's zone, disintegration of plasmic membrane and cytoplasm in-homogeneously.

Analysis of Oocyte viability

Viabillity test would be done by staining method use Hoechst 33342 and Propidium Iodide The oocyte was washed in PBS solution then will be put to Hoechst 33342-PI solution for 30 minutes in the dark room. The colored oocyte put on object glass and covered by cover glass, searched under fluorescent microscope (Nikon, Japan). The blue and the red color in nucleus, each of them showed the survivability or death oocyte.

In vitro fertilization

Frozen thawed oocytes would be fertilized with frozen-thawed semen. The oocytes results on vitrification were further used for IVF process. To make preparation of semen and oocytes fot it process, according to Djati (1999) with slighly modification, the methods employed was that the oocytes were be inseminated by spermatozoon with 2 x 10^6 spermatozoons/ml. After being inseminated, they were be incubated into CO_2 incubator at 5% and 38^{0}C, and maximum humidity for 24 h. In this research was

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searched the penetrated oocytes, the forming of female/male pronucleus and the obtaining of polyspermia with 1% of aceto orcein.

Statistical analysis:

Data was be analyzed using analysis of variance. If there were differences among groups of treatment then they were be analyzed by Duncan`s test for further analysis.

RESULT AND DISCUSSION

1. Oocyte Morphological Analysis

The result of this work morphologically showed that the kinds of vitrification solution given implies to the rate of normality of oocyte morphological after they had been vitrificated and thawed (Table 1).

Table 1. Oocyte's morphology after been vitrificated using three of kinds of vitrification solution.

The kinds of vitrification	Morphology (%)	
Solution	**Normal**	**Abnormal**
Control	$90,35 \pm 1,97^{a}$	$9,65 \pm 0,5^{a}$
VS_1	$78,75 \pm 1,22^{b}$	$21,25 \pm 1,13^{b}$
VS_2	$69,33 \pm 1,34^{c}$	$30,67 \pm 1,39^{c}$
VS_3	$67,56 \pm 1,18^{c}$	$33,44 \pm 1,23^{c}$

A value in a column with different superscripts was significantly different ($p<0,05$).

The percentage of oocyte with normal morphology after that had been vitrificated in VS_1 solution was obviously higher ($p < 0,05$) than if it was in VS_2 and VS_3 solution. Whereas, the rate of abnormality was significantly lower ($p < 0,05$) than in VS_2 and VS_3 solution. The morphological abnormality that is due about vitrification was fracture of pelucide zone and lysis of plasmic membrane.

Park and Ruffing (1992) declared that vitrication could seem the destroy on pelucide zone, plasmic membrane and cytoplasm so that it could due the degenerating on oocyte. The exposure of oocyte to the cool temperature respected to vitrification, it could change the form pelucide zone and structure of membrane and it's function, respectively. The change that happened to lipid-protein-double layer on plasmic membrane cause destabilization of membrane when the thawing was operated.

In this work, it's destroying could be minimized by using together sucrose and intercellular cryoprotectant when vitrification was performed. Performing of sucrose could facilitate dehydrating process and keep oocyte viability to the bad result of vitrication (Tada *et al.* 1994). On the other hand, it is also to avoid destroy is caused by hypo-osmotic pressure and over swelling the oocyte when cryoprotectant was carried out (Hamano *et al.* 1992).

2. Effect of vitrification solution to oocyte viability

The searching in oocyte viability on nucleus maturation stages, consist of GV, GVBD, Mt-I and Mt-II had been performed after vitrification in VS_1, VS_1 and VS_3. The

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rate of oocyte viability after vitrification process, each of them could be seen on the Table 2.

Table 2. Oocyte viability on nucleus maturation stages after vitrification in three vitrification solution

Treatment	Oocyte Viability in nucleus maturation stages (%)			
	GV	GVBD	MT-I	MT-II
Control	49/55(89,09)[a]	40/45(88,89)[a]	54/68(85,29)[a]	57/63(90,48)[a]
VS_1	23/68(33,82)[b]	52/68(76,47)[b]	54/68(79,42)[b]	57/68(83,82)[b]
VS_2	18/67(26,86)[c]	48/68(70,59)[c]	55/69(79,71)[b]	48/66(72,73)[c]
VS_3	15/65(23,08)[d]	48/69(69,57)[c]	48/68(70,59)[c]	48/68(70,59)[c]

The value in a column with different superscrips were significantly different (P<0,05)

The vitrification of oocyte within a solution with 40% of etylen-glicol (VS1) showed higher viability ($p < 0.05$) than another of two solution of vitrification. This result showed that the kinds of cryoprotectant given an effect toward oocyte viability at the all of maturation stages.

There are some factors, that they could cause the decreasing of oocyte viability, those are kinds of and concentration of cryoprotectant, oocyte characteristics and cool temperature. The material of cryorotectant could protecting a cell since freezing process, but if the concentration and cryoprotectant kind used is not confirmed with its relating cell, this material will be a toxic and destroying cell apparatus. The presence of different weight of molecule and chemical stucture of intracellular cryoprotectant materials used are causing the presure ability of this materials to cell's membrane is also different (Vicente and Garcia-Ximenez, 1994). A positive effect of ethylen-glycol had been the best proof in freezing system, conventionally (Wahjuningsih et al. 2002) or vitrificationally (Rayos, *et al* , 1994). It is confirmity with molecule weight from each cryoprotectant material, oocyte permeability to ethylen-glycol, it is bigger than glyserol and DMSO.

The number of oocyte that it showed normal morphology was lower than the rate of oocyte viability. Some of oocyte that they were fracturing on pelucide zone, it seems it's survivability after had been colored by bisbenzimidazole(Hoechst 33342)

After vitrification process into VS_1, VS_2, or VS_3, GV-oocyte showed a lower viability ($P<0.05$) than GVBD, metaphase I(Mt I) and metaphase II(Mt II). Compared with control-oocyte, the vitrification process of GV-oocyte to VS_1, VS_2 or VS_3 was able to decrease it's viability ($p < 0.05$) significantly; and then the oocyte viability at GVBD, MT I and MT II was decreasing after vitrification process to VS2 and VS_3.

This result showed that GV-oocyte was more sensitive to vitrification solution than it was at GVBD, MT I and MT II because GV-oocyte has lower permiability than it is at MT I and MT II (Agca *et al* , 1997).

There were feature and procedure used which those were involving the kinds and cryoprotectant concentration, temperature and process. It had been known that the nature of membrane showed the differences between stages of Oocytes (Agca, et al.,1997). GV and GVBD oocyte had been assessed having lower permeability than Mt-I and Mt-II oocyte plus the presence of compacted cumulus cells layer could inhibit including cryoprotectant to the oocyte so they were not enough to protect oocyte toward vitrification process. Whereas, Mt-I and Mt-II's oocyte had the higher membrane's

permeability and the longer cumuli cell, relatively than GV oocyte. This condition may be better of permeation of cryoprotectant that it had been supported with presence of sucrose in vitrification solution. The deficiency of viability rate after been vitrification may be resulted by vitrification and *warming* process so that appearing the destroying of oocyte's physics. The previous work showed that oocyte after been vitrification was more sensitively to hypotonic stress than the fresh oocyte. The hypotonic's stress could result physic damage on cell membrane (Pedro *et al.*, 1997).

Based on this work's above, could be concluded that the rate of viability of Mt-I and Mt-II Oocyte after been vitrification were the higher than GV and GVBD's ones.

3. The fertilization rate of MT II-oocyte after vitrification process

To current the fertilization rate of oocyte was done based on obtainability of male and female pronucleic. the present of 2 pronucleic (2PN) or more than it (>2PN) showed that in each oocyte, they had been penetrated by one or more than one of spermatozoa.

Tablle 3. Fertilisation rate after vitrification process in VS_1, VS_2 and VS_3

Vitrification	The development of pronucleus (%)				Viability of
Solution	2PN (%)	>2PN (%)	Fertilized (%)	Unfertilized (%)	Oocyte[2] (%)
Control	62/80(77,5)[a]	8/10(10)[a]	70/80(87,5)[a]	10/80(12,5)[a]	59/80(73,75)[a]
VS_1	60/80(75)[a]	5/80(6,25)[b]	65/80(81,25)[b]	15/80(18,75)[b]	54/80(66,25)[b]
VS_2	40/80(50)[b]	15/80(18,75)[c]	55/80(68,75)[c]	25/80(31,25)[c]	42/80(52,5)[c]
VS_3	39/80(48,75)[c]	14/80(17,5)[c]	53/80(66,25)[d]	27/80(33,75)[d]	41/80(51,25)[c]

[1]fertilisation was accounted from monosperm plus polysperm. [2]Oocyte viability at 18th hours after *in vitro* insemination. Value in one coloumn with different superskripts were significantly different (P<0,05)

The vitrification process of MT II oocyte in VS_1(40 % etilen glikol) was showing differents significantly (P<0,05) by control oocyte to viability of oocyte rate and fertilization rate.The *in vitro* fertilization rate of MT-II oocyte after vitrification in VS_1(81.25%) was higher than VS_2 (68.73%) and VS_3 (66.25%)

The oocyte viability after 18th hours of *in vitro* insemination was different significantly ($P < 0.05$) among three of above treatments include 66.25%, 52.5% and 51.25%, each of them at VS_1, VS_2 and VS_3, and significantly lower ($p<0.05$)than control ones (73.75%). This shown that although oocyte is morphologically seems normally after had vitrification process and re-warming, some of them may have had destroying intracellularly that this are not seemed under light microscope. Some structures, like the role oolemma, pellucids zone and cortext grains in inhibiting to penetrate polysperm may have reformed a long vitrification process (Rayos *et al,* 1994; Hochi, *et al.*, 1998).

These shown that vitrification process could decrease the ability of Mt-II oocyte in supporting fertilization. Vitrification and *warming* process caused reformation cell biologically so that they caused decreasing the fertilization rate and further embryonic development.

The rate of fertilisation from Mt-II oocyte after given vitrification showed the higher values than fertilisation rate of Mt-II cattle oocyte which have been given freezing in conventional method (Wahjuningsih et al., 2002; Lim *et al.,* 1992). It has been assessed that the vitrification process was able to keep the Mt-II oocyte than usual freezing process. Then, the high polyspermia may be caused by destroying at cortext

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grains so that the oocyte was not able to protect it from polyspermya (Hyttel, *et al.*, 2000).

CONCLUSION

Normal oocyte's morphology, viability and the rate of oocyte's fertilisation after vitrification at in VS1solution(40% EG + 0.5 M) was better than those were in VS_2 (40% Gly + 0.5 M) and VS_3 (40% DMSO + 0.5 M).The vitrification's solution of 40% EG + 0.5 M could keep the oocyte's structure of minimize the damage resulted in vitrification process so that it's viability, morphology and the fertilisation's rate could be maintained. GV-oocyte was more sensitive to vitrification solution than it was at GVBD, MT I and MT II.The rate of viability of Mt-I and Mt-II Oocyte after vitrification were higher than GV and GVBD's ones.

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