Effects of Viability of Canine Oocytes Vitrified-Warmed by the EDS and EDT Methods

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ABSTRACT

In the present studies, we have intended to compare the EDS (20% EG + 20% DMSO + 0.4 M sucrose + 10% FCS) and EDT (20% EG + 20% DMSO + 0.3 M trehalose 10% FCS) methods for vitrification of canine oocytes, in order to improve the vitrification methods. The survival rate of vitrified-thawed oocytes using the EDS method was 15.1±1.8% (p<0.05), which was lower than that of the control group (66.7±2.5%). About 45~55% of the vitrified-warmed oocytes showed normal morphology, as assessed by PI staining. However, the ratio of survival rate of oocytes showed lower than that of normal morphology in comparison between EDS method and control group. The survival and developmental rates of vitrified-warmed oocytes by the EDS and EDT methods were 16.7±1.4% and 11.1±0.8% and 8.3±1.4% and 4.4±1.8%, respectively (p<0.05). The results were significantly lower than the control group (66.7±2.5% and 16.7±3.7%). However, the survival rate of vitrified-warmed oocytes using EDS method showed higher than that in the ETS group.

(Key words : Canine oocytes, Vitrification, Survival rate, EDS, EDT methods)

INTRODUCTION

Despite attempts improvement the efficiency of *in vitro* maturation (IVM) of canine oocytes remains very low compared with that of other domestic animals (Hewitt and England, 1999; Saint-Dizier *et al.*, 2001; Otoi *et al.*, 2004; Abe *et al.*, 2005). However, it can be useful for improved breeding of companion and guide dogs. Although cleavage rates ranging between 8% and 37% have been reported (Hewitt and England, 1997; Songsasen *et al.*, 2002; Rodrigues *et al.*, 2004; Otoi *et al.*, 2005), only one morula (Otoi *et al.*, 2004) and one blastocyst (Otoi *et al.*, 2005) have developed in culture. Vitrification has been widely developed to apply to cryopreservation of mammalian oocytes. However, the suitability of both vitrification methods for canine oocytes has not been investigated. After vitrification the factors that effect canine oocytes are the developmental stage, the toxicity of the cryoprotectant, composition of the vitrification solution and freezing and thawing speed etc. (Cuello *et al.*, 2004). Cryoprotectants like glycerol (Iwasaki, *et al.*, 1994; Saito *et al.*, 1994), ethyleneglycol (EG) (Ishimori *et al.*, 1991; Voelkel and Hu, 1992; Saha *et al.*, 1994) propylene glycol (Kasai *et al.*, 1991), Dimethylsulfoxide (DMSO) were being used for vitrification. Each cryoprotectant has different toxicities or has poor permeabilities which are their weak points. Although, when permeable cryoprotectants like glycerol, DMSO, ethyleneglycol (EG) etc. are mixed with non-permeable cryoprotectants sucrose and trehalose, less cell damage occurs during the steps of equilibrium and thawing (Massip *et al.*, 1993; Saha *et al.*, 1996; Saito *et al.*, 1994). The vitrification method that has been reported is the E30S (Fujuhira *et al.*, 2004) and ovary (Ishijima *et al*, 2006) method. These researchers gained high survival rates by treating the vitrified embryos with high concentrations of cryoprotectants which prevents ice crystal formation.

The objective of the present study was to compare the EDS and EDT methods for vitrification of canine oocytes to improve the vitrification methods.

MATERIALS AND METHODS

Collection and Incubation of Oocytes Comlexes (COCs)

Ovaries within the ovarian bursa from bitches of mixed breed at random stages of the estrous cycle were collected at slaughterhouses and transported to the laboratory in a thermos flask containing sterile saline at approximately 38℃. Each ovary was cleaned of fat and blood vessels and placed in a petri dish containing TCM-199 medium (Gibco, U.S.A.) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin

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G and 100 μg/ml streptomycin sulfate. Ovarian tissue was sliced by a surgical blade repeatedly to collect COCs. Afterwards morphologically excellent oocytes were recovered under a stereo microscope (40 x). The recovered oocytes were cultured in TCM-199 medium supplemented with 10% (v/v) FCS, 1 μg/ml follicle stimulating hormone (FSH), 2 IU/ml human chorionic gonadotrophin (hCG), 1 μg/ml β-estradiol, 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate in an incubator (37℃, 5% CO_2). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO U.S.A.) except for those specifically described.

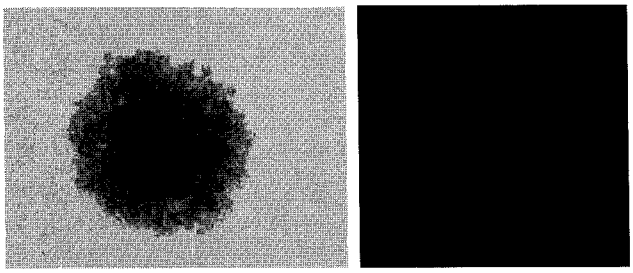
Vitrification and Thawing

The COCs pretreated with TCM-199 medium. The COCs were transferred into a 1 ml cryotube containing 5 ul of 1 M DMSO room temperature (25±2℃), which was then placed in ice water for 5 min. Subsequently, 95 ul of EDS and ETS, maintained at 0℃, were added to each cryotube. After the cryotubes had been placed in ice water for 5 min, they were plunged directly into LN_2 and stored until use. For thawing, the samples were taken from the LN_2 and allowed to stand at room temperature (25±2℃) for 60 sec, and then diluted with 500 ul of TCM-199 medium containing 0.25 M sucrose. The recovered COCs were transferred to TCM-199 medium and washed 5 times.

Survival Judgement of Vitrified-Warmed Oocytes

After thawing, the oocytes were denuded of cumulus cells in TCM-199 medium using a pipette by repeated aspiration and expulsion. The cumulus-free oocytes were stained with 20 ug/ml propidium iodide (PI) in PBS containing 0.1% polyvinyl alcohol and incubated for 15 min. The oocytes were examined under ultraviolet light using an epifluorescence microscope (Nikon, Japan) and plasma membrane integrity of oocytes was assessed. The oocytes with disrupted plasma membrane were dyed red with PI.

Statistical Analysis



(A) (B)

Fig. 1. Morphological appearance of canine oocytes after vitrification. (A) Fresh COCs, (B) Normal appearance of oocytes after vitrification were stained with PI.

Data on oocytes survival were compared using the chi-square test as presented by SAS package of General Linear Model procedures (SAS institute, 2005). Differences were considered significant at a level of $p< 0.05$.

RESULTS

Viability of Vitrified Oocytes

The viability of canine oocytes vitrified-warmed by the EDS method are shown in Table 1. The survival rate of oocytes vitrified-thawed by the EDS method was 15.1±1.8% ($p<0.05$). This result was lower than the control group (66.7±2.5%). About 45~55% of the vitrified-warmed oocytes showed normal morphology. However, the survival rates of oocytes were lower than normal morphology, as assessed by PI stain.

Viability of Canine Oocytes after Vitrification

The viability of canine oocytes vitrified-warmed by EDS and EDT methods are shown in Table 2. The survival and developmental rates of vitrified-warmed oocytes by the EDS and EDT methods were 16.7±1.4% and 11.1±0.8% and 8.3±1.4% and 4.4±1.8%, respectively ($p<0.05$). The results were significantly lower than the control group (66.7±2.5% and 16.7±3.7%). However, the viability of oocytes vitrified-warmed with EDS was higher than those in the ETS group.

DISCUSSION

It is generally accepted that the rate of IVM and IVF in canine oocytes is significantly lower than that in domestic animals. The IVM rate of canine oocytes is reported to be 8~35% and the IVF rate of canine oocytes is about 10~20%. Therefore, the development rate to morula and blastocyst is markedly low (Otoi *et al*, 2005). Among many reasons for lower IVM and IVF rates of dog oocytes, the critical point seems to be hard to study due to limitation of available oocy-

Table 1. Viability of canine oocytes vitrified-warmed with EDS method

Treatment	No. of oocytes examined	No. of oocytes survived (%)
Fresh	30	20(66.7±2.5)[a]
Vitrified	93	14(15.1±1.8)[b]

[ab] Values with different superscript are significantly different ($p<0.05$).

Table 2. Viability of canine oocytes vitrified-warmed with EDS and ETS methods

Vitrification	No. of oocytes vitrified	No. of oocytes examined(%)	No.(%) of normal oocytes(%)		No. of oocytes matured(%)
			Morphology	PI stain	
Fresh	-	30	26(86.7±2.4)	20(66.7±2.5)[a]	5(16.7±3.7)[a]
EDS	52	48(92.3)	25(52.1±2.7)	8(16.7±1.4)[b]	3(6.3±1.4)[b]
EDT	50	45(90.0)	22(48.9±2.6)	5(11.1±0.8)[b]	2(4.4±1.8)[b]

* EDS (20% EG + 20% G + 0.3 M sucrose), EDT (20% EG + 20% DMSO + 0.3 M trehalose).
[ab] Values with different superscript are significantly different ($p<0.05$).

tes. However, in order to develop guide dogs with superior genetic traits, it is absolutely necessary to obtain oocytes and their vitrification. We, therefore, intend to get information to increase the IVM and IVF rates obtained from developing oocyte vitrification and storage. Vitrification has been widely developed to apply to cryopreservation of mammalian oocytes. However, the suitability of both vitrification methods for canine oocytes has not been investigated. After vitrification the factors that effect canine oocytes are the developmental stage, the toxicity of the cryoprotectant, composition of the vitrification solution and freezing and thawing speed etc. (Cuello *et al.*, 2004). In order to vitrify canine oocytes, we have to obtain enough amounts of oocytes. And then, it is necessary that establish vitrification methods such as temperature speed, the proper concentration of cryoprotectants, and the toxicity of cryoprotectant.

The survival rate of oocytes vitrified-thawed by the EDS method was 15.1±1.8% ($p<0.05$). This result were lower than the control group (66.7±2.5%). About 50~55% of the vitrified-warmed oocytes showed normal morphology. However, there was showed the survival rate of oocytes lower than normal morphology, as assessed by PI stain (Table 1). This result was lower than that of Abe *et al.* (2008) who reported that the survival rates of oocytes vitrified-warmed was 17.6%. Also, this result was lower than that of Saha *et al.* (1996) who reported that the survival rate of vitrified porcine oocytes was 65~70%. Canine oocytes and spermatozoa were compared to other mammalians. Thus the IVM and IVF of canine oocytes remain very low compared with that of other mammalian animals. Vitrification has been widely developed to apply to cryopreservation of mammalian oocytes. However, the suitability of both vitrification methods for canine oocytes has not been investigated. Further study is required to examine the IVM and subsquent IVF in vitrified-warmed canine oocytes.

The survival and developmental rates of vitrified-warmed oocytes by the EDS and EDT methods were 16.7±1.4% and 11.1±0.8% and 8.3±1.4% and 4.4±1.8%, respectively ($p<0.05$). The results were significantly lower than the control group (66.7±2.5% and 16.7±3.7%) (Table 2). However, the viability of oocytes vitrified-warmed with EDS was higher than that in the ETS group ($p<0.05$). The results were lower than that of Abe *et al.* (2008) who reported that the ability of oocytes vitrified-warmed by the E30S method were 17.6 % (fresh oocytes, 100%). Also, Abe *et al.* (2008) reported that the oocytes with integral plasma membrane as measured by PI stain in the E30S group was higher than that in the DAP213 group ($p<0.05$). However, Also, Lee *et al.* (2008) reported that the *in vitro* developmental rates of the vitrified-thawed (with EDS and EDT) porcine oocytes following ICSI were 18.5± 2.5%, 16.4±2.1%, respectively. Isachenko *et al.* (1998), who reported the effect of Cytochalasin B (CB) to reduce injury during the vitrification of porcine oocytes, reported that the maturation rate of the vitrified-warmed oocytes treated with 7.5 μg/ml CB for 10~15 min was compared with the non-treated oocytes (22.0 % vs. 5.6%). Oocytes vitrified with EDT supplemented with trehalose prevents toxicity of the cell, detrimental temperature and cell damage but also prevents excess penetration to increase survival rates (Clark *et al.*, 1984; Fahy *et al.*, 1984; Sutton, 1982; Utsumi *et al.*, 1982). After vitrification the factors that effect canine oocytes are the developmental stage, the toxicity of the cryoprotectant, composition of the vitrification solution and freezing and thawing speed etc (Cuello *et al.*, 2004). Thus, further study seems to be required to develop an optimal vitrification concentration, speed and method for canine oocytes. We found that the survival rate of EDS method in canine oocyte vitrification is superior to other methods. In order to increase the survival rate after thawing, more studies have to be done in the future. These results were recommended as the best vitrification method for canine oocytes, to improve the vitrification methods.

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