EFFECT OF eCG DOSE AND VITRIFICATION METHOD ON *IN VITRO* SURVIVAL RATE OF RABBIT EMBRYOS

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ABSTRACT

Forty one New Zealand White multiparous rabbit does with an average live body weight of 3.78±0.15 kg were used as embryo donors in two experiments. In the 1st experiment, twenty donors were intramuscularly administered with 20 or 40 IU eCG/kg live weight, 68 h before artificial insemination (AI). Immediately prior to insemination, the does were intravenously injected with hCG at 40 IU/kg live weight. In a 2nd experiment, 21 does were injected with 20 µg GnRh intramuscularly and inseminated. The embryos were collected from slaughtered does 48-50 h post AI, vitrified using one of the two vitrification methods. In the 1st method, the cryoprotective solution was a 1:1:2 solution (v/v/v) of 3.5 M DMSO, 4.4 M EG in DPBS+0.132 g $CaCl_2$/L+0.2 (w/v) BSA/L. Vitrification was carried out in two steps. In the 2nd procedure, an one-step loading process in the vitrification solution containing 40% EG, 18% Ficoll 70+0.3 M/l sucrose. Irrespective of the vitrification method, the percentage of morphologically normal embryos recovered from does injected with 40 IU eCG was lower compared to 20 IU, nevertheless, the difference was not significant. In contrast, the embryo diameter was significantly smaller when the eCG dose increases. In 40 IU eCG treated does, only 42/147 embryo vitrified-thawed reached hatching blastocysts stage (28.6%) *vs.* 95/186 (51.1%) in 20 IU group. Irrespective of the hormonal treatments in the 1st and 2nd experiments, one-step vitrification procedure with EG+Ficoll+sucrose improved insignificantly post-thaw morphologically normal embryos and development to hatching blastocysts stage compared to two-step vitrification procedure with EG+DMSO. It could be concluded that treating rabbit does with 40 IU eCG/kg live weight delays embryo development and reduced quality scores and embryo diameters compared to 20 IU. One-step vitrification method (EG+Ficoll+sucrose) appears to be appropriate for cryopreservation of rabbit embryos

Key words: Vitrification methods, eCG dose, rabbit Embryos.

INTRODUCTION

Cryopreservation enables banking of embryos for future use in medicine and in animal breeding. It also enables protection of germ plasm of endangered species and unique strains or lines of laboratory animals (Papis *et al.,* 2005). Hormonal treatments have been largely used these last 15 years, particularly PMSG which is now called eCG (Equine Chorionic Gonadotrophin). This hormone is often used by breeders who have chosen to use AI and a single batch breeding system for improving the receptivity and fertility of does (Theau-Clément *et al*., 2008). A single injection of eCG at dosages from 40 to 150 IU has been frequently used to synchronize estrus and to increase the number of oocytes or embryos (superovulation) for various embryo manipulation studies (Schmidt *et al*., 1992). For most domestic animals, the responses to superovulation treatments are not controlled as a consequence of the lack of knowledge on exogenous gonadotrophins effects on the ovarian function (Salvetti *et al*., 2007).

The survival rate after vitrification differs depending on type and concentration of cryoprotective agent. During vitrification embryo exposure to high levels of cryoprotectant additives (CPAs) can have deleterious effects on the development of the embryo after devitrification. Cryoprotectants are organic solutes that help to protect cellular organelles during cryopreservation although they may

damage the cytoskeletal system as they can be toxic and cause disruptive osmotic damage to the cell. Dimethylsulfoxide (DMSO), propylene glycol (PG), or ethylene glycol (EG), alone or as mixtures, have been used as cryoprotective additives for freezing or vitrification of rabbit embryos. Mixtures of cryoprotectants may have some advantages over solutions containing only one solute. Therefore the aim of our work was to investigate on donors, the effect of PMSG dose and vitrification method on the post-warming *in vitro* survival rate and diameter of rabbit embryos.

MATERIALS AND METHODS

This experimental work was carried out at the laboratory of Reproduction and Biotechnology, Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. Forty-one New Zealand White multiparous rabbit does with an average live body weight of 3.78±0.15 kg were used as embryo donors in two experiments. In the 1st experiment, twenty donors were intramuscularly injected with 20 or 40 IU eCG/kg live weight (Folligon, Intervet, International B.V. Boxineer-Holland), 68 h before artificially insemination (AI). Immediately prior to insemination, the does were intravenously injected with hCG (Pregnyl, Organon, Nile Co., Egypt) at 40 IU/kg live weight. In the 2nd experiment, twenty one does were intramuscularly injected with 20 µg GnRh for induction of ovulation and inseminated. The does in the 1st and 2nd experiments were inseminated with semen collected from a male of proven fertility from the same breed. The embryos were flushed from the oviduct and uterine horns of slaughtered animals 48-50 h post AI, with Dulbecco´s Phosphate Buffered Saline (®DPBS, Gibco, Cat. No 21300-017, UK) supplemented with 0.132 g $CaCl_2$/L, 0.2% (w/v) BSA at room temperature (20-25 ºC). After recovery, embryos were washed twice in fresh DPBS+10% FCS, counted and morphologically evaluated under stereoscopic microscope.

In the 1st and 2nd experiments, embryos with no abnormalities in mucin coat, *zona pellucida* and with homogenous blastomeres were vitrified using one of the two vitrification methods and media compositions. In the 1st method, described by Vicente *et al*. (1999). The cryoprotective solution was a 1:1:2 solution (v/v/v) of dimethyl-sulfoxide (3.5 M DMSO, Sigma), ethylene glycol (4.4 M EG, Sigma), in DPBS supplemented with 0.132 g $CaCl_2$/L and 0.2 (w/v) BSA/L of cryoprotective solution. Vitrification was carried out in two steps. First, embryos were pipetted into 0.2 ml of PBS medium and placed in a culture dish and then 0.2 ml of the cryoprotective solution was added and agitated. Embryos were kept in this medium for 2 minutes. In the second step, 0.6 ml of the cryoprotective solution was added and quickly agitated. Then, embryos suspended in the final vitrification solution were loaded into 0.25 ml plastic straws (IMV, L'Aigle, France), sealed with polyvinyl-alcohol sealing powder and plunged directly into liquid nitrogen. The exposure time of embryos to the final vitrification solution did not exceed 1 minute. In the 2nd procedure, an one-step loading process in the vitrification solution containing 40% EG, 18% Ficoll 70 and 0.3 M/l sucrose (Papis *et al.*, 2005) dissolved in a basic medium (HEPES-buffered CIM, Gibco BRL supplemented with 20% FCS) was used. Embryos were loaded into 0.25 ml plastic straws, sealed, identified and plunged directly into liquid nitrogen. In both procedures, the straws were stored in liquid nitrogen for two days. The warmed embryos were morphologically scored and cultured in a standard *in vitro* culture condition for 72 h in 50 µl microdrops of Ham's F10 medium+ 20% FCS under mineral oil at 38.5 °C in an incubator containing 5% CO2 and humidified air. Only embryos with homogenous cell mass and intact zonae pellucida were *in vitro* cultured. Other non-invasive parameter for the testing of embryo viability seems to be the embryo diameter (Makarevich *et al.*, 2006). After warming, embryo diameters excepting zona pellucida, were measured from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a ×40 objective and ×10 eyepieces.

Data for embryo diameters were assessed by analysis of variance using GLM procedure of SAS® Program (1998) with the fixed effect of eCG doses, (2 levels), the vitrification method (2 levels) and their interaction in the 1st experiment and in the 2nd experiment, the vitrification method (2 levels). Significance of the differences in the results was tested by Duncan's New Multiple Range Test (Duncan, 1955). For percentage a chi square was used.

RESULTS AND DISCUSSION

eCG dose treatment (experiment 1)

Irrespective of the vitrification method, the percentage of morphologically normal embryos recovered from does injected with 40 IU eCG/kg was lower compared to 20 IU eCG/kg (table 1) nevertheless, the difference was not significant. In contrast, the embryo diameter was significantly smaller when the eCG dose increases. In 40 IU eCG treated does, only 42 from 147 embryo vitrified-thawed reached hatching blastocysts stage (28.6%) compared to 95/186 (51.1%) in 20 IU eCG treated does. These results indicate that *in vitro* development of embryos vitrified-thawed was reduced ($P<0.05$) mainly by increasing the dose of eCG. These results are in accordance with those reported by Mehaisen *et al.* (2005) who found that the injection of rabbit does with 200 IU gonadotropin hormones had a negative effect on the *in vitro* development of embryos after thawing when compared to 50 IU. In earlier studies, Carney and Foote (1990) observed that superovulation reduced rabbit embryo cells number (slower rates of cell division) and size compared with embryo recovered from normally ovulated donors. Furthermore, Kauffman *et al.* (1998) reported that embryos recovered from superovulated donors, although may have normal morphological appearance, exhibiting lower developmental potential than embryos recovered from non-superovulated donors. The higher sensitivity of embryos recovered from superovulated does to low temperatures leads to a decrease in their subsequent potential capacity for development after vitrification. In addition, superovulation may cause the cytogenesis defects and chromosomal alterations of recovered embryos (Tsiligianni *et al.* 2004). Meshreky *et al.* (2007) also found that using superovulation, 63/175 vitrified embryos (80 IU eCG/doe) were developed after thawing (36%) compared to 89/174 control embryos (51.15%). However, Salvetti *et al.* (2007) and Viudes De Castro *et al.* (2009) reported that no significant differences were observed between superovulated does and control group for *in vitro* embryos development rate after a classic freezing-thawing process.

Vitrification methods (experiment 1 and experiment 2):

Regardless of the hormonal treatments in the 1st and 2nd experiments, results in table 1 showed that the one-step vitrification procedure with EG+Ficoll+sucrose used as cryoprotective agents (Papis method) gave insignificantly better results of post-thawing morphologically normal embryos and development to hatching blastocysts stage compared to the two-steps vitrification procedure with EG+DMSO (Vicente method). In Papis method, 44.9% (74/165) of embryos recovered from does treated with eCG/hCG reached hatching blastocysts stage *vs.* 37.5% (63/168) vitrified by Vicente method and 64.8% (35/54) *vs.* 57.9% (33/57), respectively, of embryos collected from does injected with GnRh. These results are in agreement with Kasai *et al.* (1992) who found that of 235 morulae vitrified by a simple method using EG+Ficoll+sucrose, 89% developed into blastocysts. Also, Popelkova *et al.*, (2009) observed that embryo rabbit vitrified by two steps in EG group showed significantly lower ($P<0.05$) hatching rate (15%) than those vitrified with one step in EG+Ficoll+sucrose (63%). Vicente *et al.* (1999) using the same vitrification procedure used in this study, observed an embryo survival of 70-71%. The composition of the vitrification solution (permeating and non-permeating macromolecules or saccharides) is among the factors influencing the cryosurvival of embryos. The improved results of post-thawing quality and developmental potential of rabbit embryos vitrified by Papis method (EG+ Ficoll+ sucrose) in this work may be due to EG and may have the advantage of lower toxicity (Kasai *et al.*, 1996). EG has a lower molecular weight than DMSO, which facilitates a rapid permeation into the cell during short-term exposure and a rapid removal from the cell after warming. These properties prevent both toxic and osmotic injuries (Massip, 2001). Moreover, addition of macromolecular substance Ficoll (mol. wt 70,000) to vitrification medium improved the survival and blastocyst rate of vitrified/warmed rabbit embryos (Popelkova *et al.*, 2009). This polymer is less toxic and can protect embryos against cryoinjury by mitigating the mechanical stress occurring during cryopreservation, and by building a viscous matrix around these embryos, which prevents crystallization during cooling and warming (Kuleshova *et al.*, 2001). Ficoll is known to have higher solubility with lower viscosity than other compounds. Kasai *et al.* (1992) suggested that Ficoll dehydrates the zona and the mucin coat of embryos. Moreover, sucrose with EG and Ficoll

significantly decreases the toxic effect of EG on mouse embryos (Kasai *et al*., 1990), and is also known to prevent osmotic swelling of blastomeres during removal of intracellular cryoprotectants after warming.

Table 1: Effect of does hormonal treatments and vitrification methods on post-thawing morphologically normal embryos, *in vitro* embryos development and embryo diameter.

eCG dose (IU)	Vitrification method	No. Embryos vitrified	Morphologically normal embryos post-thaw n (%)[1]	Embryo diameter (µm) (±SE)	Hatching blastocysts n (%)[1]
Experiment 1. Does treated with eCG/hCG:					
20	Papis (1 step and EG+Ficol+sucrose)	89	63 (70.8)	127^{a}±1.7	49 (55.1)^{a}
	Vicente (2 steps+ EG + DMSO)	97	65 (67.0)	123^{a}±1.8	46 (47.4)^{a}
40	Papis (1 step and EG+Ficol+sucrose)	76	48 (63.2)	117^{b}±2.3	25 (32.9)^{b}
	Vicente (2 steps+ EG + DMSO)	71	44 (62.0)	116^{b}±2.6	17 (23.9)^{b}
Experiment 2. Does treated with 20 µg GnRh:					
	Papis (1 step and EG+Ficol+sucrose)	54	41 (75.9)	134±2.5	35 (64.8)
	Vicente (2 steps+ EG + DMSO)	57	40 (70.2)	131±2.6	33 (57.9)

[1] Percentage based on the number of embryos vitrified.
EG = Ethylene glycol; DMSO = Dimethylsulfoxide
[a,b] Values with different superscripts in the same column in each factor differ significantly (P<0.05).

CONCLUSIONS

In vitro developmental potentialities of rabbit embryos were harmfully affected according to the eCG dose. Whereas, one-step vitrification procedure with 40% EG, 18% Ficoll 70 and 0.3 mol/l sucrose (Papis method) appears to be appropriate for cryopreservation of rabbit embryos.

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