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THE EFFECT OF VITRIFICATION PROCEDURES AND CRYOPROTECTANT TYPES ON POST-THAW SURVIVAL RATE OF RABBIT OOCYTES.

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ABSTRACT

Aim of the present study was to determine the effect of some factors affecting oocyte vitrification of rabbit does including, three cryodevices [Straws (S), Open pulled straws (OPS) and Sealed Open pulled straws (SOPS)] using three cryoprotectant types [40% ethylin glycol (EG), 40% dimethyl sulfoxide (DMSO) and their combination, 20% EG + 20% DMSO). Total of 36 New Zealand white (NZW) rabbit does with (5 mo old and 3.25±0.25 kg LBW) and were used in this study. Ovaries were collected and oocytes were harvested. The oocyte yield was recorded and the number of oocyte/ovary was counted, and oocyte recovery rate was calculated. Results indicated that total number of follicles averaged 36.0/doe and 18.0/ovary. Yield of oocytes averaged 31.3/doe and 15.5/ovary and oocyte recovery rate was 86.2%. Oocyte recovery rate was for 61.96, 11.65, 6.48, and 6.10% for compact, denuded, partial denuded and expanded oocytes, respectively. The corresponding frequency distribution was 71.89, 13.52, 7.52 and 7.07%, respectively. Relative to total number of vitrified oocytes, total survival rate of oocyte was not affected significantly by cryodevice, being 87.30, 83.09 and 85.77% for S, OPS and SOPS, respectively. Post-thaw survival rate of normal oocytes was the highest (68.33%, $P<0.05$) and abnormal oocytes was the lowest (17.44%, $P<0.05$) with SOPS and the opposite was obtained for straws (59.43 and 27.87%, respectively, $P<0.05$). While, OPS showed moderate values (63.31 and 19.78%, respectively), but did not differ significantly from each of straw and SOPS. Total survival rate of oocyte was not affected significantly by cryoprotectant type, being 85.38, 83.45 and 87.22% for EG, DMSO and their combination, respectively. Post-thaw survival rate of normal oocytes was the highest (72.56%, $P<0.05$) and abnormal oocytes was the lowest (14.66%, $P<0.05$) with EG+DMSO and the opposite was obtained for EG alone (56.92 and 28.46%, respectively, $P<0.05$). While, DMSO showed moderate values (61.97 and 21.48%, respectively).

In conclusion, using sealed open pulled straws and combination of 40% ethylin glycol and 40% dimethyl sulfoxide as cryoprotectant solution as vitrification procedures of rabbit oocytes showed the best results of post-vitrification survival rate.

INTRODUCTION

A large number of oocytes are required for various biotechnological techniques including oocyte maturation, cloning and the production of transgenic animals. Thus cryopreservation of mammalian oocytes may be used to establish an ova bank for reproductive biotechnology.

Cryopreservation of oocytes is more difficult than embryos. Many problems are associated with the cooling and freezing of *in vitro* matured or *in vivo* ovulated oocytes, namely spindle disorganization (Magistrini and

Szollosi, 1980), loss or clumping of microtubules resulting in scattering of chromosomes (Sathananthan *et al*., 1988), increased polyploidy during fertilization (Carroll *et al*., 1989) and a decreased fertilization rate (Wood *et al*., 1992).

The difficulty, in obtaining acceptable rates of survival and functionality for oocytes after cryopreservation, is due to the size of this cell and its unique morphologic characteristics. Vitrification is a process whereby a solution with a high solute concentration will solidify into a glass-like structure upon cooling, without any ice crystal formation. The process may be thought of as simply increasing viscosity until a solid state is reached (Shaw, *et al*., 2000a). Vitrification is an alternative that may increase oocyte survival after thawing. Vitrification is the solidification of a solution at low temperatures without ice crystal formation. This phenomenon requires either rapid cooling rates (Rall, 1987) or the use of concentrated cryoprotectant solutions, which depress ice crystal formation and increase viscosity at low temperatures (Vajta, 2000).

The present study was carried out to study the effect of vitrification procedures {straws (S), open pulled straws (OPS) and sealed open pulled straws (SOPS) and cryoprotectant types {ethylin glycol (EG), dimethyl sulfoxide (DMSO) or its combination) on quality of vitrified rabbit oocytes.

MATERIALS AND METHODS

This study was carried out at International Livestock Management Training Center, (ILMTC), Sakha, Kafr El-Sheikh Governorate belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture during the period from August 2008 to August 2009.

Animals:

Total of 36 New Zealand white (NZW) rabbit does with average of 5 mo old and 3.25±0.25 kg live body weight (LBW) were used in this study. All rabbit does were subjected as donors of oocytes and 5 rabbit does were used for blood collection for preparing rabbit doe serum (RDS).

All does were kept under the same conditions of feeding and management in the special farm, being individually housed in metal cages (40 x 50 x 60 cm) provided with feeders and water nibble in each cage. Does were fed *ad. libitum* on a commercial pelleted concentrate diet.

Ovarian collection:

Immediately after slaughtering, ovaries were removed, washed by saline solution (0.9% NaCl) and dried by cleaning paper. Ovaries were collected and excised, submerged in a flacon plastic tissue culture dishes (60 x 15 mm) containing saline solution supplemented with (Sodium penicillin G, 100 IU/ml and streptomycin, 100 µg/ml) and transported to the laboratory within 5 minute.

Oocyte collection:

All visible follicles on the ovarian surface with ≥1 mm in diameter were counted. Oocytes were collected using slicing technique into glass Petri dishes containing 4 ml of harvesting medium (Phosphate buffer saline, PBS)

supplemented with sodium penicillin G (100 IU/ml) and streptomycin (100 µg/ml). Ovaries were held by a forceps and by a scalpel blade were made incisions along the whole ovarian surface. Each ovary was washed three times with harvesting medium in dishes containing 2 ml medium for washing and searching oocytes using stereomicroscopy.

Preparation of harvesting medium:

Phosphate buffer saline (PBS) medium was prepared according to Gordon (1994). Two mg/ml of bovine serum albumin (BSA) was added to PBS. The pH value of the medium was adjusted to 7.2-7.4 using pH-meter and to osmolarity of 280-300 mOsmol/kg using osmometer. Then, the medium was filtered by 0.22 µm millipore filter (milieux GV, millepore, Cooperation Bedford MOA.).

Yield and recovery rate of oocytes:

The oocyte yield was recorded and the number of oocyte/ovary was calculated. The recovery rate was determined as percentage of oocytes in proportional to total number of visual follicles presented on the ovarian surface of each doe using the following formula of recovery rate (RR%):

RR (%) = (Number of recovered oocytes/number of follicles) x 100

Oocyte categories:

After collection, oocytes were washed three times in harvesting medium, counted, evaluated under inverted microscope and classified into six categories, *compact*, *expanded, denuded, partial denuded and degenerated oocytes.*

Preparation of doe rabbit serum:

Doe rabbit serum was prepared from blood collected from 5 rabbit does. The collected blood was centrifuged two times at 3000 rpm for 15 minutes. Clear sera were aspirated by pasture pipette and placed in another 15 ml-sterile tubes. These tubes were placed into water bath at 56^{o}C for 30 minutes, and then left to cool. Thereafter, sera were placed into 1.5 ml eppendorf tubes and frozen until used.

Vitrification procedures:

Only morphologically high quality immature oocytes (COCs), as determined by uniform granular, homogeneously distributed cytoplasm surrounded by compact layers of cumulus cells were vitrified in this study. Oocytes were vitrified by different vitrification procedures (S, OPS and SOPS) with each of three cryoprotectants (40% EG, 40% DMSO or combination of 20% EG +20% DMSO) using three carrier systems.

The vitrification procedures employed throughout this experiment were based on the methods originally designed by Silvestre et al. (2003). The basal medium used for vitrification (VS) was TCM-199 (Sigma) supplemented with 20% (*v:v*) of DRS.

Types of cryodevice:

Conventional straws:

Plastic insemination straws (0.25 ml, IVM L' Aigle, France) were used for oocyte vitrification. In this method, ten COCs were transferred in 50 µl of VS then, oocytes with 3 µl loading into a column located between two columns of vitrification solution separated by air bubbles into the center of

0.25 ml plastic insemination straws using a fine glass capillary pipette. After heat-sealing, the straws were plunged immediately into LN_2 (-196°C).

Open pulled straws (OPS):

Conventional plastic insemination straws (0.25 ml, IVM L' Aigle, France) were heat-softened over a Bunsen burner and pulled manually. The straws were cooled in air and then cut at the tapering end with a blade. The inner diameter of the tip decreased from 1.7 mm to 0.8 mm, and the wall thickness diminished from 0.15 to 0.07 mm according to Vajta et al. (1998).

In this method, ten COCs were transferred in 50 µl VS then, the COCs were transferred to a 3 µl droplet of and loaded into a OPS by capillary action. The OPS were plunged immediately into LN_2 (-196°C).

Sealed open pulled straws (SOPS):

The preparation of SOPS was similar to that described previously for OPS. The straws were cut at the narrowest point with a razor blade. Only half with the cotton plug was used (Lopez-Bejar and Lopez-Gatius, 2002).

In this method, ten COCs were transferred in 50 µl VS then, the COCs were aspirated into a column located between two columns of vitrification solution separated by air bubbles. The other end of the straw is sealed with heat-sealing before plunged immediately into LN_2 (-196°C).

In all methods, three VS containing different tested cryoprotectants were used and oocytes were exposed to VS for 20–25 seconds prior to immersion in LN2.

Warming method:

After storage for at least 3 weeks in LN2, vitrified oocytes were warmed by holding the straws for 6 sec in air and then agitating them in water bath at 20 °C for at least 10 sec. The contents of each straw were expelled into Petri dish. To remove of intracellular cryoprotectants, oocytes were transferred to solution of TCM-199 supplemented with 10% DRS and 0.25 M sucrose, then in the same medium with 0.125 M sucrose and finally in the same medium without sucrose, for 5 min per solution,.

Evaluation of post-warming oocyte viability:

Oocyte viability was evaluated morphologically based on the integrity of the oolemma and zona pellucida; loss of membrane integrity (lysis) was obvious upon visual inspection as the sharp demarcation of the membrane disappeared and the appearance of the cytoplasm changed. The criteria used for assessing the pot-thaw morphology of vitrified/warmed oocytes were normal and abnormal oocytes.

Statistical analysis:

Data were analyzed using analysis of variance using computer program of SAS (2000). The significant differences among group means were preformed using Duncan Range Test (Duncan, 1955).

RESULTS

Characteristics of follicles and oocytes:

Number of follicles:

Total numbers of follicles and average number of follicles per doe or ovary are shown in Table (1). Results indicated that total number of follicles

including count of vesicular follicles, small follicles < 3 mm, medium follicles 3-10 mm and large follicles >10 mm, was 1296 follicles, averaging 36.0/doe and 18.0/ovary, regardless the ovarian side.

Table (1): Total and average number of follicles per doe or ovary observed on the ovarian surface of doe rabbits.

Number of does	Number of ovaries	Number of follicles	Number of follicles	
			Per doe	Per ovary
36	72	1296	36.0	18.0

Yield and recovery rate of oocytes:

Results shown in Table (2) revealed that total of 36 doe rabbits yielded a total number of 1117 oocytes, averaging 31.03 and 15.5 oocytes per doe and ovary, respectively. This reflected in higher oocyte recovery rate, being 86.2%.

Table (2): Total and average number of oocytes per doe or ovary recovered from ovarian follicles of doe rabbits.

Number of follicles	Number of oocytes	Number of oocytes		Recovery rate (%)
		Per doe	Per ovary	
1296	1117	31.03	15.5	86.2 %

Recovery rate of different oocyte categories:

Results presented in Table (3) show that the total oocyte recovery rate (86.2%) was the highest for compact oocytes (61.96%), followed by denuded and partial denuded (11.65 and 6.48%, respectively), while the lowest rate was obtained for expanded oocytes (6.10%).

Examination of oocytes recovered from the ovaries of doe rabbits in all groups revealed that all oocytes were immature and were evaluated into compact, denuded, partial denuded and expanded oocytes (Plates 1-4, respectively).

Table (3): Number and recovery rate (%) of rabbit oocytes at different categories.

Number of follicles	Total oocytes	Oocyte category							
		Compact		Denuded		P. denuded		Expanded	
		n	%	n	%	n	%	n	%
1296	1117	803	61.96	151	11.65	84	6.48	79	6.1

Frequency distribution of different oocyte categories:

Results shown in Table (4) revealed the highest frequency distribution was obtained for compact oocytes (71.89%), followed by denuded (13.52%) and partial denuded (7.52%), while the lowest distribution was recorded for expanded oocytes (7.07%). Different oocyte categories recovered from all visual follicles on the ovarian surface of doe rabbits are illustrated in Plates (1-4).

Table (4): Number and frequency distribution (%) of oocytes at different categories.

Total numbe of oocytes	Oocyte category							
	Compact		Denuded		P. denuded		Expanded	
	n	%	n	%	n	%	n	%
1117	803	71.89	151	13.52	84	7.52	79	7.07

N= Number

Post-thaw survival rate of vitrified oocytes:

Effect of cryodevice:

Data in Table (5) show that total survival rate of oocyte was not affected significantly by cryodevice, being 87.30, 83.09 and 85.77% for S, OPS and SOPS, respectively. However, post-thaw survival rate of normal and abnormal oocytes relative to total vitrified oocytes was affected significantly (P<0.01 and P<0.001, respectively) by cryodevice, being significantly (P<0.05) the highest (68.33%) with SOPS and the lowest (59.43%), while OPS showed moderate values (63.31%), but did not differ significantly from each of straw and SOPS.

Table (5): Effect of cryodevice on survival rate of different types of oocytes relative to total vitrified rabbit oocytes.

Cryodevice	N	Post-thawing survival oocytes					
		Total		Normal		Abnormal	
		n	%	n	%	n	%
Straw	244	213	87.30	145	59.43^{b}	68	27.87^{a}
OPS	278	231	83.09	176	63.31^{ab}	55	19.78^{b}
SOPS	281	241	85.77	192	68.33^{a}	49	17.44^{b}

a and b: Means denoted within the same column with different superscripts are significantly different at P<0.05. N= Total number of vitrified oocytes.

On the other hand, survival rate of oocytes in abnormal type was significantly (P<0.05) higher with S than those with OPS and SOPS (27.87 *vs.* 19.78 and 17.44%, respectively. These results indicated the beneficial effect of SOPS as cryodevice on survival rate of rabbit oocytes.

Effect of cryoprotectant type:

Results presented in Table (6) revealed that the effect of cryoprotectant type on total survival rate of oocytes relative to vitrified oocytes was not significant, ranging between 87.22% with the combination of DMSO and EG and 83.45% with DMSO. However, post-thaw survival rate of oocytes as normal and abnormal oocytes relative to total number of vitrified oocytes was affected significantly (P<0.05) by cryoprotectant type.

Table (6): Effect of cryoprotectant type on survival rate of different types of oocytes relative to total vitrified rabbit oocytes.

Type of cryoprotectant	N	Post-thawing survival oocytes					
		Total		Normal		Abnormal	
		n	%	n	%	n	%
EG	253	216	85.38	144	56.92^{b}	72	28.46^{a}
DMSO	284	237	83.45	176	61.97^{b}	61	21.48^{b}
EG+DMSO	266	232	87.22	193	72.56^{a}	39	14.66^{c}

A and b: Means denoted within the same column with different superscripts are significantly different at P<0.05. N= Total number of vitrified oocytes.

DISCUSSION

Oocyte recovery rate:

Based on the present results of recovery rate and frequency distribution of different oocyte categories, higher yield of oocytes was obtained from rabbit ovaries. Al-Hasani *et al*. (1987) found that the average number of oocytes recovered from ovarian follicles by puncture was 11.7 oocytes/doe versus 31.03/doe as obtained in the present study. Hulot and Matheron (1981) reported that average number of rabbit oocytes that are shed after mating varies according to the strain, and is correlated with live body weight. The wide variation in number oocytes/ovary recovered from rabbits may be attributed to pronounced differences in animal ages, reproductive status, site of the ovary and their interactions (Khalil, 2005). Also, diameter of needle used in oocyte harvesting by aspiration was found to affect recovery rate of oocyte (Abdoon, 2001). Moreover, the differences in number of oocytes/ovary may be related to size of follicles (Shamiah, 1997). In accordance with the present results, data of El-Ratel (2008) clearly revealed that frequency distribution of oocytes at compact category was the highest in NZW rabbits, being 55.6%. However, frequency distribution of oocytes was 30.5, 8.3 and 5.6% at denuded, partial denude and expanded categories.

Effect of cryodevice:

A variety of new techniques and types of holders/devices have been tested with the aim of improving the overall survival of oocytes post-cryopreservation. Several open-carrier systems and closed carrier methods for oocyte vitrification were developed. Until recently, 0.25 ml standard insemination straws were used almost exclusively for vitrification of oocytes and embryo with a relative cooling speed of 2,500°C/min (Palasz and Mapletoft, 1996) by direct plunging into liquid nitrogen (LN2). Methods using cryodevices such as OPS (Vajta *et al*., 1998), cryoloops (Lane *et al*., 1999) and cryotops (Kuwayama and Kato, 2000) allow oocyte vitrification in a volume as little as few microliters. Open pulled straws rendered a high cooling-warming rate of more than 20,000°C/min. Dropping oocytes containing vitrification solution directly into LN2 (Papis *et al*., 1999) successfully eliminated the insulation effects of the container wall. Yet, plunging a warm object into LN2 resulted in boiling the liquid and creating an isolating layer of nitrogen vapor around the object.

A vitrification device was developed by Arav *et al*. (2000) using LN2 slush by reducing the temperature of LN2 to -205°C. These methods use very high cooling rates coupled with minimal volume of freezing medium containing high concentration of cryoprotectants that permits rapid traversal of temperature zones, corresponding to chilling injury. As applied in this study on rabbit oocytes, Vajta *et al*. (1998) reported vitrification using open pulled straws (OPS) for bovine embryos as well as oocytes. Later on the improved OPS methods using glass capillary (Hochi, *et al*., 2000 & 2001).

Type of cryoprotectant:

The primary components in successful freezing and vitrification solutions are cryoprotectants (CPAs). Permeating CPAs are essential for dehydrating intracellular water. Additionally, they lower the freezing point, thus giving more time for dehydration. Careful selection of CPAs must be made however, first for toxicity and second for permeability. The CPAs are toxic at high concentrations, but toxicity is minimized at low temperatures, and with short periods of exposure (Agca *et al*., 1998). The CPAs protect slowly frozen cells by one or more of the mechanisms such as suppressing high salt concentrations, reducing cell shrinkage at a given temperature, reducing the fraction of the solution frozen at a given temperature and minimizing intracellular ice formation. Ice formation can be eliminated entirely, both within the cells and the extra-cellular matrix, when CPAs are used in extremely high concentrations (at least 50% volume/volume) as reported by Fahy (1988). The CPAs used in early experiments are glycerol (Willadsen, 1977). Glycerol and DMSO are the most commonly used CPAs. To achieve high cooling rates requires the use of high concentration of the CPA solution, which depresses ice crystal formation. There is critical concentration required for vitrification. Kanno *et al*. (1975) were able to demonstrate that the temperature at which crystallization begins can be reduced through an increase in the hydrostatic pressure. Finally, reducing the cooling rate can be achieved by reduction of minimal concentration through the additional use of polymers which are non-permeable and therefore remain in the extra-cellular area (Fahy *et al*., 1984).

Ethylene glycol is currently the most commonly used permeating CPA for the vitrification of oocytes including human oocytes and embryos, because of its low molecular weight, high permeation ability, and low toxicity. Its low molecular mass permits fast influx and efflux into and out of cells (Kuwayama *et al*., 2005). O'Neill *et al*. (1997) observed that addition of polyethylene glycol (PEG) resulted in greatly improved viability of oocytes following cryopreservation. Minimizing the toxicity of the CPAs can also be achieved by using a combination of two CPAs, and a stepwise exposure of cells to pre-cooled concentrated solutions. In agreement with the present results, some studies mixed other permeating agents, such as DMSO, to reduce the concentration of single CPAs (Vajta *et al*., 1998; Yokota *et al*., 2000). Also, Valdez *et al*. (1992) showed that EG was the least toxic to mouse embryos and successfully used in combination of 20% EG, 20% DMSO and 10% 1, 3-butanediol. Moreover, Valdez *et al*. (1992) found that EG is a very weak glass forming molecule, whereas DMSO is a better glass forming molecule, so often times the two are combined in vitrification solutions.

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تأثيرطرق التجميد بالتزجج وانواع المواد الواقية على معدل الحيوية أثناء الإسالة لبويضات الأرانب٠

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تهدف هذه الدراسة الى تحديد تأثير ٣ انواع من المواد الواقية ٤٠% اثيلين جليكول – ٤٠% داى ميثيل سلفوكسيد وخليط منهما معا (٢٠% اثيلين جليكول + ٢٠% داى ميثيل سلفوكسيد) مع ٣ أنواع من الأدوات المستخدمة فى اجراء التجميد بالتزجج (القشات العادية – القشات مفتوحة الطرفين – القشات ذات طرف واحد مفتوح) ٠ تم اجراء هذه الدراسة على عدد ٣٦ أنثى نيوزيلاندى بيضاء متوسط عمرها ٥ شهر تقريبا ومتوسط الوزن الحى ٣,٢٥ كجم تم استخدام الإناث فى هذه الدراسة للحصول على البويضاتتم جمع البويضات من المبايض وتم حساب معدل استرداد البويضالت من المبايض ٠وقد اشارت النتائج الى ان العدد الكلى للحويصلات المرئية التى تم الحصول عليها من المبايض هى ١٢٩٦ حويصلة بمتوسط ٣٦ حويصلة لكل أم و١٨ حويصلة لكل مبيضبينما كان العددالكلى للبويصات التى تم الحصول عليها من الحويصلات المبيضية ١١١٧ بويضة بمتوسط ٣١,٠٣ لكل أم و١٥. ٥ لكل مبيض ٠ وكان المعدل الكلى للبويضات المستردة هى ٨٦,٢% حيث سجلت البويضات الجيدة اعلى معدل استرداد بنسبة ٦١,٩٦% تعقبها البويضات المعراه كليا ثم البويضات المعراة جزئيا بنسبة(١١,٦٥-٦,٤٨%) على الترتيب٠ بينما سجلت البويضات المتوسطة اقل معدل استرداد بنسبة ٦,١٠%٠ وكانت نسبة التوزيع النسبى للبويضات الجيدة أعلى من البويضات المعراه كليا ثم المعراه كليا ثم المتوسطة بنسبة (٧١,٨٩- ١٣,٥٢- ٧,٥٢- ٧,٠٧%)٠ وقد اوضحت النتائج ان طرق الحفظ بالتزجج باستخدام القشات ذات طرف واحد مفتوح اظهرت اعلى نتائج على معدل الحيوية للبويضاتبعد التجميد وكان العدد الكلى للبويضات الطبيعية وغير الطبيعية هى (٨٥,٧٧- ٦٨,٣٣-١٧,٤٤%) على الترتيب مقارنة بالطرق الأخرى٠ وكان العدد الكلى للبويضات التى تم حفظها بالتزجج اعلى معنوية عند استخدام خليط من اثيلين جليكول + داى ميثيل سلفوكسيد (٢٠% اثيلين جليكول + ٢٠% داى ميثيل سلفوكسيد)٠عن البويضات التى تم حفظها باستخدام اثيلين جليكول بمفرده أو داى ميثيل سلفوكسيد بمفرده

قام بتحكيم البحث

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