aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Asian Journal of Animal and Veterinary Advances

ISSN 1683-9919
DOI: 10.3923/ajava.2016.608.619

Research Article
Effect of Cryoprotective Solutions, Ethylene Glycol, Dimethyle-sulfoxide and Ficoll 70 with Different Combination Ratios on Vitrification of Bovine Oocytes and Embryos Produced *in vitro*

[1]E.M.M. Abdel-Gawad, [1]B.R. Abdel-Halim, [2]N.A. Helmy and [1]A.F. Badr

[1]Department of Theriogenology, Faculty of Veterinary Medicine, Beni-Suef University, Egypt
[2]Department of Physiology, Faculty of Veterinary Medicine, Beni-Suef University, Egypt

Abstract

Objective: The objective of the present study was to compare cryoprotective solutions such as Ethylene Glycol (EG), dimethyle-sulfoxide (DMSO) and ficoll 70 with different combination ratios for vitrification of mature bovine oocytes and embryos produced *in vitro*. In addition to the demonstration of the effect of the straw diameter on post thawing viability of the thawed matured oocytes and embryos. **Materials and Methods:** A total of 509 oocytes were collected from 175 ovaries by slicing technique. Matured oocytes frozen in solutions containing 20% EG+20% DMSO+0.3 M trehalose had mean survival rate of (44.43±4.98%). Mature oocytes frozen in solutions containing 40% EG and 18% ficoll 70 by a ratio of 1:1, 2:1 and 3:2 in volume had a mean survival rate of 49.22±1.66, 54.33±3.11 and 62.00±3.71%, respectively. **Results:** The present study revealed that blastocysts cryopreserved in media containing EG+ficoll (3:2) had a significantly ($p<0.01$) higher recovery rate (79.28±13.08) compared to 45.00±16.24 blastocyst in embryos cryopreserved in DMSO, respectively. Moreover, recovery rates of blastocysts cryopreserved in media containing EG+ficoll (2:1) and in media containing EG+ficoll (3:1) were numerically higher than those cryopreserved in DMSO group (50.00±3.74 and 63.49±6.83, respectively). Also using of ministraw for the cryopreservation of mature bovine oocytes had post-thawing viability significantly higher (<10%) than using midistraw. **Conclusion:** By this study it can be concluded that using of ministraw for the cryopreservation of mature bovine oocytes had post-thawing viability significantly higher (<10%) than using midistraw. So, combination of 40% EG+18% ficoll 70 by the ratio of 3:2 act as a good cryoprotectant combination for vitrification of mature bovine oocytes.

Key words: *In vitro* maturation, cryopereservation, bovine, oocytes, ficoll 70, EG, DMSO, vitrification, embryo transfer

Received: April 27, 2016 Accepted: August 02, 2016 Published: September 15, 2016

Citation: E.M.M. Abdel-Gawad, B.R. Abdel-Halim, N.A. Helmy and A.F. Badr, 2016. Effect of cryoprotective solutions, ethylene glycol, dimethyle-sulfoxide and ficoll 70 with different combination ratios on vitrification of bovine oocytes and embryos produced *in vitro*. Asian J. Anim. Vet. Adv., 11: 608-619.

Corresponding Author: B.R. Abdel-Halim, Department of Theriogenology, Faculty of Veterinary Medicine, Beni-Suef University, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Vitrification is the rapid cooling of cells in liquid medium in the absence of ice crystal formation. Vitrification can be achieved when the intracellular concentration of cryoprotective agents (CPAs) is higher[1] than 6 mol L^{-1}. The benefits of a two-step vitrification method are that it allows establishment of a relatively complete equilibrium, while reducing exposure of the oocyte to potential toxic effects of CPAs. Previously, oocytes or embryos were first exposed to non-vitrifying solutions containing permeating CPAs[2]. Next, the oocytes were exposed for a short time (45-60 sec) to a Vitrifying Solution (VS) containing high concentrations of penetrating (4.8-6.4 mol L^{-1}) and non-penetrating (0.5-0.75 mol L^{-1}) CPAs before being plunged into liquid nitrogen (LN2)[3]. Since, the 1st successful vitrification of mouse embryos by Rall and Fahy[1], this method has been used widely for oocyte and embryo cryopreservation. Numerous studies have focused on CPA permeability and the rate at which it enters cells[4]. Other studies have investigated incubation times in both the pre-treatment and vitrification solutions and found that the temperature used during the handling procedure is also important for successful vitrification[5]. Open Pulled Straw (OPS) method originally described by Vajta *et al.*[6] allows for faster heat transfer between the solution and the environment, achieving cooling/warming rates on the order of 20,000°C min^{-1}. In other studies, compared three approaches (standard 0.25 mL straw, OPS and micro drop) for cooling a vitrification solution containing bovine oocytes, the highest cleavage rate was achieved with the traditional straw[7]. These variations make the vitrification method seem difficult to master, which has limited the application of this technology in the field of reproductive biology[8]. Cells react to changes in extracellular osmolarity by altering their volume. Cells exposed to hypotonic or hypertonic solutions initially react either by swelling (hypotonic solutions) or shrinking (hypertonic solutions) due to water exchange but later recover as permanent solutes equilibrate across the cell membrane[9]. The final intracellular concentration of cryoprotectant (ICCP) after incubation in vitrification solutions by exposing cells to sucrose solutions with defined molarities was estimated by Vanderzwalmen *et al.*[3]. The ICCP was calculated from the sucrose concentration that produced no change in cell volume, i.e., when intra and extracellular osmolarities were equivalent. Mouse oocytes were successfully cryopreserved[10]. Bovine oocytes were also vitrified and remained viable for offspring production after *in vitro* fertilization and embryo transplantation[11]. Vitrified buffalo oocytes with 51.1% glycerol via the straw method, obtained a maturation rate of 23.5% after thawing[11]. When glycerol was used with EG, which increased permeability of the cell membrane during oocytes vitrification and maturation rates of 30 ses exposure groups did not differ from those of controls[12]. Additionally, the Open Pulled Straw (OPS) method results in a better survival rate during cryopreservation than the straw method[13]. However, unlike other methods, the straw method is safer for oocytes vitrification because the oocytes are free of bacterial contamination due to a lack of direct contact with liquid nitrogen. Oocytes were exposed to the cryoprotectant composed of 40% (v/v) ethylene glycol, 18% (w/v) ficoll 70 and 0.3 M sucrose (EFS40) in three stepwise dilutions. Thawing was conducted with a series of 0.5, 0.25 and 0.125 M sucrose dilutions in 20% Fetal Bovine Serum (FBS). Thawing resulted in 98.9% morphological survival with intact cumulus cells in both populations of oocytes[14]. The present study was designated to compare cryoprotective solutions, such as Ethylene Glycol (EG), dimethyle-sulfoxide (DMSO) and ficoll 70 with different combination ratios for vitrification of mature bovine oocytes. In addition to the demonstration of the effect of the straw diameter on post thawing viability of the thawed matured oocytes.

MATERIALS AND METHODS

Animals and slaughterhouse materials: A total of 509 oocytes were collected from 175 ovaries of mature cows (3-9 years age). Ovaries were transported in container containing saline 0.9% at 25 approximately 45 min after the animal was slaughtered at a local abattoir in Beni-Suef governorate. Ovaries were rinsed 3 times with PBS to make the ovaries neat in the washing solution[15].

Chemicals and cryoprotectant agents: Ficoll 70, DMSO and mineral oil were purchased from Sigma Aldrich, while Tissue Culture Medium-199 (TCM-199), Fetal Calf Serum (FCS) and Bovine Serum Albumin (BSA) were purchased from Biomed. Phosphate Buffer Saline (PBS) was used freshly prepared at the lab.

Collection of oocytes: Ova were collected through slicing technique of the ovaries in sterile 9 cm petri dishes contain PBS supplemented with 10% BSA[16].

Selection of oocytes for maturation: Under stereomicroscope the oocytes were washed 3 times with TCM-199 supplemented with 50 mg mL^{-1} gentamycin sulfate.

According to Ganguli *et al.*[17], the recovered oocytes were classified based upon their morphological criteria into 4 categories:

Grade I: Oocytes with evenly granulated cytoplasm and completely surrounded by multiple layers of cumulus cells "cCOCs".

Grade II: Oocytes which were surrounded by scanty layers of cumulus cells "sCOCs".

Grade III: Nude oocytes that were devoid of cumulus cells. This grade was excluded from culturing, while grade I and II oocytes were included to be cultured.

Grade IV: Mature bovine oocytes.

***In vitro* maturation (IVM) of selected oocytes:** For maturation of oocytes, COCs were washed twice in TCM-199 supplemented with 10% FCS, 50 mg mL^{-1} gentamycin sulfate[18] then transported to 50 µL droplets of the maturation medium supplemented with 0.2 IU Follicular Stimulating Hormone (FSH), 2.0 IU human chorionic gomadotropin (hCG) per milliliter[19,20]. The oocytes-containing-droplets (10 cells per droplet) were covered with 4 mL sterile mineral oil to prevent evaporation. The cells were incubated for maturation in CO_2 incubator at temperature of 38.5°C, 5% CO_2 tension for 24 h[18]. After which the oocytes were examined under stereomicroscope for evaluation of cumulus cell expansion[21]. Accordingly, the oocytes were classified into 3 classes:

- Excellent which have evenly granulated ooplasm with multiple expanded layers of undegenerated cumulus cells
- Good oocytes showing evenly granulated ooplasm surrounded by some layers of expanded cumulus cells leaving 2 or 3 inner more unexpanded cumulus layers
- Poorly mature oocytes in which the cell exhibited uneven cytoplasm with unexpanded and/or degenerated cumulus cells. Excellent and good mature COCs were used for cryopreservation in different cryopreservation media while poorly mature oocytes were excluded

Cryopreservation and ultra-rapid freezing: Cryoprotectants for vitrification were DMSO, EG, ficoll 70 and trehalose and these were applied to make up various kinds of freezing media. All cryoprotectants were stocked by a version of PBS containing 18% FCS except the control group, which was stocked by TCM-199. Four kinds of cryoprotectant solutions were used in the present study as follows: The DMSO group (control group)[16]: 20% DMSO+20% EG+0.3 M trehalose. The ficoll 70 group[22]: 40% EG+18% ficoll 70 (volume ratio of 1:1 mL), 40% EG+18% ficoll 70 (volume ratio of 2:1 mL) and 40% EG+18% ficoll 70 (volume ratio of 3:2 mL).

***In vitro* capacitation of cattle spermatozoa:** Motile spermatozoa were selected using swim-up technique[23]. For this purpose, 2 straws of frozen bull semen received from Artificial Insemination Centre, Beni-Suef were thawed in a water bath at 37°C for 30 sec then the semen was pooled in a sterile warm tube. Six conical sterile eppendorf tubes each contained 1.0 mL S-TALP medium was prepared. In each conical tube, 50 µL of semen was layered under 1.0 mL of the medium. The tubes held at 45°C angle for 1 h at 39°C after which 200 µL of the upper most supernatant of each tube (that contain highly motile spermatozoa) were pooled in centrifugation tube. The pooled semen was centrifuged at 1800 rpm for 10 min after which the supernatant was discarded and the sperm pellet was resuspended in 1.0 mL of F-TALP medium for 10-15 min for capacitation[24]. Evaluation of sperm capacitation can be determined under the microscope to detect the hypermotility and clumping of sperms by head due to acrosomal reaction. Sperm concentration was measured by haemocytometer and a sufficient medium was added to yield the final concentration of 2×10^6 sperm mL^{-1} (4000 sperm cell per droplets).

***In vitro* fertilization of mature oocytes:** Following maturation, excellent and good matured COCs were washed thrice by F-TALP medium then placed in 50 µL droplets of the same medium then incubated in CO_2 incubator for 1 h after which oocytes were inseminated with sperm suspension (2 µL per droplet). If some cumulus cells were still surrounding the oocytes, it is necessary to nude the cells by repeated gentle pipetting 24 h post-fertilization. Twenty four hours later, the inseminated oocytes were washed 3 times using H-TCM-199 medium s upplemented with 10% FCS, 50 µg mL^{-1} gentamycin sulfate and 5 µL mL^{-1} L-glutamine then transferred to droplets of the same supplemented medium (5 oocytes/100 µL) and incubated in the CO_2 incubator. Fertilization was detected by the appearance of the peripherally located second polar body and confirmed by cleavage of oocytes[25].

***In vitro* culture:** The oocytes were cultured in the supplement H-TCM-199 and placed in the CO_2 incubator with change of the medium every 48 h for 7 successive days[26].

Embryo identification and evaluation: Cleaved embryos were classified according to their cell number during a specific time as 1-cell on day 1, 2-cells on day 2, 4-cells on day 3, 8-cells on day 4 and 16-cells embryo on day 5 as well as morula and blastocyst stages on days 6 and 7, respectively[27].

Cryopreservation of mature oocytes and embryos: Vitrification method was used for cryopreservation of cattle oocytes and embryos. For this purpose excellent and good mature COCs as well as morphologically normal compact morula and blastocysts were used[16].

Vitrification of mature oocytes and embryos by EG and DMSO (control group): Base Medium (BM) that used during vitrification was H-TCM-199 supplemented with 20% FCS and 50 μL mL^{-1} gentamycin sulfate[28,29]. The 1st Vitrification Solution (VS_1) consisted of 10% (v/v) EG+10% (v/v) DMSO as permeating cryoprotectants in BM, while the 2nd Vitrification Solution (VS_2) contained 20% (v/v) EG+20% (v/v) DMSO+0.3 M trehalose in the BM[16].

Loading and thawing of straws: Straws of 0.25 and 0.5 mL were preloaded to give the following configuration, 150 μL BM, 5 μL air, 2×10 μL of the VS_2 separated by 5 μL air then matured oocytes and embryos were loaded in the final 3rd column of the vitrification solution (10 μL) followed by 5 μL air then the reminder of the straw was filled with BM. Five matured oocytes or embryos were loaded in each straw that was heat-sealed and immediately dipped vertically in liquid nitrogen (-196°C)[28]. After the appropriate vitrification period (5-7 days), the mature oocytes and embryos (at morula and blastocyst stages) were thawed by gentile agitation of the straws for 10 sec in air followed by 20 sec at 35°C using a water bath. Immediately, the straw contents were expelled in 6 cm diameter petri dishes for examination[30].

Vitrification of mature oocytes and embryos by EG and ficoll 70: The cryoprotectants were stocked by a version of PBS medium containing 18% fetal calf serum. The EG was prepared with DPBS into a fraction of 40%. Ficoll 70 was prepared to an 18% stocking solution. Three ratios of cryoprotectant solutions were used in the present study as follows: 40% EG+18% ficoll 70 (volume ratio of 3:1 mL) and 40% EG+18% ficoll 70 (volume ratio of 3:2 mL). Matured oocytes were pipetted out from 18% FCS in PBS and were then immersed in the freezing medium for 10 min. Matured oocytes were then packed into 0.25 and 0.5 mL straws and the sealed-labeled straws were plunged directly into liquid nitrogen for preservation. Straws were removed from the liquid nitrogen tank and put directly into the 25°C water bath for 1 min for thawing. Approximately 1 min later, water onthe surface of thawed straw was wiped away to avoid cell collapsing due to an imbalance of water electrolytes. One end of thawed straw was then cut and let thawed oocytes be squeezed into 0.5 M sucrose solution for eluting out cryoprotectants. Ten minutes later, ova were put into 18% FCS in PBS for observing the integrality of frozen-thawed oocytes[22].

Evaluation of post-thawing viability of mature oocytes and embryos: The oocytes and embryos were morphologically examined under stereomicroscope (X100) and the cells were considered normal if they have spherical and symmetrical shape with no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content. On the other hand, abnormal cells form appeared to have a rupture zona pellucida or fragmented cytoplasm with signs of degeneration[31]. The post-thawing viability of mature oocytes and embryos can also determine by the use of trypan blue stain (0.05% in PBS) for 2 min to differentiate the live and dead cells without adverse effects on the cells[32]. It is worth mentioning that *in vitro* survival rate is defined as the ratio of viable cells after thawing to the total vitrified cell number[33].

Statistical analysis: The obtained data were subjected to statistical analysis as outlined by Snedecor *et al*.[34].

RESULTS

The obtained results showed that, a total of 175 ovaries were sliced to yield 728 oocytes which were recovered and graded into 4 different grades. The mean percentages of recovered grade I and II oocytes related to total recovered oocytes per replicate were 38.73±2.92 and 33.51±2.42%, respectively. Moreover, the average maturation rate of recovered grade I and II oocytes was 80.62±3.14 with a range of 66.66-96.15% (Table 1, Fig. 1, 2). Regarding the effect of straw type on post-thawing viability of cryopreserved mature oocytes, data presented in Table 2 show that out of 251 oocytes which were preserved in midistraws, 116 oocytes appeared morphologically normal post-thawing representing a mean percentage of 47.16±2.62%. A significantly higher ($p<0.01$) percentage of post-thawing morphologically normal oocytes (57.83±2.59%, 147/258) was recorded for mature oocytes cryopreserved in ministraws (Fig. 3-5). Concerning effects of type of cryoprotectant on post-thawing viability of preserved mature oocytes, results obtained in Table 3 showed that the mean percentage of post-thawing

Table 1: Descriptive statistics of slaughterhouse ovaries, recovered and matured oocytes in different trials of experiment one

		Recovered immature oocytes (N = 728)								
		Grade I oocytes		Grade II oocytes		Grade III oocytes		Grade IV oocytes		
Statistics	Ovaries	No.	Percentage of total oocytes	No.	Percentage of total oocytes	No.	Percentage of total oocytes	No.	Percentage of total oocytes	Maturation rate* (%)
Total	175	295		256		128		49		
Minimum	9	10	24.39	8	21.05	9	11.3	0	0	66.67
Maximum	24	55	50.0	43	45.74	19	42.86	9	14.63	96.15
Mean	17.5	29.5	38.73	25.6	33.51	12.8	20.49	4.9	7.26	80.62
SE	1.78	5.15	2.92	4.31	2.42	1.1	3.17	0.77	1.41	3.14

*Maturation rate: Number of recovered grade I and II immature oocytes, which was successfully matured divided by total number of recovered grade I and II immature oocytes, SE: Standard error

Table 2: Effect of straw type on post-thawing viability of preserved mature cattle oocytes

Variables	Midistraw	Ministraw
No. of preserved oocytes	251	258
No. of morphologically normal oocytes	116	147
Mean percentage of morphologically normal oocytes	47.16±2.62[a]	57.83±2.59[b]

[a,b]Means with different superscript lower case letters significantly differ (p<0.01)

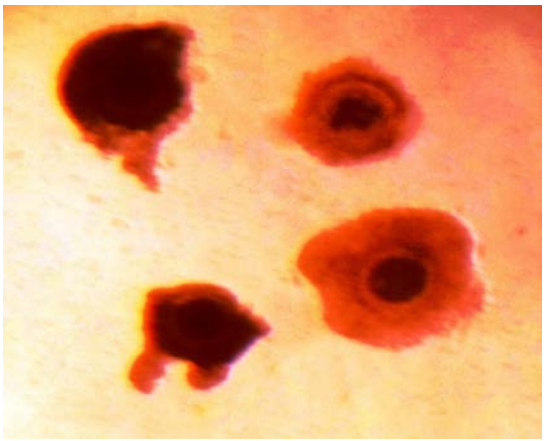

Fig. 1: Grade I and II immature bovine oocytes ready for maturation

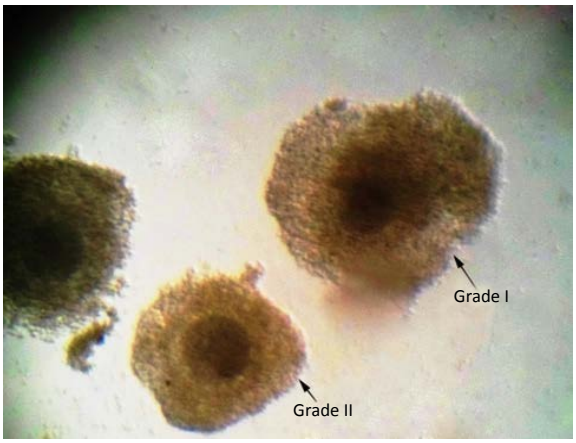

Fig. 3: Post-thawing mature oocytes

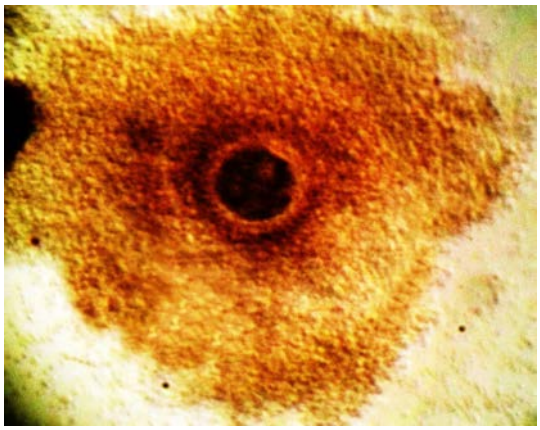

Fig. 2: Mature bovine oocytes show excellent cumulus cells expantion

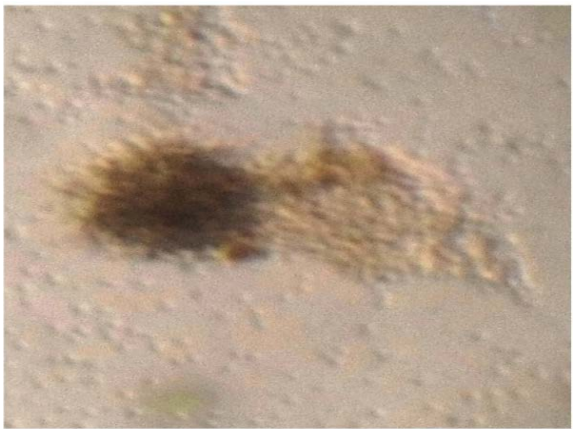

Fig. 4: Post-thawing degenerated cattle oocyte

Table 3: Effect of type of cryoprotectant on post-thawing viability of preserved mature cattle oocytes

Variables	Control	EG+ficoll (1:1)	EG+ficoll (2:1)	EG+ficoll (3:2)
No. of preserved oocytes	155	118	118	118
No. of morphologically normal oocytes	68	58	64	73
Mean percentage of morphologically normal oocytes	44.43±4.98[a]	49.22±1.66[ab]	54.33±3.11[ab]	62.00±3.71[b]

[a,b]Means with different superscript lower case letters significantly differ (p<0.01), EG: Ethylene glycol

Table 4: Interaction between straw and cryoprotectant type on post-thawing viability of preserved mature cattle oocytes

	Midistraw				Ministraw			
Variables	Control	EG+ficoll (1:1)	EG+ficoll (2:1)	EG+ficoll (3:2)	Control	EG+ficoll (1:1)	EG+ficoll (2:1)	EG+ficoll (3:2)
No. of preserved oocytes	76	58	58	59	79	60	60	59
No. of morphologically normal oocytes	27	26	30	33	41	32	34	40
Mean percentage of morphologically normal oocytes	35.41±4.2[a]	44.88±1.43[a]	52.65±4.77[ab]	55.71±5.15[ab]	53.46±7.32[ab]	53.56±0.94[ab]	56.01±4.4[b]	68.31±3.96[b]

[a,b]Means with different superscript lower case letters a significantly differ (p<0.01), EG: Ethylene glycol

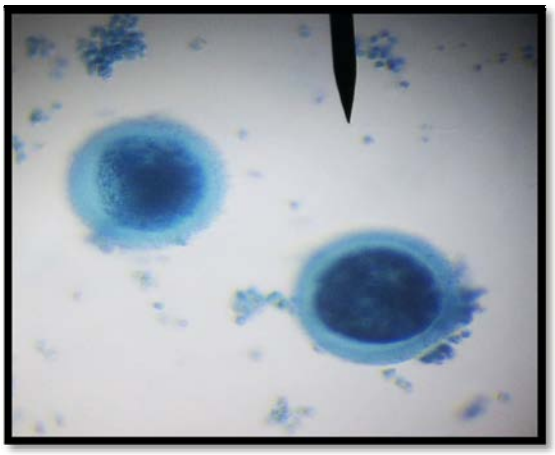

Fig. 5: Post-thawing mature oocytes stained by trypan blue

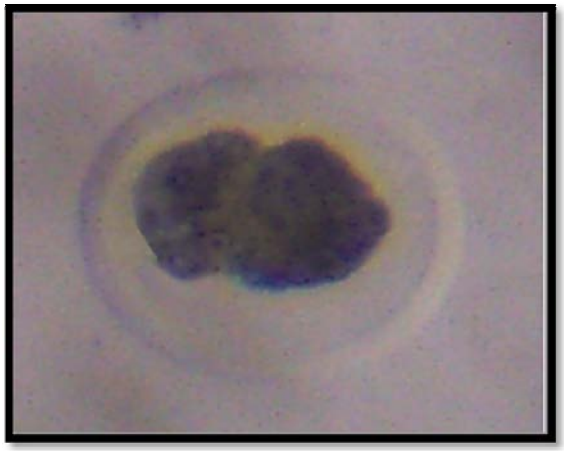

Fig. 6: Two-cell stage embryo

morphologically normal oocytes was significantly higher (p<0.01) (62.00±3.71%) for those preserved in EG+ficoll 70 3:2, compared to oocytes preserved in control medium containing 20% EG+20% DMSO (44.43±4.98%). Additionally, numerical but not significant increases in the percentages of post-thawing morphologically normal mature oocytes were observed for oocytes preserved in EG+ficoll 70 1:1 and EG+ficoll 70 2:1. As depicted in Table 4, mature oocytes preserved in media containing EG+ficoll 70 at ratios of 3:2 showed the highest post-thawing viability (55.71±5.51 and 68.31±3.96% in midistraw and ministraw, respectively), followed by EG+ficoll 2:1 (52.65±4.77 and 56.01±4.40%, respectively). However, within each straw type, the increase in post-thawing viability of mature oocytes was not significant between different cryoprotectants. In regards to the cleavage rate in experimental media containing ficoll 70 in different combinations with EG in comparison to control (DMSO) medium. Table 5 and Fig. 6 and 7 show the total number of oocytes submitted to *in vitro* fertilization ranged between 122 (DMSO group) and 209 in media containing EG+ficoll (3:1). The lowest cleavage rate (p<0.05) was recorded for containing EG+ficoll (2:1) (39.83±0.61), compared to 51.93±2.21 for DMSO group. Moreover, media containing EG+ficoll (3:1) and containing EG+ficoll (3:2) showed intermediate cleavage rates of 43.28±3.24 and 46.51±2.38, respectively. In addition, morula rate in experimental media containing ficoll 70 in different combinations with EG in comparison to control (DMSO) medium was demonstrated in Table 5 and Fig. 8 illustrates morula rate in different groups of embryos cultured for further cryopreservation. As depicted, there were no significant differences in morula rates between the four experimental groups ranging between 23.41±2.75 morulae in media containing EG+ficoll (2:1) group and 32.59±3.01 morulae in DMSO group. It is clear from the results that blastocyst rates in the four groups of embryos were not statistically different i.e.,

Table 5: Cleavage rate, morula rate, blastocyst rate and recovery rate of frozen-thawed blastocysts in experimental media containing ficoll 70 in different combinations with EG in comparison to control (DMSO) medium

Variables	EG+DMSO+Trehalose	EG+ficoll (2:1)	EG+ficoll (3:1)	EG+ficoll (3:2)	p-value
Replicates	5	5	5	5	NA
Mature oocytes submitted to IVF	122	146	209	162	NA
*Cleavage rate	51.93±2.21[b]	39.83±0.61[a]	43.28±3.24[ab]	46.51±2.38[ab]	0.012
*Morula rate	32.59±3.01	23.41±2.75	26.16±2.17	29.84±3.31	0.152
*Blastocyst rate	14.93±5.46	13.95±1.57	15.15±1.72	17.48±2.43	0.88
*Recovery rate of frozen-thawed blastocysts	45±16.24[a]	50±13.74[ab]	63.49±6.83[ab]	79.28±13.08[b]	0.003

*Cleavage rate: No. of two cell stage embryos at 24 h post-IVF divided by total No. of mature oocytes submitted to IVF, *Morula rate: No. of morula stage embryos at 6 days post-IVF divided by total No. of mature oocytes submitted to IVF, * Blastocyst rate: No. of blastocyst stage embryos at 7 days post-IVF divided by total No. of mature oocytes submitted to IVF, *Recovery rate: No. of post-thawing viable blastocyst divided by total No. of fresh blastocysts submitted to vitrification

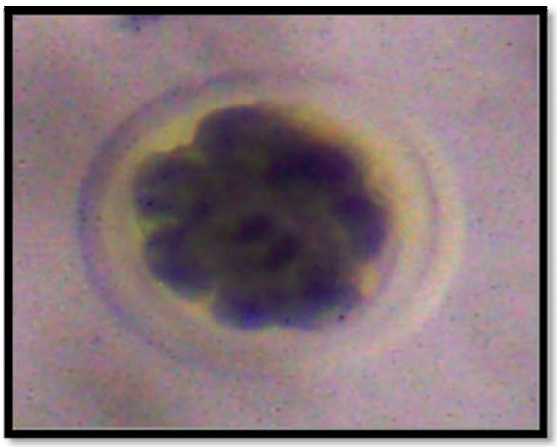

Fig. 7: Eight-cell stage embryo

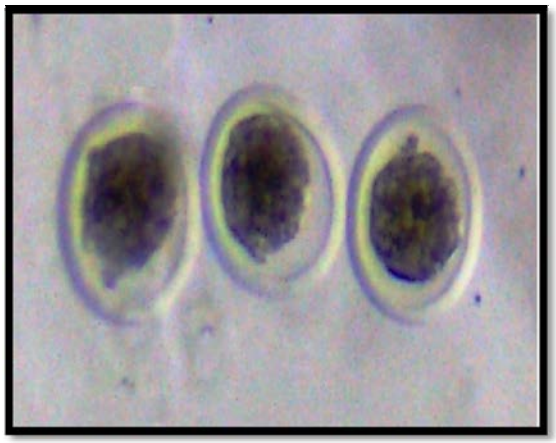

Fig. 8: Morula stage

having the same developmental potential (Fig. 9). The minimal blastocyst rate was observed for embryo groups prepared to be cryopreserved in media containing EG+ficoll (2:1) (13.95±1.57), while the maximal blastocyst rate was observed for those prepared to be preserved in media containing EG+ficoll (3:2) (17.48±2.43). Regarding the

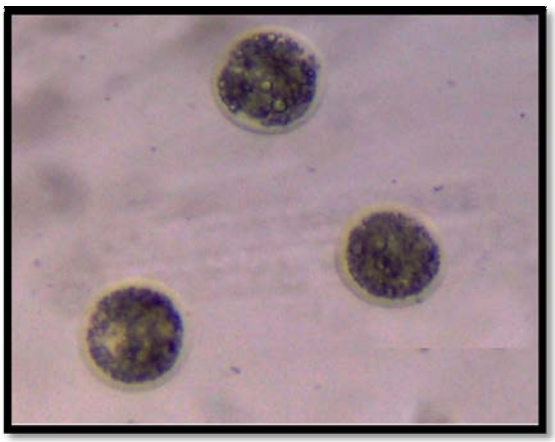

Fig. 9: Blastocyst stage

recovery rate of frozen-thawed blastocyst, blastocysts cryopreserved in media containing EG+ficoll (3:2) had a significantly ($p<0.01$) higher recovery rate (79.28±13.08), compared to 45.00±16.24 blastocyst in embryos cryopreserved in DMSO, respectively. Recovery rates of blastocysts cryopreserved in media containing EG+ficoll (2:1) and in media containing EG+ficoll (3:1) were numerically higher than those cryopreserved in DMSO group 50.00±3.74 and 63.49±6.83, respectively (Table 5, Fig. 10).

DISCUSSION

Several methods are used for recovery of immature oocytes from abattoir ovaries including aspiration, slicing the ovaries and puncture of the visible surface follicles[35]. In the present study, immature oocytes were recovered by slicing methods with an average 3-4 oocytes/ovary, while an average of 5-10 oocytes/ovary were collected by Mermillod *et al.*[36]. Moreover Shirazi *et al.*[37] compared between slicing and aspiration methods in goat and found that slicing procedure

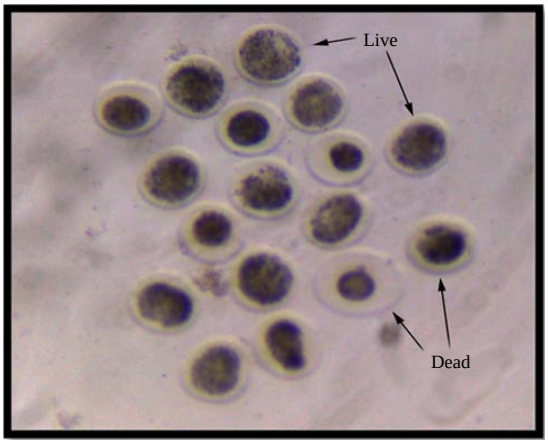

Fig. 10: Post-thawing blastocyst showing live and dead embryos

yielded significantly higher percentage of grade I oocytes than aspiration (52 versus 22%). These findings come in disagreement of the earlier study of Martino *et al.*[38] reported a lower recovery rate of good quality oocytes by slicing compared to aspiration. Thus, it could be concluded from the present study that slicing procedure in bovine predominates over other ways for retrieval of a higher recovery rate and grade I oocytes. The IVM utilized in the present study was TCM-199 supplemented with FCS, FSH and E_2 which found to yield the highest maturation rates. These findings agree with the results of Younis *et al.*[24] and Nakagawa and Leibo[39], revealed that addition of LH singly or in combination with FSH and E_2 had a significant enhancing influence on the IVM rate in bovine. Serum added to the oocytes culture media provided a source of albumin that balance the osmolarity and act as a free radical scavenger[40]. In the present study, only grade I and II were utilized for IVM and subsequent IVEP as well as vitrification as recommended by Sirard and Blondin[41] as cumulus cells provide nutrients to oocytes during their growth. The IVM of oocytes were performed in incubator in the presence of 5% CO_2 tension at 39°C for 24 h as described by Atef[20] and Ward *et al.*[42]. In the present stydy separation of morphologically normal and high motile sperms were selected using swim up separation technique. In this respect, Somfai *et al.*[43] have recorded a lower recovery with a better quality of spermatozoa using swim up Percoll separation resulted in a higher recovery rate. The sperm capacitation was performed by heparin (200 IU mL^{-1}). However, Tatham *et al.*[44] reported that treatment of spermatozoa by 5 mM caffeine and 5 μg heparin mL^{-1} resulted in a higher fertilization rate than did treatment with 5 μg heparin mL^{-1} alone. Therefore, the sperm concentration of the present study was 2×10^6 spermatozoa mL^{-1} as described by Dalvit *et al.*[45]. The reduction of sperm concentration into ($0.016\text{-}0.125\times10^6$) leading to lowering the cleavage rate[42]. The fertilization medium used in this study was TALP medium, in this concern, many studies recorded better results with TALP medium in comparison with BO medium[46]. Moreover, Coy *et al.*[47] reported that the percentage of cleavage were higher in oocytes cultured in TCM-199 and TBM than TALP. This study revealed that cleaved oocytes as well as morula and blastocysts developmental rates/mature cells were 45.39±2.11, 28.00±2.81 and 15.29±2.79%, respectively. Previously, Anderiesz *et al.*[48] obtained a higher blastocyst rates (25.00%) following addition of FSH and LH into maturation media in bovine. In addition, Choi *et al.*[19] by adding FSH, LH and E_2 recorded 84.3±2.1, 48.6±4.5 and 15.3±2.2% for cleavage, morula and blastocyst development, respectively. It was shown that the most important step affecting the quality of blastocyst is post-fertilization culture condition[49]. Therefore, suboptimal *in vitro* culture environment can seriously affect the developmental potential of *in vitro* produced embryos. In the present study, according Dalvit *et al.*[45], the fertilized oocytes were cultured in the supplemented H-TCM-199 and placed in the CO_2 incubator with change of the medium every 48 h for 7 successive days. Therefore, it is worth denoting that the success of *in vitro* embryo production in bovine is a multifactorial process that depends upon choice of convenient cell type undergoing further development, type of the selective media, incubation condition as well as providing the environment with additives particularly hormones and sera to enhance cellular developmental activities. The present article studied the effect of sugars including trehalose and macromolecules as ficoll 70 additions to cryoprotectant solutions on the post-thawing viability of matured cattle matured oocytes and embryos. As well as determined the effect of the straw diameter on the post-thawing viability of mature cattle oocytes. Two procedures are well known to induce cellular cryopreservation. The conventional slow freezing, which exposed the cells at various phases of freezing and thawing to physical, chemical and biological hazard particularly intracellular ice crystals formation[50]. On the other hand, vitrification is a method that suppresses both intra and extracellular ice formation thus producing instead a glass-like state[51]. Throughout the present study, the method used for cryopreservation of bovine oocytes and embryos was vitrification which has been successfully applied in several mammalian species. Despite the importance of penetrating cryoprotectants to avoid intracellular ice crystals formation, the high concentration of these substances is toxic and may

cause osmotic injury[52]. So using of less toxic substances, association of different cryoprotectants, gradual exposure and reduction of exposure time to cryoprotectant solution are required[53]. In the present experment, a combination of 20% EG and 20% DMSO was used for cells vitrification. Incorporating of DMSO and EG-containing medium has at least to advantages, vitrification is facilitated because of the greater glass forming characteristics of DMSO[54] as well as the permeability of each of the cryoprotectants is enhanced by the presence of second[55]. Similarly, Yamada *et al.*[12] found that *in vitro* maturation rate of bovine oocytes vitrified in EG+DMSO (29.2%) was significantly higher than that after vitrification in EG+glycerol (4.30%). The addition of trehalose to embryo vitrification medium containing DMSO offered significantly better results than those obtained with sucrose[56]. In this respect, trehalose has greater stabilizing effects on cell membrane than sucrose[57]. In relation to trehalose concentration, it was found that the best concentration that achieved the best results was 11.3% (0.3 M)[58]. This study showed that the mean percentage of post-thawing morphologically normal oocytes was significantly higher ($p<0.01$) (62.00±3.71%) for those preserved in EG+ficoll 70 3:2, compared to oocytes preserved in control medium containing 20% EG+20% DMSO+0.3 M trehalose (44.43±4.98%). Additionally, numerical but not significant increases in the percentages of post-thawing morphologically normal mature oocytes were observed for oocytes preserved in EG+ficoll 70 (2:1) and EG+ficoll 70 (2:1). While, the blastocysts cryopreserved in media containing EG+ficoll (3:2) had a significantly ($p<0.01$) higher recovery rate (79.28±13.08), compared to 45.00±16.24 blastocyst in embryos cryopreserved in DMSO, respectively. Recovery rates of blastocysts cryopreserved in media containing EG+ficoll (1:1) and in media containing EG+ficoll (2:1) were numerically higher than those cryopreserved in DMSO group. These results run with previous studies, which concluded that the EG and ficoll 70 cryoprotectants combination would maintain the survival rate of frozen-thawed porcine oocytes at 81% or more. In addition, the survival rate of oocytes in the cryoprotectant medium of 3.5 M DMSO plus 40% EG and 18% ficoll 70 was 75%, although the exposure time was 10 min. Oocytes frozen in solutions containing of 40% EG and 18% ficoll 70 by a ratio of 2:1, 3:1 or 3:2 had a mean survival rate of 81, 82 and 97%, respectively[22]. The addition of trehalose in the cryoprotectant medium containing of EG and ficoll 70 increase the intact rate to almost 100% but did not improve the survival rate. Results of the present study supported the concept of McWilliams *et al.*[59] in their study non-permeating solutes, such as ficoll 70 and sucrose served as osmotic buffers for the recovery of cryopreserved oocytes as EG or DMSO permeating cryoprotectant was used. Polysaccharides like ficoll 70 could influence the viscosity of the vitrification solution and reduce the toxicity of the cryoprotectant through lowered concentration to prevent cells from cryoinjury by reducing mechanical stress which occurs during cryopreservation[60].

A typical low toxicity vitrification solution, EFS40 contains three cryoprotectants: 40% EG-rapidly permeating, low toxicity agent, ficoll 70 at 18%, a macromolecule and sucrose-a non-permeating hexose sugar as described[61]. The cattle embryos were incubated in the EFS40 for 1 min before plunging into LN2. A stepwise pre-equilibration procedure, in which the amount of penetrating cryoprotectant was gradually increased was very effective for human oocytes[62] and bovine oocytes[6,63]. According to the results of the present study, regarding the effect of straw type on post-thawing viability of cryopreserved mature oocytes, show that out of 251 oocytes which were preserved in midistraws, 116 oocytes appeared morphologically normal post-thawing representing a mean percentage of 47.16±2.62%. A significantly higher ($p<0.01$) percentage of post-thawing morphologically normal oocytes (57.83±2.59%, 147/258) was recorded for mature oocytes cryopreserved in ministraws. Using 0.25 mL straws was previously applied in studies concerned with cryopreservation of mature oocytes and embryos of different species[11,64-66]. In this respect, Dattena *et al.*[64] suggested that by using traditional 0.25 mL straws, the maximum cooling rate was 2500°C min which allows embryos to pass through certain critical temperature zones quickly and decreases the chilling injuries. Several new techniques have been developed recently including Open Pulled Straw (OPS), electron microscope grids, cryoloop as well as cryotop methods[67,68]. All these devices achieved 10-fold faster cooling rates than those obtained in standard straws. However, the major limit for application of these vitrification techniques is the direct contact between the medium containing cells and liquid nitrogen which may introduce infections[69]. A possible solution to the problem is the minimum volume cooling method whereas the embryos are loading in an extremely low volume (20 µL) into 0.25 mL insemination straw, which is sealed before cooling[70].

CONCLUSION

It can be concluded that using of ministraw for the cryopreservation of mature bovine oocytes had post-thawing viability significantly higher (<10%) than using

midistraw. As well as, it was proved that matured oocytes frozen in solutions containing 20% EG+20% DMSO+0.3 M trehalose had mean survival rate of (44.43±4.98%). While mature oocytes frozen in solutions containing 40% EG and 18% ficoll 70 by a ratio of 1:1, 2:1 and 3:2 in volume had a mean survival rate of 49.22±1.66, 54.33±3.11 and 62.00±3.71%, respectively. So, combination of 40% EG+18% ficoll 70 by the ratio of (3:2) act as a good cryoprotectant combination for vitrification of mature bovine oocytes.

ACKNOWLEDGMENT

Sincere gratitude is to Department of Physiology, Faculty of Veterinary Medicine, Beni-Suef University for their kind supervision and his helpfulness during this study and for their kind cooperation, faithful advice and careful revision of this study.

REFERENCES

1. Rall, W.F. and G.M. Fahy, 1985. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. Nature, 313: 573-575.
2. Vanderzwalmen, P., F. Ectors, L. Grobet, Y. Prapas and Y. Panagiotidis *et al*., 2009. Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM. Reprod. BioMed. Online, 19: 700-707.
3. Vanderzwalmen, P., D. Connan, L. Grobet, B. Wirleitner and B. Remy *et al*., 2013. Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions. Hum. Reprod., 28: 2101-2110.
4. Wang, L., J. Liu, G.B. Zhou, Y.P. Hou, J.J. Li and S.E. Zhu, 2011. Quantitative investigations on the effects of exposure durations to the combined cryoprotective agents on mouse oocyte vitrification procedures. Biol. Reprod., 85: 884-894.
5. Campos-Chillon, L.F., D.J. Walker, J.F. de la Torre-Sanchez and G.E. Seidel, Jr., 2006. *In vitro* assessment of a direct transfer vitrification procedure for bovine embryos. Theriogenology, 65: 1200-1214.
6. Vajta, G., P. Holm, M. Kuwayama, P.J. Booth, H. Jacobsen, T. Greve and H. Callesen, 1998. Open Pulled Straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51: 53-58.
7. Le Gal, F., R. De Roover, B. Verhaeghe, D. Etienne and A. Massip, 2000. Development of vitrified matured cattle oocytes after thawing and culture *in vitro*. Vet. Rec., 146: 469-471.
8. Zhou, Y., X. Fu, G. Zhou, B. Jia, Y. Fang, Y. Hou and S. Zhu, 2014. An efficient method for the sanitary vitrification of bovine oocytes in straws. J. Anim. Sci. Biotechnol., Vol. 5. 10.1186/2049-1891-5-19.
9. Wang, X., A. Al Naib, D.W. Sun and P. Lonergan, 2010. Membrane permeability characteristics of bovine oocytes and development of a step-wise cryoprotectant adding and diluting protocol. Cryobiology, 61: 58-65.
10. Whittingham, D.G., 1977. Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at-196° C. J. Reprod. Fertil., 49: 89-94.
11. Wani, N.A., A.K. Misra and S.N. Maurya, 2004. Maturation rates of vitrified-thawed immature buffalo (*Bubalus bubalis*) oocytes: Effect of different types of cryoprotectants. Anim. Reprod. Sci., 84: 327-335.
12. Yamada, C., H.V.A. Caetano, R. Simoes, A.C. Nicacio, W.B. Feitosa, M.E.O. D'Avila Assumpcao and J.A. Visintin, 2007. Immature bovine oocyte cryopreservation: Comparison of different associations with ethylene glycol, glycerol and dimethylsulfoxide. Anim. Reprod. Sci., 99: 384-388.
13. Sharma, G.T., P.K. Dubey and V. Chandra, 2010. Morphological changes, DNA damage and developmental competence of *in vitro* matured, vitrified-thawed buffalo (*Bubalus bubalis*) oocytes: A comparative study of two cryoprotectants and two cryodevices. Cryobiology, 60: 315-321.
14. Anchamparuthy, V., 2007. Vitrification of bovine oocytes. Ph.D. Thesis, The Faculty of the Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
15. Lim, K.T., G. Jang, K.H. Ko, W.W. Lee and H.J. Park *et al*., 2008. Improved cryopreservation of bovine preimplantation embryos cultured in chemically defined medium. Anim. Reprod. Sci., 103: 239-248.
16. Atef, N., 2008. Studies on cryopreservation of *in vitro* produced bovine embryos. Ph.D. Thesis, Faculty Of Veterinary Medicine, Benisuef University, Egypt.
17. Ganguli, G., A. Indra and P. Gupta, 1998. Suitability of the follicular oocytes obtained from slaughtered buffalo ovaries and assessment of their nuclear maturation. Buffalo J., 14: 217-228.
18. Nedambale, T.L., F. Du, J. Xu, S.A. Chaubal and A. Dinnyes *et al*., 2006. Prolonging bovine sperm-oocyte incubation in modified medium 199 improves embryo development rate and the viability of vitrified blastocysts. Theriogenology, 66: 1951-1960.
19. Choi, Y.H., E.M. Carnevale, G.E. Seidel Jr. and E.L. Squire, 2001. Effects of gonadotropins on bovine oocytes matured in TCM-199. Theriogenology, 56: 661-670.
20. Atef, N., 2005. Some studies on *in vitro* fertilization in buffaloes. MVSc. Thesis, Faculty Of Veterinary Medicine, Benisuef University, Egypt.

21. Schellander, K., R.A. Fayrer-Hosken, C.L. Keefer, L.M. Brown, H. Malter, C.E. McBride and B.G. Brackett, 1989. *In vitro* fertilization of bovine follicular oocytes recovered by laparoscopy. Theriogenology, 31: 927-934.
22. Wu, M.C. and H.M. Lee, 1996. Vitrification of porcine oocytes. Contribution No. 683 from Taiwan Livestock Research Institute (TLRI), Iran.
23. Parrish, J.J., J.L. Susko-Parrish, M.L. Leibfried-Rutledge, E.S. Critser, W.H. Eyestone and N.L. First, 1986. Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology, 25: 591-600.
24. Younis, A.I., B.G. Brackett and R.A. Fayrer-Hosken, 1989. Influence of serum and hormones on bovine oocyte maturation and fertilization *in vitro*. Gamete Res., 23: 189-201.
25. Hensleigh, H.C. and A.G. Hunter, 1985. *In vitro* maturation of bovine cumulus enclosed primary oocytes and their subsequent *in vitro* fertilization and cleavage. J. Dairy Sci., 68: 1456-1462.
26. Hammam, A.M., M.M. Zabal and H.A. Sabra, 1997. Effect of types of media on *in vitro* maturation, culture and fertilization of buffalo and cattle oocytes. Beni-Swef Vet. Med. Res., 2: 242-259.
27. Linder, G.M. and R.W. Wright Jr., 1983. Bovine embryo morphology and evaluation. Theriogenol., 20: 407-416.
28. Pugh, P.A., H.R. Tervit and H. Nienmann, 2000. Effects of vitrification medium composition on the survival of bovine *in vitro* produced embryos, following in straw-dilution, *in vitro* and *in vivo* following transfer. Anim. Reprod. Sci., 58: 9-22.
29. Checura, C.M. and G.E. Seidel Jr., 2007. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. Theriogenology, 67: 919-930.
30. De Mello, M.R.B., V.S. Queiroz, A.S. de Lima, L.M.T. Tavares, M.E.O. D'Avila Asumpcao, M.B. Wheeler and J.A. Visintin, 2001. Cryopreservation of mouse morulae through different methods: Slow-freezing, vitrification and quick-freezing. Braz. J. Vet. Res. Anim. Sci., 38: 160-164.
31. Dhali, A., R.S. Manik, S.K. Das, S.K. Singla and P. Palta, 2000. Verification of buffalo (*Bubalus bubalisi*) oocytes. Theriogenology, 53: 1295-1303.
32. Nandi, S., H.M. Raghu, B.M. Ravindranatha and M.S. Chauhan, 2002. Production of buffalo (*Bubalus bubalis*) embryos *in vitro*: Premises and promises. Reprod. Domest. Anim., 37: 65-74.
33. Cuello, C., F. Berthelot, B. Delaleu, E. Venturi and L.M. Pastor *et al*., 2007. The effectiveness of the stereomicroscopic evaluation of embryo quality in vitrified-warmed porcine blastocysts: An ultrastructural and cell death study. Theriogenology, 67: 970-982.
34. Snedecor, G.W. and W.G. Cochran, 1987. Statistical Methods. 8th Edn., Oxford and Ibh, New Delhi.
35. Wang, H.F., N. Isobe, K. Kumamoto, H. August 11, 2016Yamashiro, Y. Yamashita and T. Terada, 2006. Studies of the role of steroid hormone in the regulation of oocyte maturation in cattle. Reprod. Biol. Endocrinol., Vol. 4. 10.1186/1477-7827-4-4.
36. Mermillod, P., C. Boccart, C. Wils, A. Massip and F. Dessy, 1992. Effect of oviduct-conditioned medium and of cumulus cells on bovine embryo development *in vitro*. Theriogenology, 37: 256-263.
37. Shirazi, A., N. Shams-Esfandabadi and S.M. Hosseini, 2005. A comparison of two recovery methods of ovine oocytes for *in vitro* maturation. Small Rumin. Res., 58: 283-286.
38. Martino, A., N. Songsasen and S.P. Leibo, 1996. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol. Reprod., 54: 1059-1069.
39. Nakagawa, A. and S.P. Leibo, 1997. Influence of luteineing hormone on nuclear maturation of bovine oocytes *in vitro*. Theriogenology, 47: 198-198.
40. Thompson, J.G., 2000. *In vitro* culture and embryo metabolism of cattle and sheep embryos-A decade of achievement. Anim. Reprod. Sci., 60-61: 263-275.
41. Sirard, M.A. and P. Blondin, 1996. Oocyte maturation and IVF in cattle. Anim. Reprod. Sci., 42: 417-426.
42. Ward, F., D. Rizos, M.P. Boland and P. Lonergan, 2003. Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: Work in progress. Theriogenology, 59: 1575-1584.
43. Somfai, T., S. Bodo, S. Nagy, A.B. Papp and J. Ivancsics *et al*., 2002. Effect of swim up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. Reprod. Domestic Anim., 37: 285-290.
44. Tatham, B.G., T. Feehan and R. Pashen, 2003. Buffalo and cattle hybrid embryo development is decreased by caffeine treatment during *in vitro* fertilization. Theriogenology, 59: 709-717.
45. Dalvit, G., S.P. Llanes, A. Descalzo, M. Insani, M. Beconi and P. Cetica, 2005. Effect of alpha-tocopherol and ascorbic acid on bovine oocyte *in vitro* maturation. Reprod. Domestic Anim., 40: 93-97.
46. Totey, S.M., M. Daliri, K.B.C. Appa Rao, C.H. Pawshe, M. Taneja and R.S. Chillar, 1996. Differential cleavage and developmental rates and their correlation with cell numbers and sex ratios in buffalo embryos generated *in vitro*. Theriogenology, 45: 521-533.
47. Coy, P., J. Gadea, R. Romar, C. Matas and E. Garcia, 2002. Effect of *in vitro* fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and *in vitro* development in pigs. Reproduction, 124: 279-288.

48. Anderiesz, C., A.P. Ferraretti, C. Magli, A. Fiorentino and D. Fortini *et al*., 2000. Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development *in vitro*. Hum. Reprod., 5: 1140-1148.
49. Rinaudo, P. and R.M. Schultz, 2004. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. Reproduction, 128: 301-311.
50. Matsuoka, K., S. Sakata, K. Ichino, S. Shimaya, T. Katagihara and T. Suzuki, 1995. Ultra-rapid freezing of *in vitro* produced bovine embryos. Theriogenology, 43: 274-286.
51. Liebermann, J., J. Dietl, P. Vanderzwalmen and M.J. Tucker, 2003. Recent developments in human oocyte, embryo and blastocysts vitrification: Where are we now? Reprod. Biomed. Online, 7: 623-633.
52. Arav, A., D. Shehu and M. Mattioli, 1993. Osmotic and cytotoxic study of vitrification of immature bovine oocytes. J. Reprod. Fertility, 99: 353-358.
53. Vajta, G., 2000. Vitrification of the oocytes and embryos of domestic animals. Anim. Reprod. Sci., 60-61: 357-364.
54. Ali, J. and J.N. Shelton, 1993. Vitrification of preimplantation stages of mouse embryos. J. Reprod. Fertility, 98: 459-465.
55. Vicente, J.S. and F. Garcia-Ximenez, 1994. Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. Theriogenology, 42: 1205-1215.
56. Robertson, J.L., B.S. Minhas, G.W. Randall, M.G. Dodson, T.V. Palmer and D.D. Ricker, 1989. Ultrarapid freezing of mouse embryos with DMSO and trehalose. Theriogenology, 31: 250-261.
57. Smorag, Z., Y. Heyman, V. Garnier and B. Gajda, 1990. The effect of sucrose and trehalose on viability of one-and two-cell rabbit embryos. Theriogenology, 33: 741-747.
58. Dobrinsky, J.R., S.L. Stice, P.E. Phillips, R.T. Duby and J.M. Robl, 1992. Development of IVM-IVF bovine embryos following vitrification dilution treatments. Theriogenology, 37: 202-202.
59. McWilliams, R.B., W.E. Gibbons and S.P. Leibo, 1995. Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and disaccharides. Hum. Reprod., 10: 1163-1171.
60. Dumoulin, J.C.M., J.M. Bergers-Janssen, M.H.E.C. Pieters, M.E. Enginsu, J.P.M. Geraedts and J.L.H. Evers, 1994. The protective effects of polymers in the cryopreservation of human and mouse zonae pellucidae and embryos. Fertil. Steril., 62: 793-798.
61. Kasai, M., 1996. Simple and efficient methods for vitrification of mammalian embryos. Anim. Reprod. Sci., 42: 67-75.
62. Kuleshova, L.L., D.R. MacFarlane, A.O. Trounson and J.M. Shaw, 1999. Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. Cryobiology, 38: 119-130.
63. Abe, Y., K. Hara, H. Matsumoto, J. Kobayashi and H. Sasada *et al*., 2005. Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. Biol. Reprod., 72: 1416-1420.
64. Dattena, M., C. Accardo, S. Pilichi, V. Isachenko, L. Mara, B. Chessa and P. Cappai, 2004. Comparison of different vitrification protocols on viability after transfer of ovine blastocysts *in vitro* produced and *in vivo* derived. Theriogenology, 62: 481-493.
65. Martins, R.D., E.P. Costa, J.S.C. Chagas, F.S. Ignacio, C.A.A. Torres and C. McManus, 2005. Effects of vitrification of immature bovine oocytes on *in vitro* maturation. Anim. Reprod., 2: 128-134.
66. Cetin, Y. and A. Bastan, 2006. Cryopreservation of immature bovine oocytes by vitrification in straws. Anim. Reprod. Sci., 92: 29-36.
67. Vajta, G., P.J. Booth, P. Holm, T. Greve and H. Calleson, 1997. Successful vitrification of early stage bovine *in vitro* produced embryos with the Open Pulled Straw (OPS) method. Cryo Lett., 18: 191-195.
68. Kuwayama, M., 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. Theriogenology, 67: 73-80.
69. Fountain, D., M. Ralston, N. Higgins, J.B. Gorlin and L. Uhl *et al*., 1997. Liquid nitrogen freezers: A potential source of microbial contamination of hematopoietic stem cell components. Transfusion, 37: 585-591.
70. Hamawaki, A., M. Kuwayama and S. Hamano, 1999. Minimum volume cooling method for bovine blastocyst vitrification. Theriogenology, 51: 165-174.