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Effect of Different Cryoprotectant Agents on Mitochondrial Distribution and Developmental Competence in Buffalo Oocyte (Bubalus Bubalis)

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Abstract

Abstract: Oocytes cryopreservation in mammalian species has gained a rapid pace during the past couple of decades emphasizing its importance in various assisted reproductive technologies .Aim of this work was to study effects of different cryoprprotectant agents: Ethylene Glycol (EG), Dimethyl sulfoxide (DMSO) and combination of them (EG+DMSO) on the viability, mitochondrial distribution and intensity of in vitro matured vitrified/ thawed buffalo oocytes by determine: 1) the effect of different cryoprotectants on the viability and developmental competence of vitrified/thawed in vitro matured buffalo oocytes. 2) The effect of different cryoprotectants on the mitochondria distribution and intensity of vitrified/thawed in vitro matured buffalo oocytes. Ovaries were collected from EL-Warak slaughter house, Cairo, Egypt. Excellent and good oocytes were in vitro matured in TCM-199 +IGF1 for 22 h in incubator at 38.5 ₀C in 5% CO2 and humidified atmosphere. Morphologically normal matured oocytes with first polar were placed in vitrification solution (VS1) for 1 min then oocytes were transferred to (VS2) for 30 sec. VS1 is half concentration of VS2 which contain EG 40% (EG group) or DMSO 40% (DMSO group) or EG 20%+DMSO 20% (Combination group). Oocytes were loaded in sterile 0.25 ml straws and stores in liquid nitrogen for 7-10 days. Morphological changes (normal and abnormal) of vitrified thawed in vitro mature buffalo oocyte were detected under invertd microscope. Mitotracker red stain and confocal microscope (Zeiss 710) were used to study the mitochondrial distribution and Hochest dye to study the viability of in vitro vitrified/ thawed matured buffalo oocytes. The obtained results revealed that using a combination of EG+DMSO for vitrification resulted in the best quality of in vitro vitrified thawed buffalo oocytes which was demonstrated by significantly higher percent of morphologically normal recovered oocytes and transferable embryos when compared with EG and DMSO. More mitochondria were diffusely distributed in the fresh oocytes (93.33%) than in all vitrified groups oocytes: DMSO group (63.33%), EG group (79.99%) and DMSO+ EG group (79.99%). The Mean No. of mitochondrial intensity of recovered mature oocytes vitrified in EG+DMSO group (186.22) was significantly higher ($P < 0.05$) than those vitrified in EG group (153.33) and DMSO group (146.98). In conclusion: Combination of EG+DMSO cryoprotectants improve the viability and developmental competence of in vitro matured vitrified/thawed buffalo oocytes.

Keywordds: In Vitro Embryo Production; Vitrification,;Cryoprotectant; EG; DMSO; Mitochondria and Buffalo Oocytes

1. Introduction

Buffaloes are considered as the backbone of the animal resources in Egypt. They provide meat and milk and showing a high resistance to diseases [1]. Unfortunately, the low reproductive efficiency and some inherent reproductive problems in buffaloes are still non-overcome [2]. Several assisted reproductive technologies (ART) have been improved to help overcome fertility problems in animals of high genetic merit [3]. Efficient oocyte cryopreservation protocols will widen and improve the strategic implementation of ARTs in the species like buffalo. ART still needs more research for optimization and wide application in Egypt. Oocytes' cryopreservation may have

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prospective applications in scientific research, stock raising, endangered species conservation, and the preservation of women's gametes before chemotherapy or radiation therapy [4].DNA fragmentation,chromosome disorganization, aberrant gene expression and damage to intracellular organelles, such as mitochondria, endoplasmic reticulum and lysosomes, have been observed in oocytes after cryopreservation [5] because of their large volume, high sensitivity to cooling, low surface area to volume ratio, high water content, low hydraulic conductivity [6] and high intra-cytoplasmic lipid content decrease its ability to cryopreservation [7]. Oocyte cryopreservations were performed either by slow freezing [8] or vitrification [9]. Cryopreservation of bovine oocytes and embryos by conventional slow freezing method often causes osmotic shock and intracellular ice crystallization, resulting in cell damage [10]. Vitrification offers several advantages over slow freezing such as faster and simplified freezing and thawing procedures, high oocyte/embryo survival and no requirement for an expensive freezing machine [11].

Cryoprotectants are divided into two types: membrane permeating (e.g., glycerol, ethylene glycol [EG], dimethyl sulphoxide [DMSO] and propanediol [PrOH]) and membrane non-permeating (e.g., sucrose, glucose, Ficoll, proteins and lipoproteins). Ethylene glycol and DMSO are the most commonly used cryoprotectants in vitrification, but they are considered toxic due to their cell permeating nature and the high concentrations needed to induce vitrification [12].

Numerous studies have been conducted to determine optimal meiotic stages for oocyte vitrification, yet the results are inconclusive. Maturation stages includ germinal vesicle (GV) [13], germinal vesicle breaking down (GVBD) [14] through metaphase II (MII) which have been described as the most favorable stages for vitrification [15]. Chilling injury is reported to be higher in immature oocytes, probably owing to low membrane stability and susceptibility of the cytoskeleton [16] with abnormal meiotic spindle formation and chromosomal alterations [17] as well as, high incidence of abnormal morphological changes in vitrified / thawed immature buffalo oocytes [18] that inhibit the developmental capacity of oocyte. In buffaloes, many studies found higher proportion of blastocyst in MII than germinal vesicle stage [19]. That was referred to the injury in oocytes itself, instead of fertilization failure [20]. The first success in production of blastocysts from vitrified immature buffalo oocytes was reported by wani [21] but blastocyst rates were still low (10–15%) [21].

Mitochondria have vital roles in cellular energy metabolism for most reactions occurring in oocytes [22]. Mitochondria produce ATP [23] that used in regulating apoptosis [24], calcium signaling [25]. In mammals, the copies of mitochondria increase intensely with the oocyte development. After fertilization and during the early embryonic development, mitochondrial biogenesis ceases and their number remains constant until embryo implantation [26]. Thus, it is significant that the oocyte has a decent reserve of mitochondria to provide an appropriate embryonic growth. Oocytes with low developmental capacity have shortage signs of mitochondrial organization or, alternatively, show larger mitochondrial clumps throughout the cytoplasm [26]. Mitochondria are reflected to acquisition of oocyte competence and could, therefore, be a key factor for the expression of valuable markers that reflect oocyte quality and developmental competence [27]. In oocyte, mitochondria may be damaged without any detectable morphological alterations during cryopreservation process [28]. Vitrification and warming process may impact on mitochondrial function, structure and distribution [29]. Changes in mitochondrial organization and high sensitivity of oocytes at MII stage to vitrification process reported by Shi [30]. Few studies have been conducted on the impact of cryoprotectants agents on vitrified oocytes viability in relation to mitochondrial distribution in buffalo. Therefore, the objectives of this work are to study: 1) the effect of different cryoprotectants on the viability of vitrified/thawed in vitro matured buffalo oocytes. 2) The effect of different cryoprotectants on the mitochondria distribution and intensity of vitrified/thawed in vitro matured buffalo oocytes.

2. Experimental work

All chemicals and media used in the present work were purchased from Sigma-Aldrich (Sant. Louis, MO, USA) unless otherwise mentioned. The present study was conducted in the Embryo and Genetic Resources Conversation Bank, National Research Center, Cairo, Egypt**.**

2.1. Material

1. Oocytes Collection and In vitro Maturation:

Buffalo ovaries (212) were collected from El-Warak slaughterhouse at Cairo, transported to the laboratory in a thermos containing normal saline solution (NSS) contains (0.9% NaCl + 100 IU penicillin and 100 µg/ml streptomycin). At the laboratory, ovaries were washed at least 3 times in pre-warmed saline solution (37°C) and then kept in water bath at 37°C until oocytes aspiration. Cumulus oocytes complexes (COCs) were aspirated from follicles 2-8 mm in diameter using an 18-gauge needle attached to a 10 ml sterile syringe containing 2 ml aspiration and washing medium (phosphate buffered saline; (PBS) + 6 mg/ ml bovine serum albumin F-V (BSA) + 50 µg/ml gentamicin) [31]. After aspiration, follicular contents were transferred to 15 ml Falcon tube and allowed to settle for 10 to 15 min in water bath at 37°C. Cumulus oocyte complex (COCs) were evaluated under stereo microscope (90 x) and washed 3 times in oocyte aspiration medium. Excellent and good oocytes were transferred to in vitro maturation medium consists of (TCM-199+100 ng/ml IGF1 supplemented with 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulating hormone (FSH) + 50 µg/ml gentamicin). COCs were matured for 22 h in CO_2 incubator at 38.5°C and 5% CO2 in humidified atmosphere. The assessment of in vitro maturation in buffalo oocytes was done in 15 replicates. After 22 h of incubation, the presence of first polar body in the perivitelline space (M II) was the criterion for nuclear maturation of the oocytes. Detection of first polar body was done under inverted microscope in magnification 20X [31]. Maturation rate = (No. of matured oocytes (MII)/ No. of Excellent and Good quality oocytes) X 100. In vitro matured buffalo oocytes (fresh or vitrified /thawed) were used in:

1) Detection of oocyte developmental competence through in vitro fertilization and in vitro culture.

2) Detection of oocyte viability and mitochondrial distribution and intensity through staining with Hochest dye and Mitotricker red and examination using confocal microscope.

2. Vitrification of oocytes:

Morphologically normal matured oocytes with 1st polar body were placed in basic media (BM) (9.5 ml TCM 199 + 0.5 ml FCS +50 µg/ml gentamicin), then in vitrification solution one (VS1) (BM+ Ethylene Glycol (EG) 20% or Dimethyl Sulphoxide (DMSO) 20% or combination (EG 10%+DMSO 10%)) for 1 min, which is half concentration of vitrification solution two (VS2) in EG group (EG, 40%) or DMSO group (DMSO, 40%) or combination group (EG 20% + DMSO 20%). After equilibration, oocytes were transported to the vitrification solution (VS2) for 30 sec [18] then loaded in holding media (BM +0.5 M sucrose).

3. Loading of oocytes:

Oocytes were loaded into 0.25 ml French straw using micro-classic pipette (Karl Hecht No. 558). First loading holding medium column was drawn and separated from the oocytes in the vitrification solution by air bubble chamber from two sides then drew it to the end of the straw. The loaded straw was sealed by polyvinyl powder and groups of approximately 25–30 oocytes were loaded into the straw. The straw was exposed to liquid nitrogen (LN2) vapor for 10 sec. then plunged into LN2 [18] and stored for 7 days.

4. Thawing of oocytes:

Straws contain vitrified oocytes were plunged into a water bath at 37°C for 10–15 s. The vitrified–warmed oocytes were transferred immediately to fresh thawing medium containing BM +0.5 M sucrose. A three-step procedure was used for dilution of sucrose gradually using BM + 0.5M then BM+ 0.3M then BM+0.17 M, sucrose, with 1 min equilibration in each solution. Oocytes were then washed three times with fresh BM medium [18].

5. Morphological evaluation of vitrified–thawed in vitro matured buffalo oocytes:

Recovered oocytes were examined under an inverted microscope. Normal oocytes showed spherical and symmetrical shape with no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content but abnormal oocytes showed ruptured zona pellucida (ZP) or fragmented cytoplasm with signs of degeneration [33].
Morphologically normal oocyte rate = No. of normal vitrified–thawed oocytes with 1st Polar body X 100/ Total no. of recovered vitrified/ thawed oocytes [33].

6. Detection of developmental competence of fresh or vitrified-thawed in vitro matured buffalo oocytes:

Morphologically normal vitrified-thawed in vitro matured buffalo oocytes were washed in fertilization medium (Fert-TALP + 6 mg/ml BSA+50 µg/ml gentamicin + 10 µg/ml heparin). Frozen-semen straw was thawed in water bath at 37°C for 30 seconds. Frozen /thawed semen was layered on the top of two layers of Percoll density gradient (90% and 45%) and centrifuged for 30 minutes at 2000 rpm. The supernatant and Percoll were removed and sperm pellet was suspended with 5 ml sperm-TALP medium containing 10 µg/ml heparin and 4 mg/ml BSA, then centrifuged again for 10 minutes at 1800 rpm. The supernatant was removed, and the sperm pellet was re-suspended in fertilization-TALP medium supplemented with 10 µM/ml hypotaurin, 1 µM/ml epinephrine, 20 µM pencillamine (PHE) and 6 mg/ml BSA. Sperm concentration was adjusted to 1×10^{6} sperm/ml and then allocated into 4-well culture plate. The sperm-oocytes were co-incubated for 18 h at 38.5°C and 5% CO2 in humidified air then the number of oocytes with second polar body were counted [34].

Fertilization rate = No. of normal vitrified–thawed oocytes with 2nd polar body X 100/ Total no. of normal recovered thawed oocytes

The presumptive zygotes were washed 3 times in modified synthetic oviduct fluid (mSOF) medium then incubated in mSOF medium. Incubation was performed for 7 days at 38.5ºC under atmosphere of 5% CO2 in humidified air. mSOF medium was changed every 2 days. Detection and counting of transferable embryos (morula and blastocyst) on days 5, 7 of in vitro culture (day of culture=day0) were done. Detection of the transferable embryos rate (morula and blastocyst) = No. of morula and blastocyst X 100/ Total no. of fertilized oocytes [34].

7. Detection of distribution and intensity of mitochondria of fresh or in vitro matured vitrified/ thawed buffalo oocytes:

In vitro matured buffalo oocytes either fresh or vitrified / thawed were stained using Mitochondrion-specific fluorescent probe, Mito Tracker red FM (thermo fisher). Confocal microscopy (Zeiss LSM 710) was used to determine mitochondrial distribution in the oocytes according to manufacture instruction. In vitro matured buffalo oocytes fresh or vitrified/ thawed were incubated with a final concentration of 500 nM Mito Tracker red FM in PBS for 30 min at 37 ºC in incubator. Oocytes were then washed twice in PBS and co-incubated with PBS containing 5 µg/mL bisbenzimide (Hoechst 33342) to counterstain the nucleus and enable determination of the stage of nuclear maturation. Hoechst 33342 detection in excitation 346 mm and emission in 460 mm wavelength using confocal microscope in 20X magnification. Oocytes were then washed and mounted in PBS and were visualized in glass bottom culture plate 12 mm diameter (thermo fisher) using confocal microscope (Zeiss 710).

The Mito Tracker red fluorescence was observed using an argon laser in 581nm excitation line and 644 nm emission filters. One optical section was examined for each oocyte, in the plane where the nucleus was visible. The determination of the mitochondrial distribution (Fig 1) was as follows: peripheral mitochondrial distributions were in oocyte which no mitochondria were found at the center of the oocyte; semi-peripheral distributions were in those with an in-homogeneous distribution of mitochondria in the inner region of the oocyte; and diffuse distributions were those with a uniform distribution of mitochondria across the entire inner region of the oocyte cytoplasm. The mitochondrial intensity automatic detected through the software of confocal microscope [35]. The experiment was replicated three times, with a group of 10 oocytes in each replicate.

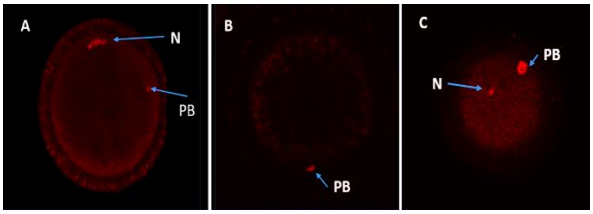

Fig 1: Mitochondrial distribution in vitrified /thawed buffalo oocytes using confocal microscope A) peripheral distribution, B) semi peripheral distribution, C) Diffuse distribution PB= Polar Body N= Nucleusles and figures

8. Statistical analysis

Data were expressed as mean ± standard error (SE). The significant of differences ($P<0.05$) was tested by one-way analyses of variance (ANOVA) followed by hoc test. Statistical analyses were performed using SPSS 16.0

2.2 Methods

Experiment 1: The effect of different cryoprotectants on morphological changes of vitrified-thawed in vitro matured buffalo oocytes

This experiment was done on 381 in-vitro matured buffalo oocytes that divided into three groups for vitrification in EG, DMSO or EG+DMSO as described before. The vitrified in vitro matured buffalo oocytes thawed after one week vitrification for morphological evaluation. The vitrified/ thawed in vitro matured buffalo oocytes classified morphologically into normal and abnormal (ruptured zona pellucida or fragmented cytoplasm with signs of degeneration) [18].

Experiment 2: The effect of different cryoprotectants on developmental competence of in vitro matured-thawed buffalo oocytes

These experiments were done on 387 in vitro matured buffalo oocytes that divided into four groups (fresh and vitrified in EG, DMSO or EG+DMSO). The fresh and vitrified /thawed in vitro matured buffalo oocytes were in vitro fertilized and in vitro cultured as described before for evaluation the developmental competence of oocytes for detection the fertilization rate, cleavage rate and transferable embryo rate.

Experiment 3: The effect of different cryoprotectants on mitochondrial distribution and intensity of vitrified-thawed in vitro matured buffalo oocytes

A total number of 120 in vitro matured buffalo oocytes fresh or vitrified / thawed (vitrified in EG, DMSO or EG+DMSO) were stained using Mitochondrion-specific fluorescent probe, Mito Tracker red FM (thermo fisher). Confocal microscopy (Zeiss LSM 710) was used to determine mitochondrial distribution in the oocytes according to manufacture instruction. Bisbenzimide (Hoechst 33342) stain was used to counterstain the nucleus and enable determination of the stage of nuclear maturation.

3.Results:

Experiment 1: The effect of different cryoprotectants on morphological changes of vitrified-thawed in vitro matured buffalo oocytes

There were no significant differences between the percentages of recovered in vitro matured buffalo oocytes after thawing in DMSO group 97.87%, EG group 95.93% and EG+DMSO group 98.29% (Table 1) (Fig. 2).

The percentages of recovered morphologically normal in vitro matured vitrified / thawed oocytes in mixture of EG+DMSO 92.17% were significantly higher ($P < 0.05$) than oocytes vitrified in DMSO 58.69% and EG 76.27%. Also, there were significant differences ($P < 0.05$) higher percentages of recovered morphologically normal recovered in vitro matured oocytes vitrified in EG 76.27% than DMSO 58.69% (Table 1).

The percentages of recovered morphologically abnormal in vitro matured oocytes in mixture of EG+DMSO 7.82% were significantly lower ($P < 0.05$) than oocytes vitrified in DMSO 41.30% and EG 23.72%. In addition, There were significant ($P < 0.05$) lower between the percentages of recovered oocytes vitrified in EG 23.72% than DMSO 41.30% (Table 1) (Fig.2).

The percentage of vitrified oocytes with leakage of cellular content was significantly higher ($P < 0.05$) in EG group 14.28% than in EG+DMSO group 11.11% or DMSO group 5.26%. Percentage of vitrified oocytes with cracking of zona pellucida was significantly higher ($P < 0.05$) in EG+DMSO group 44.44% than in EG group 14.28% and DMSO group 5.26%. Percentage of oocytes with shrinkage of cytoplasm was significantly higher ($P < 0.05$) in DMSO group 89.47% than in EG group 71.44% and EG+DMSO group 44.44%. (Table 2) (Fig. 3&4).

Table 1: The effect of different cryoprotectants on morphological changes of vitrified/thawed in vitro matured buffalo oocytes

Items	Type of cryoprotectant								
	DMSO			EG			EG+DMSO		
	No.	Mean± SE	%	No.	Mean± SE	%	No.	Mean± SE	%
No. of mature oocytes	141	28.20±2.40	-	123	24.60±1.43	-	117	23.40±0.89	-
Recovered oocytes after thawing	138	27.60±2.03	97.87[a] (138/141)	118	23.60±1.34	95.93[a] (118/123)	115	23.00±0.63	98.29[a] (115/117)
Morphologically normal oocytes	81	16.20±1.20	58.69[a] (81/138)	90	18.00±0.50	76.27[b] (90/118)	106	21.20±1.01	92.17[c] (106/115)

Morphologically abnormal oocytes	57	11.40±1.63	41.30[a] (57/138)	28	05.60±2.19	23.72[b] (28/118)	9	01.80±0.66	7.82[c] (9/115)

***Replicates = 5**
NO.= Number, DMSO = Dimethyl sulfoxide, EG = Ethylene Glycol.
a, b c: Superscripts to be compared statistically within the same rows. Values with different letters are significantly different (P<0.05)

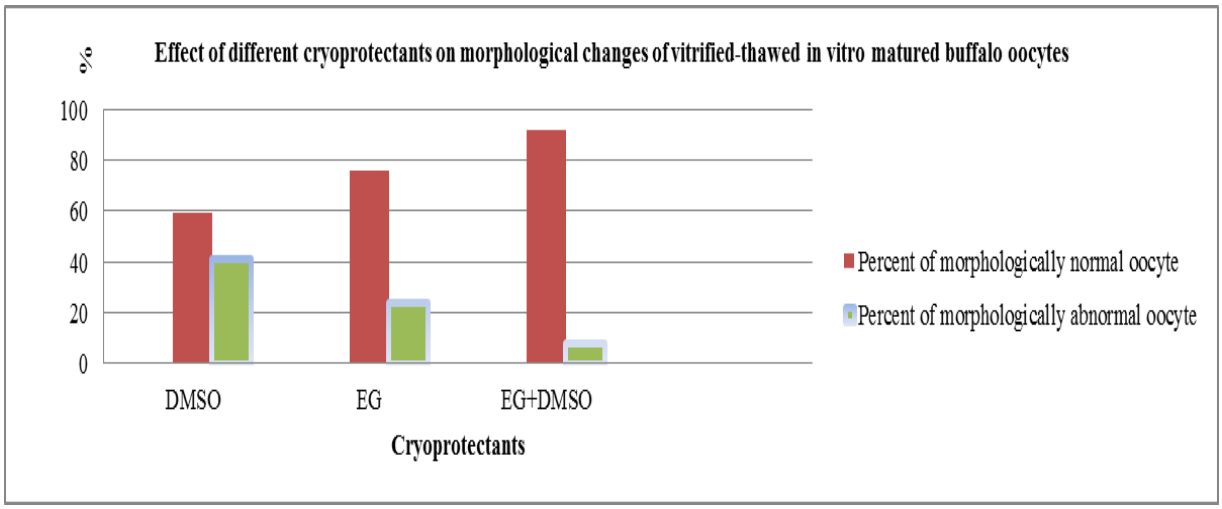

Fig. 2: The effect of different cryoprotectants on morphological changes of vitrified thawed in vitro matured buffalo oocytes

Table 2: Types of abnormalities of vitrified/thawed buffalo oocytes

Types of cryoprotectant	Leakage of cellular content (%)	Cracking of zona pellucida (%)	Shrinkageof cytoplasm (%)
DMSO	3/57 (5.26%) [a]	3/57 (5.26%) [a]	51/57 (89.47%) [a]
EG	4/28 (14.28%)[b]	4/28 (14.28%) [b]	٢0/28 (71.44%) [b]
EG+DMSO	1/9 (11.11%) [c]	4/9 (44.44%) [c]	4/9 (44.44%) [c]

***Replicates = 5**
DMSO = Dimethyl sulfoxide, EG = Ethylene Glycol.
a,b,c supscripit within columns with different letters are significantly different; p < 0.05.

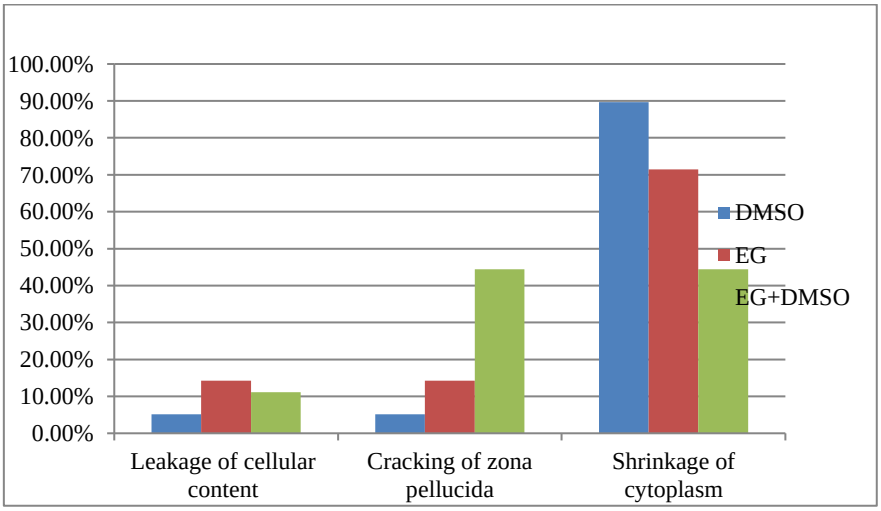

Fig. 3: The effect of different cryoprotectants on morphological changes of vitrified/thawed in vitro matured buffalo oocytes

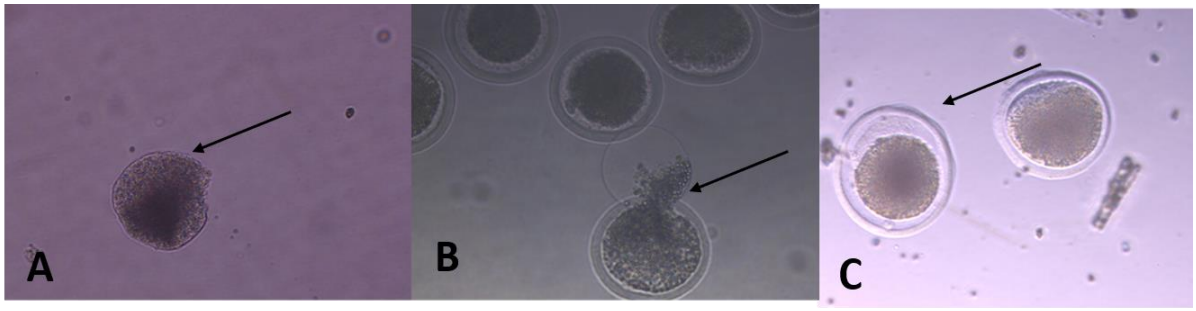

Fig. 4: Types of oocyte abnormalities A: Cracking of zona pellucida, B: Leakage of cellular content, C: Shrinkage of cytoplasm

Experiment 2: The effect of different cryoprotectants on developmental competence of in vitro matured-thawed buffalo oocytes

The fertilization rate of mature oocytes vitrified in EG+DMSO group 58.49% was significantly higher (P < 0.05) than those vitrified in EG group 48.89% or DMSO group 38.27%. Fertilization rate of EG+DMSO 58.49% was significantly lower (P < 0.05) than fresh group 91.81% (Table 3) (Fig.5).

Cleavage rate of mature oocytes vitrified in EG+DMSO group 56.60% was significantly higher (P < 0.05) than those vitrified in EG group 47.77% and DMSO group 35.80%. The percentage of cleavage rate of EG+DMSO 56.60% was significantly lower (P < 0.05) than fresh group 90.90% (Table 3) (Fig.5).

Transferable embryos rate of mature oocytes vitrified in EG+DMSO group 25.00% was significantly higher (P < 0.05) than those vitrified in EG group 20.93% or DMSO group 17.24%. The percentage of Transferable embryos rate of EG+DMSO 25.00% was significantly lower (P < 0.05) than fresh group 53.00% (Table 3) (Fig.5).

Experiment 3: The effect of different cryoprotectants on mitochondrial distribution and intensity of vitrified-thawed in vitro matured buffalo oocytes

More mitochondria were diffusely distributed in the fresh oocytes 93.33% than in all vitrified groups oocytes DMSO group 63.33%, EG group 79.99% and DMSO+ EG group 79.99%; peripheral and semi-peripheral distributions were significantly (P<0.05) higher in DMSO group 20% and 16,67% than EG group 10% and10%, DMSO+ EG group 6.67% and 13.33% and the fresh in vitro matur group 3.33% &3.33%, respectively. (Table 4) (Fig.6 &7). The intensities of florescent of fresh mature oocytes 282.66 was significantly higher (P < 0.05) than those vitrified in EG+DMSO group 186.22. The Mean No. of mitochondrial intensity of recovered mature oocytes vitrified in EG+DMSO group 186.22 was significantly higher (P < 0.05) than those vitrified in EG group 153.33 or DMSO group 146.98. There was no-significant difference between EG group and DMSO group (Table 5, Fig.8).

Table 3 The effect of different cryoprotectants on developmental competence of in vitro matured-thawed buffalo oocytes

Type of Cryo-Protectant	Normal mature oocyte	Morphologically normal oocyte	Fertilized oocyte NO.	Mean Fertilized oocyte	Fert. rate%	Cleaved zygote NO.	Cleavage rate%	(Tr.Emb.) NO.	(Tr.Emb.) rate%
DMSO	**81**	**58.69%** (81/138)	**31**	**6.20 ± 0.58**	**38.27%^{a}** (31/81)	**29**	**35.80%^{a}** (29/81)	**5**	**17.24%^{a}** (5/29)
EG	**90**	**76.27%** (90/118)	**44**	**8.80 ± 0.58**	**48.89%^{b}** (44/90)	**43**	**47.77%^{b}** (43/90)	**9**	**20.93%^{b}** (9/43)
EG+DMSO	**106**	**92.17%** (106/115)	**62**	**6.20 ± 0.66**	**58.49%^{c}** (62/106	**60**	**56.60%^{c}** (60/106	**15**	**25.00%^{c}** (15/60)
Fresh	**110**	-	**101**	**20.2± 0.56**	**91.81%^{d}** (101/110)	**100**	**90.90%^{d}** (100/110)	**53**	**53.00%^{d}** (53/100)

*Replicates = 5

DMSO = Dimethyl sulfoxide, EG = Ethylene Glycol, Fert= Fertilization, Tr. Emb.= Transferable Embryo

a,b,c Subscript within columns with different letters are significantly different; p < 0.05.

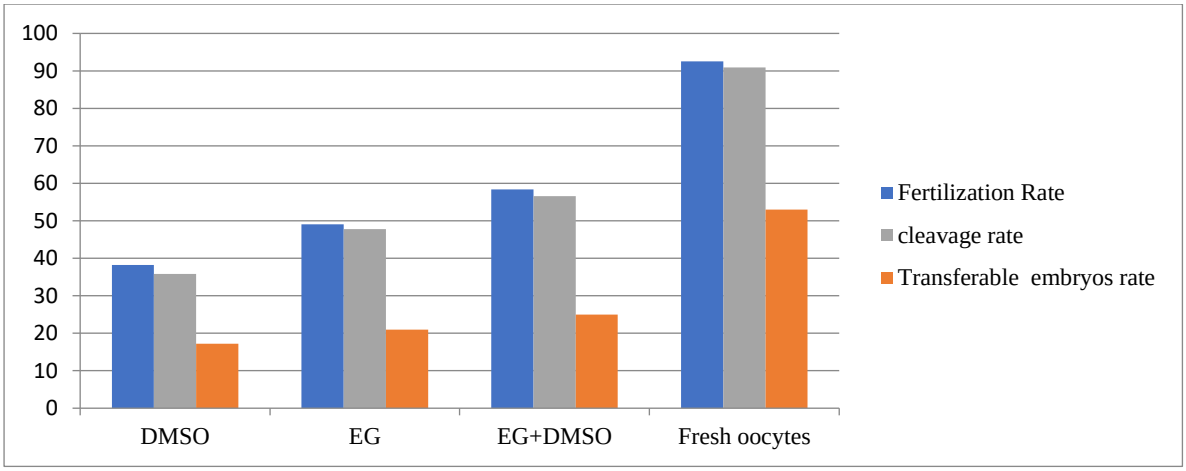

Fig. 5 The effect of different cryoprotectants on in vitro matured-thawed buffalo oocytes developmental competence of in vitro matured-thawed buffalo oocytes

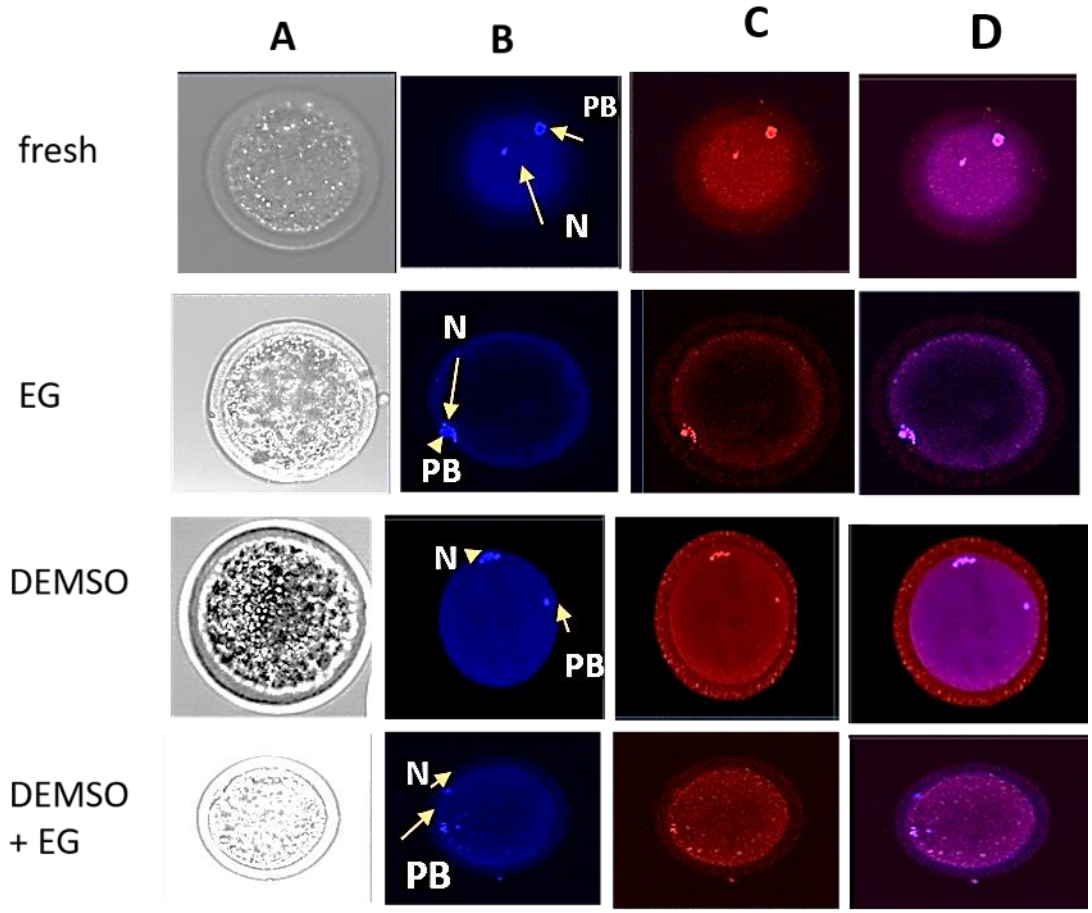

Fig. 6, Effect of different cryoprotectants on viability and mitochondrial distributions of vitrified /thawed in vitro matured buffalo oocytes using confocal microscope. A) matured oocytes without staining B) Matured oocytes stained with Hochest dye, C) Matured oocytes stained with Mitotricker red, D) Matured oocytes stained with Hochest dye and Mitotricker red

Table 4: **The effect of different cryoprotectants on mitochondrial distribution of vitrified-thawed in vitro matured buffalo oocytes**

	No of mature oocyte	peripheral (%)	Semi-peripheral (%)	Diffuse (%)
DMSO	30	6 (20%)[a]	5 (16.67%)[a]	19 (63.33%)[b]
EG	30	3 (10%)[b]	3 (10%)[b]	24 (79.99%)[b]
EG+DMSO	30	2 (6.67%)[b]	4 (13.33%)[b]	24 (79.99%)[b]
Fresh	30	1 (3.33%)[b]	1 (3.33%)[b]	28 (93.33%)[a]

***Replicates = 3**
DMSO = Dimethyl sulfoxide, EG = Ethylene Glycol
a,b Subscript within columns with different letters are significantly different; p < 0.05.

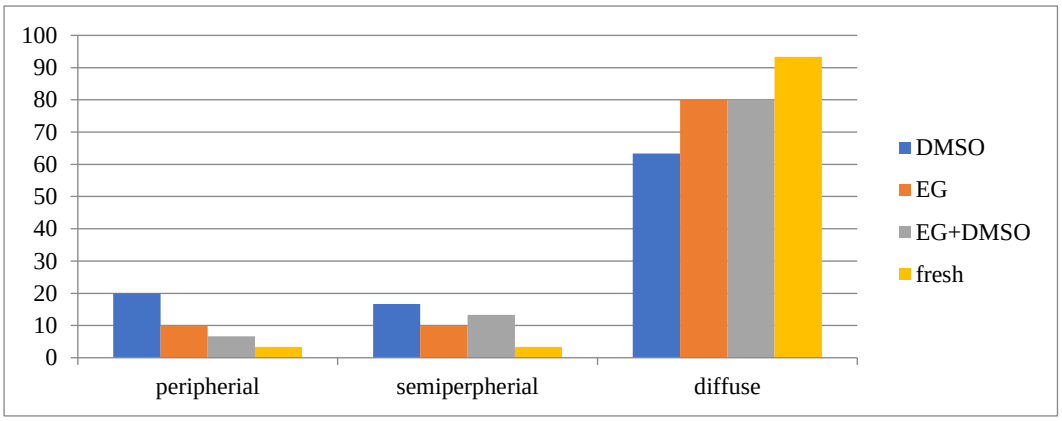

Fig. 7 The effect of different cryoprotectants on mitochondrial distribution of vitrified/thawed in vitro matured buffalo oocytes.

Table 5: **The effect of different cryoprotectants on intensity of mitochondria of vitrified/thawed in vitro matured buffalo oocytes**

	No of mature oocyte	Mean No. of florescent intensity ± SE
DMSO	30	146.98 ± 9.60[c]
EG	30	153.33 ± 17.40[c]
EG+DMSO	30	186.22 ± 7.80[b]
Fresh mature oocyte	30	282.66 ± 6.30[a]

a,b,c Subscript within columns with different letters are significantly different; p < 0.05.

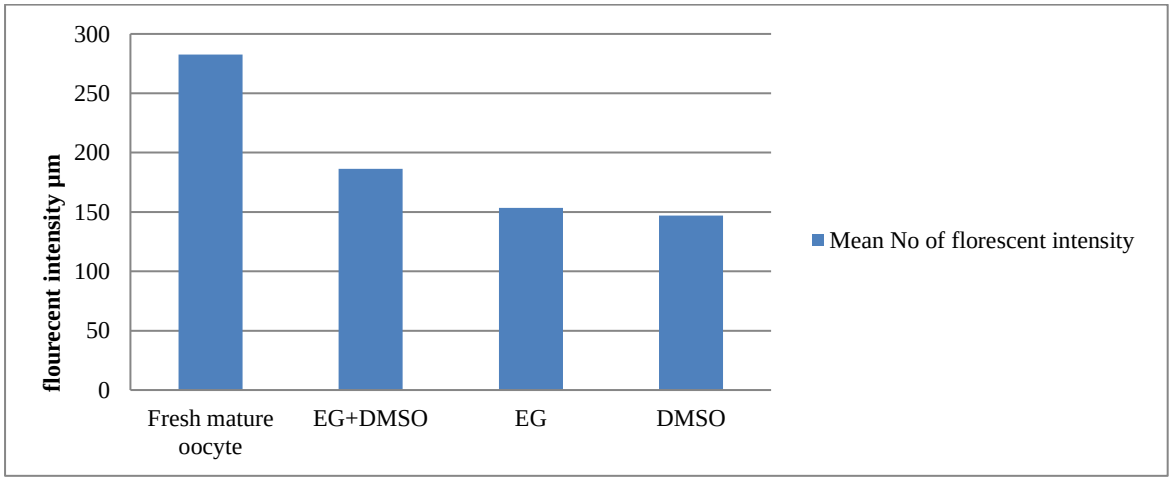

fig. 8: The effect of different cryoprotectants on intensity of vitrified/thawed in vitro matured buffalo oocytes

4. Discussion

The effect of different cryoprotectants on morphological changes of vitrified-thawed in vitro matured buffalo oocytes

In this study, there were no significant differences ($P < 0.05$) between the percentages of recovered in vitro matured vitrified/ thawed oocytes in DMSO group 97.87%, EG group 95.93% and EG+DMSO group 98.29%. Our results of in-vitro matured vitrified-thawed buffalo oocytes were similar to the results reported by Gautam [36] who had the following post thawing recovery rate of 97.8% of vitrified-thawed mature buffalo oocytes, and recovery rate ranged from 92% to 95% lower recovery rate of vitrified thawed oocytes was recorded by Sharma and loganthasamy [37]. The recovery rate ranged from 89% to 92% [38] and 92% [39]. Dolakasaria [40] who had recovery rate of 91.06%. The viability rate of vitrified thawed mature Egyptian buffalo oocytes was 89.2% [41] When the post thaw recovery of 81.35% vitrified-thawed mature buffalo oocytes [42]. Loose of the oocytes may occur due to its damage during expansion and shrinkage cycle associated with exposure to different osmotic pressure or due to sticking of the oocytes to the pipette or cryo-device [43]. This loss depends on the number of oocytes per cryo-device: as numbers increase, more time is required for the movement of cells through the vitrification solutions, which results in cells being exposed to the chemicals for a long time and subsequently results in higher damage rate [44]**.**

In this study, the percentage of morphologically normal recovered in vitro matured oocytes vitrified in mixture of EG+DMSO was significantly higher ($P < 0.05$) than oocytes vitrified in EG or DMSO. Mishra [42] had 74.28% of the normal mature buffalo oocytes. Combination between DMSO and EG cryoprotectants is significantly superior for vitrification followed by maturation of oocytes [20].

Our findings showed that the percentages of morphologically normal recovered in vitro matured oocytes vitrified in EG was 76.27% which is less than that reported by Yadav [45] who find that the survival of COCS was 86.4% and 89.6% using different concentration of EG and Glycerol and also lower than findings of Gasparrini [46] who had the following post thaw recovery of matured buffalo oocyte 84.6% using a solution of 35% ethylene glycol, 5% polyvinyl–pyrrolidone and 0.4% trehalose. Incidence of normally morphological recovered immature buffalo oocytes vitrified in EG or DMSO was 85% and 83% respectively [47]. In sheep, survival rate of oocytes after vitrification in 33% ethylene glycol was higher than in 33% DMSO or a mixture of 17.5% ethylene glycol and 17.5% DMSO 87.64 vs. 77.43 vs. 69.39%, respectively [48]. Several studies reported that EG would be the ideal cryoprotectant agent because its ability to penetrate cell membranes faster than glycerol and is less toxic than DMSO [49]. The molecular weight is of EG is 62.1Kda, lower than that of DMSO 78.1Kda which helps fast influx and efflux into and out of cells whereas EG, which has more penetrating the effect, protects all the membranes of intracellular structures, including lysosomes [50]

Our findings, also, showed that the percentage of morphologically normal recovered in vitro matured oocytes vitrified in DMSO was 58.69. Wani [21] reported survival rates of vitrified-thawed immature buffalo oocytes which was 94.6 % in DMSO and 97.1% in EG.

In this study, the percentages of morphologically abnormal recovered in vitro matured buffalo oocytes in mixture of EG+DMSO 7.82% were significantly lower ($P < 0.05$) than oocytes vitrified in in EG 23.72% and DMSO 41.30%. There were significant differences ($P < 0.05$) between the percentages of morphologically abnormal recovered in EG 23.72% and DMSO 41.30%.

Using a mixture of EG and DMSO, Mishra [42] reported damaged of vitrified-thawed mature buffalo oocytes 25.71%. Bethapudi [37] reported damaged of vitrified-thawed mature buffalo oocytes ranged from 10-22%. In the same context, Hammam [51] reported damaged of vitrified- thawed mature buffalo oocytes 27.7%. While, Gautam [36] reported damaged of vitrified-thawed mature buffalo oocytes ranged from 4% to 15%. Sharma [38] reported the lowest rate of damaged of vitrified-thawed mature buffalo oocytes 9%.

Concerning the rate of recovered oocytes, our results showed that the percentages of morphologically abnormal recovered oocytes vitrified in EG was 23.72% which more than Yadav [45] who found that the survival of COCs was 13.6% and 10.4% using different concentration of EG and Glycerol and also lower than findings of Gasparrini [46] who had the following post thaw recovery of matured buffalo oocyte 15.4% using mixture of 35% ethylene glycol, 5% polyvinyl–pyrrolidone and 0.4% trehalose. Incidence of normally morphological recovered immature buffalo vitrified in EG or DMSO was 15% and 17%, respectively [47].

The oocytes have relatively complex sub-cellular structure, within which many of the subcellular components are particularly temperature sensitive [52]

and osmotically and ionically sensitive [53]. Cooling of oocyte affects spindle fiber integrity [54] and depolymerization of the spindle fiber is likely lead to aneuploidy [55] Cooling affects cortical granule vesicles and premature release of the cortical granule vesicles is likely to lead to ZP hardening [56].

In this study, the percentage of oocytes with leakage of cellular content was significantly higher ($P < 0.05$) in EG group 14.28% than in EG+DMSO group 11.11% and DMSO group 5.26%. The percentage of oocytes with cracking of zona pellucida was significantly higher ($P < 0.05$) in EG+DMSO group 44.44% than in EG group 14.28% and DMSO group 5.26%. Percentage of oocytes with shrinkage of cytoplasm was significantly higher ($P < 0.05$) in DMSO group 89.47% than in EG group 71.44% and EG+DMSO group 44.44%.

Using a mixture of EG and DMSO, Hammam [51] recorded zona crack 16.0%, shrinkage of cytoplasm 50% and leakage of cellular content 33.3% after thawing of in-vitro matured buffalo oocytes. It was recorded that recorded zona crack 42.9%, splitting 14.3%, change in shape 17.1% and leakage of cellular content 25.7% after thawing of in-vitro matured buffalo oocytes [38]. The damage of oocytes during cryopreservation may be because of the higher lipid content found in oocytes of many species, since lipid removal or lipid polarization reduces chill and cryoinjury [57].

According to Abd El-Raheem [18], recovered oocytes that affected by zona crack or changes in shape or leakage ranged between 65, 5%, 73.3% and 72.4%, respectively) according to oocyte quality. There was wide difference in the results as the efficiency of buffalo oocyte vitrification depends on many factors needs to be optimized such as technicians' skills, types, concentration and combination of selected cryoprotectants, as well as strict time of exposure of oocytes to the cryoprotectants, and finally, the quality of the oocytes selected for vitrification, buffalo oocyte IVM and IVF as well as embryo culture. Moreover, the smaller sample size (vitrification solution containing oocytes) could permit an increase of cooling and thawing rates which can reduce the toxicity of the cryoprotectants solutions and chilling damage [58]. The combination of cryoprotectant agents (CPAs) for the vitrification process may have induced a lower relative concentration and also lower toxicity of CPAs [59].

The effect of different cryoprotectants on developmental competence of in vitro matured-thawed buffalo oocytes

In this study, the percentage of fertilization rate of recovered mature oocytes vitrified in EG+DMSO group 58.49% was significantly higher ($P < 0.05$) than those vitrified in EG group 48.89% and DMSO group 38.27%. The percentage of fertilization rate of EG+DMSO 58.49% was significantly lower ($P < 0.05$) than fresh group 91.81%.

In addition, cleavage rate of recovered mature oocytes vitrified in EG+DMSO group 56.60% was significantly higher ($P < 0.05$) than those vitrified in EG group 47.77%) and DMSO group 35.80%. The percentage of cleavage rate of EG+DMSO 56.60% was significantly lower ($P < 0.05$) than fresh group 90.90%

Percentages of Transferable embryos of matured oocytes vitrified in EG+DMSO group 25.00% was significantly higher ($P < 0.05$) than those vitrified in EG group 20.93% and DMSO group 17.24%. Also, transferable embryos rate of EG+DMSO 25.00% was significantly lower ($P < 0.05$) than fresh group 53.00%

The fertilization rate of buffalo oocytes was 15.17% using 4.5 M DMSO with conventional straw method and 33.6% using 5.5 M EG with Open Pulled Straw (OPS)[60]. While in the solid surface vitrification (SSV) the cleavage rate of in- vitro matured buffalo oocytes were 15.2% [46], using cryoloop method, the cleavage rate of in-vitro matured buffalo oocytes was 14.2% [46]. Fertilization rates observed between the vitrification groups were 20.2% and 31.5% for calf and cow oocytes vitrified in OPS, respectively [61]. Fertilization rate of vitrified-thawed in-vitro matured buffalo oocytes ranged from 25.3% to 43.29% according to different concentrations of EG and DMSO [37]. Fertilization rate was 38.5% for vitrified-thawed mature buffalo oocytes as reported by [41]. Using 40% EG had 40.0% fertilization rate of in-vitro matured swamp buffalo oocytes [62]. Hardin found that using 20% EG+ 20% glycerol had 31.81% fertilization rate of in-vitro matured swamp buffalo oocytes [20]. Whereas no differences in blastocyst rates of vitrified thawed mature buffalo oocytes were found in different concentrations of combination of DMSO and EG 6.4%, 7.8%, 5.9% and 6.9% [63]

Cleavage rate of vitrified-thawed immature buffalo oocytes ranged from 6.9% to 29.8% on using different concentrations of DMSO and ranged from 6 % to 30.8% in different concentrations of EG [21]. In sheep, the cleavage and blastocyst rates were higher

after vitrification in mixture group than in ethylene glycol and DMSO 46.81 and 15.5 vs. 37.55 and 9.12 vs. 29.51 and 6.40%, for cleavage and blastocyst rates in different groups, respectively) [48].

Cooling affects cortical granule vesicles and premature release of the cortical granule vesicles is likely to lead to zona pellucida (ZP) hardening [56]. A reduced IVF rate due to changes in ZP has been reported in mouse oocytes exposed to 1.5 M DMSO and PG [64]. It has been documented that oocytes suffer from reversible and irreversible damage during cryopreservation [64] . This damage includes hardening of the zona pellucida, premature cortical granules release, depolymerization of the microtubules, and misalignment of the chromosomes. Vitrification negatively affects spindle morphology and other organelle morphology in mature oocytes [65]. The cortical granules seem to be very susceptible to changes due to vitrification [66]. These changes can cause a failure in the calcium release required to prevent polyspermy, which may resulted in a reduction in embryonic development [67].Another hypothesis is that the permeating cryoprotectants generating osmotic stress on the oocytes and causing formation of ice crystals which induced perforation holes through the cell membrane allowing multiple spermatozoa to enter the oocytes without oocyte activation and maternal pronuculus formation, producing multiple sperm pronuclei but not the maternal one [20]. Some spindle anomalies, induce DNA fragmentation that eventually leads to aneuploidy, which is incompatible with subsequent embryo development [67]. In addition, it was demonstrated that vitrification induces profound ultra-structural modifications to microvilli, mitochondria, oolemma and cortical granules as well as to the size and position of the vesicles in buffalo oocytes [57].

The effect of different cryoprotectants on mitochondrial distribution and intensity of vitrified-thawed in vitro matured buffalo oocytes

In the present work, higher portion of fresh oocytes showed significantly diffused mitochondria through the cytoplasm than vitrified groups while Peripheral and semiperipheral mitochondrial distributions were significantly higher in DMSO group 20% & 16.67% than EG group 10% &10%, DMSO+ EG group 6.67% & 13.33% and fresh group 3.33% &3.33%. According to Stojkovic [26] mitochondrial distribution are mainly homogenous through the cytoplasm of mature oocyte providing equal distribution of mitochondria between zygote cells after fertilization, at least in part, is likely related to their further developmental competence [26]. Moreover, it is demonstrated that higher density and distribution of active mitochondria is in correlation with faster nuclear maturation [69]. Many studies showed that vitrification and warming process may affect mitochondrial function, structure and distribution in oocytes [70]. In buffalo, mitochondria are arranged in the cortical region [3] which is also the first location damaged by freezing [7]. Much more the damage in oocytes mitochondria during vitrification procedure may be without or with visible morphological changes [28]. Cytoplasmic maturation requires high level of energy which is provided by mitochondria [71]. This organelle has vital role in production of energy for oocyte; therefore, it is an indicator of cytoplasmic maturation [69]. Competence of cytoplasm maturation omits the ability of the oocyte to block the diffusion of more than one spermatozoon [72], and also to improve the de-condensation of the sperm head during fertilization process [73]. This may explain the lower fertilization rate of vitrified-thawed mature buffalo oocytes than fresh mature oocyte in our experiment.

5. Conclusion

Combination of EG+DMSO cryoprotectants improved the viability and developmental competence of in vitro matured vitrified/thawed buffalo oocytes**.**

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