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Morphological evaluation, livability and maturation rate of post-thawed buffalo's oocytes vitrified with soybean lecithin

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ABSTRACT

Current work aims to improving survival rate, quality and maturation rate of buffalo oocytes after vitrification using soybean lecithin (SL) to vitrification solution (VS) media of immature, previously matured oocytes and maturation rate after thawing. Three separate trials were held in this study using fresh COC's were collected from slaughtered buffalo's ovaries which divided into three major groups, first group underwent direct vitrification (control) the second group was treated with addition of 1, 2.5, 5 and 10% soybean lecithin to vitrification media and the last group recruited for *in vitro* maturation and then stepwise vitrification with or without SL.

Total post-thawed normal or abnormal oocytes morphological appearance did not significantly affected by addition of soybean lecithin for both immature and previously matured oocyte vitrification trials. The best viability after thawing for immature (57.1%) *vs.* matured oocytes (42.9%) was observed for 5% SL and 2.5% addition to VS respectively. Similarly, subsequent maturation of vitrified-thawed oocytes with SL; was not affected. However the best maturation rate (46.5%) was observed for 5% SL addition to VS. Generally, soybean lecithin has no effect on vitrified-thawed buffalo's oocytes.

Key words: lecithin, buffalo, oocytes, vitrification, maturation

Introduction

Genetic improvement of buffalo can be achieved by the application of assisted reproductive technologies. However, in buffaloes, those methods are not as efficient as in bovine. Cryopreservation of oocytes and embryos is the key of preserving genetic resources for future protection and managing species population integrity and heterozygosity (Parnpai *et al.,* 2016). The major problem with oocyte cryopreservation is low survival and/or poor developmental competence (Szurek and Eroglu, 2011).

The critical site of cryopreservation-induced damage is known to be the oocyte membrane Phospholipids (PL) that affects thermal phase behavior and several physicochemical properties such as fluidity and permeability (Pitangui-Molina *et al*., 2017). Stability of membrane PL improves the oocytes cryotolerance, meanwhile the loss of membrane integrity leads to cell death by a process resembling necrosis. Survival of mature *vs.* immature oocyte after cryopreservation is different, controversial and require further investigation to decide each of which is suitable (Rodriguez-Villamil *et al.*, 2016). Furthermore, preservation of immature oocytes is essential when conditions for *in vitro* maturation (IVM) are not present (Mavrides and Morroll, 2002).

On the other hand, use of nonpermeating macromolecules in vitrification has become more applicable (Prentice and Anzar, 2011), as it increases viscosity, less toxic than other cryoprotectants (CPA), avoids formation of extracellular ice crystal (Mahmoud and El-Sokary, 2013) and protect oocytes against Zona pellucida (ZP) cracking (Moore and Bonilla, 2006). Additionally, lipids play both structural and metabolic roles in mammalian oocytes. Hence, in response to vitrification, 31 type of oocyte phospholipids decreased and 5 increased with a decrees to several triglycerides.

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Additionally, nine phospholipids showed a significant reduction in mouse (Jung *et al.* 2014) and bovine oocytes (Aono *et al.*, 2013) after vitrification-thawing processes.

Hence, current research work aims to modify the vitrification solution of both immature and mature buffalo's oocytes by addition of soybean lecithin (SL), as a nonpermiating macromolecule containing phospholipids and examine its cryoprotective potential on post-warming oocytes membrane stability, viability and maturation rate.

Materials and Methods

Current study was carried out at Animal Reproduction Research Institute, Egypt. Soybean lecithin (SL, MC041) was purchased from Lab M Ltd. Co., Lancashire, UK. Meanwhile, the rest of all chemicals used in this study were purchased from Sigma Aldrich Co. St. Louis, Mo, USA. The pH value for all media was adjusted at 7.3-7.4 and osmolarity at 280-300 mOsmol/L and filtrated twice by 0.22-µm filter (Millipore, Germany) before usage.

Collection of oocytes

Ovaries were collected from slaughtered buffaloes and placed in physiological saline (NaCl, 9 mg/ml), containing antibiotics (penicillin, 100UI/ml and streptomycin sulphate, 100 µg/ml); that maintained at 25-30˚C until oocyte recovery. The collected ovaries were washed twice in saline and rinsed in 70% ethyl alcohol for a few minutes. Compact cumulus-oocytes complexes (COC's) were aspirated from follicles at 2 to 8 mm in size using 18-gauge needle in 5ml Dulbecco's phosphate buffer solution (PBS) supplemented with 3% bovine serum albumin (BSA) and 50 µg/ml gentamicin. The oocytes enclosed in a compact, more than three layers, of cumulus cells, and uniform cytoplasm were selected under stereomicroscope for the current study. Fresh COC's were divided in into three groups, first group underwent direct stepwise vitrification (control). The second group vitrified with addition of different concentrations SL. Meanwhile the third group was used for oocyte maturation trials.

Vitrification and thawing of oocytes

The vitrification-warming procedure was done according to Mahmoud *et al.,* (2013). The basal medium (BM) of the vitrification solution (VS) consisted of TCM-199+ 2.5 mM HEPES + 20% fetal calf serum(FCS) +100 IU/ml penicillin +100 µg/ml streptomycin. Using a stepwise vitrification oocytes were loaded into VS1 (BM + 1.5 M EG + 1.5 M DMSO) in disposable sterile Petri dish for 45 sec then underwent the second step by loading oocytes into VS2 (BM+ 3 M EG + 3 M DMSO) for 25 sec. About 3-7 oocytes were loaded into 0.25 ml French straws with VS2, sealed and plunged directly into liquid nitrogen. The warming procedure was done after one week of storage period by transferring straws into 37°C water bath for 30sec., then rehydrated in 1ml. of BM+0.25% and BM+0.15% sucrose for one min and 5 min respectively. The rehydrated oocytes were washed four times and transferred to fresh BM.

Morphological and viability assessment

Warmed oocytes were evaluated under stereomicroscope for normal and abnormal appearance according to Yadav *et al.*, (2008) as follows; Normal: oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content; Abnormal: oocytes with a ruptured ZP or ruptured vitelline membrane, and fragmented cytoplasm with signs of degeneration. On the other hand, livability was estimated according to El-Sokary *et al.* (2013) using trypan blue dye solution (0.05%, PH=7.0 2min) at room temperature. Hence, exclusion of dye by COCs is an indicative of viability; viable oocytes with viable or dead cumulus were considered viable while dead oocytes (blue color) with viable or dead cumulus were considered dead.

In vitro maturation (IVM)

Both post-thawed and fresh oocytes with normal morphology were used for IVM trials. All oocytes undergoing IVM were cultured in 100 µl of TCM-199+20% FCS+1 µg/ml oestradiol-17β + 50 µg/ml gentamicin covered with mineral oil in four-well culture plates for 22-24 h in a Co_2 incubator (38.5ºC, 5% Co2 and 95% relative humidity). Maturation was assessed by expansion of cumulus cell mass (El-Shahat and Hammam, 2014).

Experimental design

Current study was carried out three separate experiments to evaluate the impact of different concentrations of soybean lecithin (SL) supplementation to the vitrification solution (VS) media on buffalo oocytes morphology, viability and maturation after warming.

Collected COC's were divided into three major groups. The first major group (control) underwent direct stepwise vitrification without adding SL to VS. The second major (treated) was divided into four subgroups that underwent direct stepwise vitrification with different concentrations of SL at 1, 2.5, 5 and 10% to the vitrification media respectively. Post-thawed oocytes from the first and second major groups with normal morphology were recruited for *in vitro* maturation trails (IVM).

In the third group, all fresh oocytes with normal morphology were went first to IVM then the one with normal morphology were divided into five subgroups that underwent stepwise vitrification without (control) or with adding different concentrations of SL at 1, 2.5, 5 and 10% to vitrification solution respectively.

Statistical analysis

The experiment was replicated three times. The proportion of morphologically normal oocytes recovered, viability and maturation rate were compared between various groups by the Chi-square test according to Snedecor and Cochran, (1976).

Results

1-Post-thawed immature oocytes morphology and viability

Addition of SL did not affect total post-thawed normal or abnormal oocytes (Table 1). However, the percent of total immature oocytes with normal morphological appearance were higher for SL addition than control group. The best performance was observed for 5% SL addition to VS that showed higher ($p<0.05$) morphologically normal and viable oocytes (66.7 and 57.1%resp.) with lower percent ($p<0.05$) of morphological abnormalities (33.3%) after thawing.

Table 1: Morphological evaluation and viability of post-thawed immature oocytes vitrified with soybean lecithin.

Treatment	N*	Normal oocytes		Abnormal oocytes		
		Total (%)	Live (%)	Broken Zona pellucida (%)	Cytoplasm shrinkage (%)	Total Abnormalities (%)
Control	41	19(46.3)[a]	14(34.1)[a]	9(22.0)	13(31.7)	22(53.7)[a]
Lecithin 1%	46	22(47.8)[a]	17(37.0)[a]	8(17.4)	16(34.8)	24(52.2)[a]
Lecithin 2.5%	44	21(52.3)[a]	17(43.2)[a]	7(20.5)	10(27.3)	19(47.7)[a]
Lecithin 5%	42	28(66.7)[b]	24(57.1)[b]	6(14.3)	8(19.0)	14(33.3)[b]
Lecithin 10%	42	20(47.6)[a]	14(33.3)[a]	11(26.2)	10(23.8)	21(50.0)[a]

*N number of post-thawed immature oocytes.
a and b: Means within each column followed by different letters differ significantly at *P<0.05*.

2-*In vitro* maturation of post-thawed immature oocytes

Same trend was noticed for subsequent IVM of vitrified-thawed oocytes with SL; no significant effect was found on maturation rate. However the best maturation rate (46.5%) was observed for 5%SL vitrified oocytes than other groups (Table 2).

Table 2: Maturation of post thawed immature oocytes vitrified with soybean lecithin

Treatment	N*	Normal oocytes		Maturation Rate
		Live (%)	Dead/Abnormal (%)	
Control	45	34(75.6)	11(24.4)	12(26.7)
Lecithin 1%	46	36(78.3)	10(21.7)	15(32.6)
Lecithin 2.5%	42	34(81.0)	8(19.0)	15(35.7)
Lecithin 5%	43	38(83.7)	7(16.3)	20(46.5)
Lecithin 10%	39	29(74.4)	10(25.6)	12(30.8)

*N the number of total normal immature oocytes after vitrification-thawing process.

3-Post-thawed mature oocytes morphological assessment and viability

As presented in Table (3) neither oocyte morphology nor viability was affected by SL supplementation to VS. However, the concentration of 2.5% SL has the best performance on post-thawed oocyte morphology and livability (52.4 and 42.9% resp.) which recorded the lowest oocyte morphological abnormalities (47.6%) than other groups.

Table 3: Morphological evaluation and viability of post-thawed of fresh and matured oocytes vitrifided with soybean lecithin

Treatment	N*	Normal oocytes		Abnormal oocytes		
		Total (%)	Live (%)	Broken Zona pellucida(%)	Cytoplasm shrinkage(%)	Total Abnormalities(%)
Control	52	22(42.3)	17(32.7)	16(30.8)	14(26.9)	30(57.7)
Lecithin 1%	43	21(48.8)	15(34.9)	10(23.3)	14(32.6)	24(55.8)
Lecithin 2.5%	42	22(52.4)	18(42.9)	9(21.4)	11(26.2)	20(47.6)
Lecithin 5%	48	21(43.8)	15(31.3)	12(25.0)	15(31.3)	27(56.3)
Lecithin 10%	54	21(38.9)	13(24.1)	14(25.9)	19(35.2)	33(61.1)

Discussion

Major vitrified-thawed oocyte cryoinjuries noted in previous researches were ZP breakage, crack in ZP, leakage of the cellular contents and cytoplasmic shrinkage (Mishra *et al.,* 2012).Immature buffalo's oocytes vitrified with SL have better morphologically normal (66.7 *vs.* 52.4%) and live percent (57.1 *vs.* 42.9%) than mature oocytes compared to control groups of both experiments after warming. Previous results confirmed those reported by Yang *et al.,* (2003) and Cajucom *et al.,* (2017) that post-thawing survival rate of buffalo oocytes is between 65% and 89.0%.On the other hand, immature oocyte showed better performance than mature ones after thawing; because of that genetic material of the germinal vesicle stage (GV) is more resistant to cold stress-induced damage once the chromatin is still protected by nuclear envelope and no spindle apparatus is present (Luciano *et al.*, 2009). Meantime, matured oocytes are subjected to meiotic spindle damage that cause successive chromosomal aberration and aneuploidy resulted in low survival rates after thawing.

Further, Hegab *et al.*, (2009), Kumar and Anand, (2012) and Waheed *et al.*, (2016) reported high post-warming survival and poor maturation rates (45-70%) in vitrified buffalo GV; which is according to oocyte quality, protein additives, hormonal supplement and type of capacitating agents.

Many research reported the correlation of SL addition to cryopreserved bovine (Badr *et al.*, 2012), ram and goat (Khalifa and Abdel-Hafez, 2013) and buffalo (El-Sherbieny, 2014) spermatozoa with high viability after thawing, but no further research studied that effect on vitrified oocytes.

The teamwork of the current study theoretically assumed that SL might maintain buffalo's oocytes morphology and livability after thawing since that SL is known as complex mixture of typical membrane phospholipids (Shurtleff and Aoyagi, 2016). It is well known that, oocytes with more

flexible membranes are permeable to water and CPA, much tolerant to low temprature and likely to suffer less damage that those with more rigid less permeable membranes (Arcarons *et al.*, 2017).

Current experimental results revealed that vitrified-thawed buffalo's oocytes did not significantly affected by SL addition to VS compared to the control groups. In agreement with Pitangui-Molina *et al.*, (2017) who found that selective incorporation of unsaturated phospholipids Phosphatidylcholine (PC) and Phosphatidylglycerol (PG) in bovine oocyte maturation process however addition of PC to IVM media has no potential detrimental effect on maturation rate as well it cannot prevent the oxidative stress of frozen-thawed boar spermatozoa (Alvarez-Rodriguez *et al.,* 2017). That might be due to the negative effects of spontaneous lipid peroxidation that occurred during incubation (Moore and Bonilla, 2006). Alternatively, SL induced serious mitochondrial damage of frozen-thawed ram spermatozoa (Del-Valle *et al.,* 2011), possibly by displacement of cardiolipin which is the main acidic phospholipid of the inner mitochondrial membrane, leading to the release apoptotic factors.

Furthermore, Shchipunov, (2002) cited that polar solvents (i.e.EG, glycerol (GL) and Dimethyl formarnide DMFA) are incapable of inducing jellification of SL solutions. Nevertheless, 1, 3-PD-tike GL forms hydrogen bonds with lecithin phosphate group, though they are weaker.

Conclusion

Although the advantages of SL in cryopreservation of spermatozoa, it did not show a significant enhancement to buffalo oocyte morphology and viability or even *in vitro* maturation in the current experiment. These data results gives the respect to the difference of oocyte compared to spermatozoa because of its substantial volume changes due to water and cryoprotectant movement during cryopreservation. Finally, our teamwork found that SL is not suitable for buffalo oocyte vitrification.

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