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Effect of in Vitro Culture Media on Embryo Developmental Competence and Vitrification Methods in Blastocyst Viability in Buffalo

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Abstract: Embryos derived by *In vitro* production would allow more efficient utilization of superior genetic females without compromising donor fertility. Moreover, vitrification is a promising procedure that is relatively rapid and inexpensive and it has been shown to be beneficial for embryos produced *In vitro*. The developmental potential of *In vitro* produced embryos can be affected by various factors including the maturation system, culture system, oocyte quality and embryonic growth factors. The objectives of this work were, improving *In vitro* embryo production through using different *In vitro* culture maturation mediums and study the effect of virtification method in post thawing survival rate of buffalo embryo. Good and excellent oocytes were cultured in TCM-199 vs. TCM-199+ EGF (20 ng/ml), CR1aa vs. CR1aa + EGF (20 ng/ ml)) 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 a CO_2 incubator at 38.5°C in 5% CO_2 in humidified atmosphere, matured oocytes fertilized with frozen thawed semen (washed by fertilization TALP) and incubated for 18 hours, then *In vitro* culture by SOF and co culture using buffalo oviduct epithelial cells for 7 days. Number of the Blastocysts fixed for cell count using Giemsa stain and other of the rest of morula and blastocyst for cryopreservation. Vetrification of morula and blastocyst using 10% ethylene glycol (EG) + 10% dimethylesulfoxide (DMSO) or 10% glycerol (G) + 10% EG in base medium for 4 min then 25% EG + 25%DMSO+ 0.3 M sucrose or 25% G+25%EG +0.3M sucrose for 45 second. The results showed that, *In vitro* maturation, cleavage, transferable embryos rate of buffalo oocytes showed highly significant ($P < 0.01$) increase in using CR1aa medium when compared with TCM-199 medium. Addition of EGF 20 ng/ml either to TCM-199 or CR1aa improved the developmental competence of embryo. The *In vitro* buffalo embryo development matured in the presence of EGF showed significantly ($P< 0.01$) higher rate of morula and blastocyst (transferable embryos) (40.9-42.7 % respectively) than that without EGF (20-33.5% resp) and showed higher significance ($p<0.01$) in the cell number of the blastocyst. Cryosurvival rate after vitrification-thawing were significantly higher ($P< 0.01$) in embryos that cryoprotected in using 25% EG + 25% DEMSO + 0.3 M sucrose 50% (16/ 32) VS 25% G+25% EG+ 0.3M sucrose 21% (6/29). In conclusion: The addition of epidermal growth factor to *In vitro* maturation mediums improved embryo viability with increasing transferable embryos rate and blastocyst cell number. The using of EG + DMSO cryoprotectants in vitrification of buffalo oocytes showed high cryosurvival post thawing buffalo embryo development. Therefore, *In vitro* produced buffalo embryo and vitrification techniques were suitable for future embryo transfer in buffalo.

Key words: *In vitro* embryo production · Epidermal growth factor · Cell count · Vitrification · Buffalo

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INTRODUCTION

Buffalo is considered as the backbone of the animal resources in Egypt. However, they suffer from low fertility and production, that has been attributed to the development of relatively smaller numbers of primordial follicles and to high levels of follicular atresia compared to bovine [1], due to the lack of selection of breeding stock [2]. This collectively leads to less reproductive efficiency and limit the productivity of this species [3]. Assisted reproductive technologies (ART) have been introduced to overcome the reproductive inefficiency [4]. Embryo transfer (ET) is an accepted technique for both animal breeding improvement and disease control. It is widely used in beef and dairy cattle. In buffalo only a few successful embryo transfers have been reported [5]. In Egypt, the first report on ET in water buffalo was done by Kandil *et al*. [6]. Pregnancy rate, following non-surgical transfer in buffalo was 18% [7] and recently it increased to 35 to 40 % [6-9] because embryo transfer technique becomes well established in buffalo. There were attempts [10] to enhance genetic improvement in the buffalo, more particularly for the dairy buffaloes, through the use *In vitro* embryo production technology as potential methods for propagation of superior animals. In buffaloes, the production of transferable embryos (TE) from *In vitro* embryo production (IVP) is 1.8-to 2.8-fold less than that in cattle [11]. Embryos derived by IVP would allow more efficient utilization of superior genetic females without compromising donor fertility or the inter-calving interval. IVP technique was well established in Egypt, Kandil *et al*. [12] produced high percentage (40.2 %) of transferable buffalo embryos. The developmental potential of *In vitro* produced embryos can be affected by various factors including the maturation system, culture system, oocyte quality and embryonic growth factors [13, 14]. Culture medium plays an important role in the maturation process. TCM-199 and CR1 media can be used successfully for the induction of *In vitro* maturation of oocytes [15]. Also, the culture employed in IVM not only affect the proportion of bovine oocytes that reach metaphase II (MII) and become cable of undergoing *In vitro* fertilization, but can also influence subsequent embryonic development [16].

The cryopreservation of oocytes and embryos has become a powerful tool in the assisted reproduction of several mammalian species and has been widely used commercially for bovine embryos [17]. Vitrification is a promising procedure that is relatively rapid and inexpensive and it has been shown to be beneficial for embryos that have low cryosurvival rates such as those produced *In vitro* [18]. The *In vitro* and *in vivo* survival rates of vitrified embryos have been reported to be reasonable in cattle [19]and buffaloes [20]. Cryoprotectants are organic solutes that help to protect cellular organelles during cryopreservation although they may damage the cytoskeletal system as they can be toxic and cause disruptive osmotic damage to the cell. Mixtures of cryoprotectants may have some advantages over solutions containing only one solute [21]. Different cryoprotectants like dimethylesulfoxide (DMSO), ethylene glycol (EG),1,2-peopaediol PROH, Glycerol, have been used in different combinations for vitrification of mammalian oocytes and embryos [22]. Ethylene glycol (EG) has been effectively employed as a cryoprotectant for cattle embryo preservation [23] but information on cryopreservation of IVP buffalo embryos by vitrification is scarce and intrinsic biological problems such as high chilling sensitivity and high embryo lipid content have impeded progress in this species [24].

The present study was undertaken to determine 1) Effect of culture mediums and addition of Epidermal growth factor (EGF) on the *in-vitro* embryo development and assessment of viability of blastocyst by cell counting in buffalo. 2) Effect of different vitrification medium compositions on the ability of compact morulae and blastocysts produced *In vitro* to expand and hatch following vitrification.

MATERIALS AND METHODS

All chemical used in this work were purchased from Sigma (STL, MO, USA) unless otherwise mentioned.

Oocytes Collection and *In vitro* Maturation: Buffalo ovaries were collected from El-Monib slaughterhouse at Cairo, transported to the laboratory in a thermos containing normal saline solution (NSS, 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 + 50 µg/ml gentamicin. After aspiration, follicular content was transferred to 15 ml Falcon tube and allowed to settle for 10 to 15 min in water bath at 37°C.

COCs were evaluated under stereo microscope (x 28) and washed 3 times in oocyte aspiration medium then divided into three groups of 50 excellent and good oocytes. COCs were transferred to *In vitro* maturation medium (TCM-199, TCM-199+ EGF (20 ng/ml), CR1aa and CR1aa + EGF (20 ng/ ml)) 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 a CO_2 incubator at 38.5°C in 5% CO_2 in humidified atmosphere.

***In vitro* Embryo Production:** Matured oocytes with full cumulus expansion and presence of 1st polar body) were washed in fertilization medium (Fert-TALP supplement with 6 mg/l BSA). Frozen-semen from the same bull and the same batch was thawed in water bath at 37°C for 30 seconds. Motile spermatozoa were 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 with10 µM/ml hypotaurin, 1 µM/ml epinephrine, 20 µM pencillamine (PHE) + 1µg/ml heparin 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 18-20 h at 38.5°C under 5% CO_2 in humidified air.

The presumptive zygotes were washed at least 3 times then cultured in culture medium (IVC, modified synthetic oviduct fluid, mSOFaa medium) supplemented with 5 mg/ml BSA, 5 µg/ml insulin and 50 µg /ml gentamycin and a monolayer cells of oviduct epithelial cells (OEC, previously prepared 3 days before using) and incubated at 38.5°C under 5% CO_2 in humidified air. Cleavage rate and embryo development to the morula and blastocyst stages were checked on Days 2, 5 and 7. Culture medium was changed every 48 h. Part of the blastocysts were fixed for cell count and other for the rest of morula and blastocyst for cryopreservation.

Cell Numbers: Blastocysts from each group were fixed in methanol: acetic acid: water (3:2:1) for one minute. They were mounting on glass slid and then stained with 5% Giemsa stain for 10 minutes. Total cell count of the embryos was determined under a microscope at × 400.

Vitrification: The morula and blastocysts were vitrified. All vitrification solutions were prepared in 25mM Hepes-buffered TCM199 supplemented with 10% fetal calf serum and 50 µg/ml gentamicin (base medium). Embryos were held at 25 °C in 500 ml TCM199 until vitrification, which was performed in two steps as follow. Briefly, embryos were equilibrated at 25 °C in 10% Ethylene glycol (EG) + 10% Dimethylesulfoxide (DMSO) or 10% glycerol (G) + 10% EG in base medium for 4 min and subsequently, transferred to a 7µl drop (1-3 embryos/drop) of 25% EG + 25%DMSO+ 0.3 M sucrose in basal medium or 25% G+25%EG +0.3M sucrose in base medium for 45 second. During the second exposure step, the embryos were loaded into 0.25 ml straws in between 180 ul of dilution medium (0.5 M sucrose in TCM 199). The straw was then heat sealed and immediately after a lapse of 45 s, the straws were plunged vertically, sealed end first, into liquid nitrogen to cover the embryos. After one week of storage, the straws were warmed in air (25 °C) for 10 second and then in water horizontally at 20 °C until the cryopreservation medium melted. The straw was held at the sealed end and shaken to mix the columns, 30 s later the embryos were expelled into a 30-mm Petri dish and maintained at 25 °C for 5 min, then the embryos were washed three in 200 µl drops of embryo culture medium. Blastocysts were cultured for further development on 7day old oviductal epithelial monolayer in a CO_2 incubator for 48 h. The numbers of morula and blastocysts that could develop to expanded blastocyst or hatched blastocyst stage were recorded

Statistical Analysis: The obtained data were subjected to analysis using STAT program.

RESULTS

***In vitro* Embryo Production in Buffalo:** Total number of buffalo oocytes recovered from 345 buffalo ovaries was 1034 oocytes with 749 (72.4 %) excellent and good quality oocytes. *In vitro* maturation, cleavage, transferable embryos rate of buffalo oocytes showed highly significant ($P < 0.01$) increase in using CR1aa medium when compared with TCM-199 medium. Addition of EGF 20 ng/ml either to TCM-199 or CR1aa improved the developmental competence. The *In vitro* buffalo embryo development matured in the presence of EGF showed significantly ($P< 0.01$) higher rate of transferable embryo (40.9-42.7 %) than that without EGF (20-33.5%). (Table, 1 and Fig, 1).

Table 1: Developmental competence of *In vitro* produced buffalo embryos using different *In vitro* maturation medium:

			Transferable (%)	
Treatment	Maturation rate (%)	Cleavage rate (%)	Morula rate	Blastocyst rate
TCM199	86/173 (49.7)^{c}	35/86 (40.7)^{c}	5/35 (14.3)^{c}	2/35 (5.7)^{c}
TCM19+ EGF	150/221 (67.9)^{b}	105/150 (70)^{a}	23/105 (21.9)^{ab}	20/105 (19)^{a}
CR1aa	153/245 (62.4)^{b}	80/153 (52.3)^{b}	15/80 (18.8)^{b}	12/80 (15)^{b}
CR1aa + EGF	171/210 (81.4)^{a}	122/171 (71.3)^{a}	28/122 (23)^{a}	24/122 (19.7)^{a}

Means of different letters superscript (a, b and c) in the same column are significantly different ($p<0.01$).

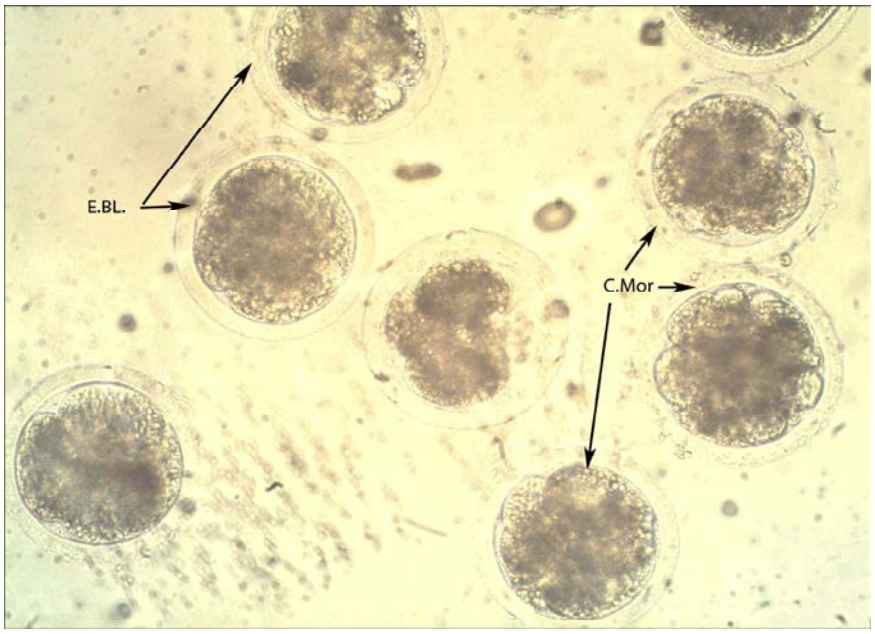

Fig. 1: Different developmental stages of *In vitro* produced buffalo embryos cultured in buffalo oviduct epithelial cells C.Mor = compact morula E.Bl =Early blastocyst

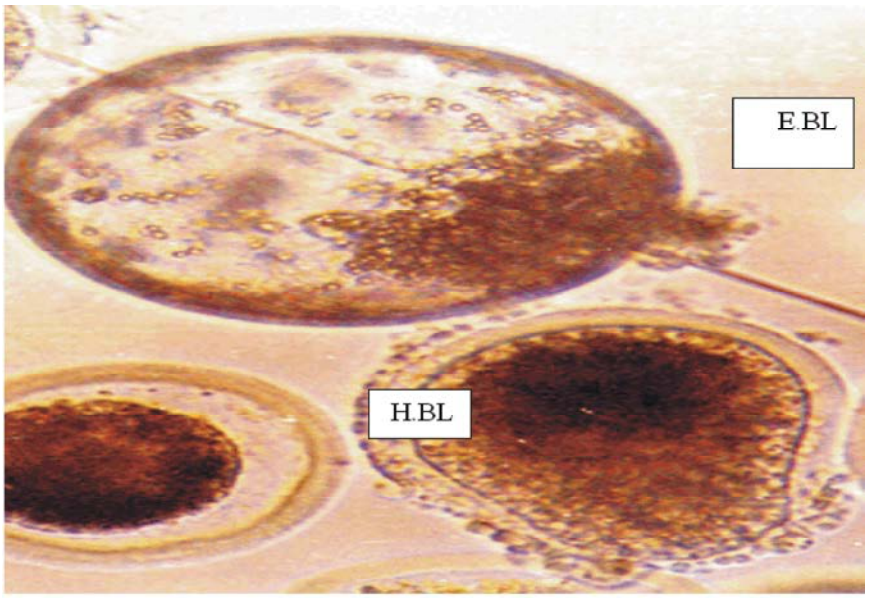

Fig. 2: Expanded and hatched blastocyst stages of Frozen-thawed *In vitro* produced buffalo embryos

Table 2: Effect of different culture media on the viability of buffalo blastocyst

Treatment	Cell number
	M ± SE
TCM199+FSH	60.75±1.46 [d]
TCM199+FSH+EGF	98.02±0.78 [b]
CR1aa+FSH	85.82±0.74 [c]
CR1aa+FSH+EGF	114.10±0.95 [a]

Means of different letters superscript (a, b, c and d) in the same column are significantly different ($p<0.01$).

M = Mean. SE = Standard Error.

Table 3: Number and survival rate of *In vitro* buffalo embryos after vitrification using different cryoprotectants (G + EG vs EG + DEMSO)

Vitrification medium	Thawing embryo (n)	Survival rate after thawing % (n)
25% EG+25% DEMSO	32	50 (16)*
25% G+25% EG	29	21 (6)

Buffalo Blastocyst Total Cell Count: Table 2, revealed that the buffalo blastocyst cell count were significantly ($P< 0.01$) increased in blastocyst *In vitro* produced from oocytes matured in TCM-199 + EGF or CR1aa + EGF when compared with the same medium without addition of EGF.

Cryosurvival rate (further development for 48h after thawing) of *In vitro* produced embryo after vitrification-thawing (Table 3 and Fig. 2) were significantly higher ($P< 0.01$) in embryos that vitrified in 25% EG + 25% DEMSO + 0.3 M sucrose 50% (16/ 32) when compared embryos vitrified in 25% G+25% EG+ 0.3M sucrose 21% (6/29).

DISCUSSION

Laboratory production of embryos involves three major steps: *In vitro* oocyte maturation (IVM), *In vitro* fertilization (IVF) and *In vitro* embryo culture (IVC). Five levels of oocyte developmental competence were characterized by Sirard *et al*. [25] including the ability to resume meiosis, to cleave upon fertilization, to develop into blastocysts, to induce pregnancy and to generate healthy offspring.

The present study examined the effect of *In vitro* maturation medium (TCM199 and CR1aa) with and without EGF (20 ng/ml) in developmental capacity of *In vitro* produced buffalo embryos and the quality and viability of the blastocyst by detection of their total cell count. In this work, *In vitro* maturation, cleavage rate and transferable embryos were significantly higher ($P<0.01$) in oocytes matured either in TCM-199 or CR-1aa with addition of EGF than that matured in the same medium without EGF. The cleavage rate (70 and 71.3 %) was slightly higher than our previous work (68.1 %) [13] and it is in agreement with the result of Manjunatha *et al*. [26]. who found that the cleavage rate in the good buffalo oocytes selected by brilliant crasyle blue was 71%, while it is higher than that reported by Gupta *et al*. [27] and Gasparrini *et al*. [28], those authors observed that the cleavage rate of buffalo oocytes matured *In vitro* in TCM-199 + cysteamine were 58 and 66 %, respectively. These difference may be due to the difference maturation conditions. In the present work, addition of EGF (20 ng/ml) to the *In vitro* maturation medium increased the maturation rate and the subsequently the cleavage rate and the embryo development. Similar results were previously reported by Nandi *et al.* [29]. EGF increased proteoglycan synthesis [30] and production of tissue plasminogen activator and urokinase plasminogen activator by cumulus cells and oocytes stimulated by EGF [31] and it may also be due to stimulation of DNA synthesis in cumulus cells by EGF and FSH [32].

In our results the higher transferable embryo rate of buffalo ranged from 40.9 to 42.7 %, was higher than that recorded by Gasparrini *et al*. [28] and Totey *et al*. [33], those authors observed that the transferable rate of *In vitro* produced buffalo embryos were 23.8 % and 28%, respectively. In our experiments, morula rate ranged between 21.9 to 23 and blastocyst rate were 19 to 19.7 and the total cell count were significantly high (98-114) in TCM-199+EGF and CR1aa+EGF maturation medium respectively, these results was similar in morula rate (22.6%) that recorded by Gaspmini *et al*. [34], while, it was higher than that observed by Manjunatha *et al*. [26], who recorded that the blastocyst rate of *In vitro* produced buffalo embryo was 18%. This difference may be due to the difference in culture condition, as oviduct epithelial cell co-culture enhanced the blastocyst yields [3]. Although serum is known to contain various hormones, insulin, growth factors, proteins, amino acids, trace and other essential elements. The blastocysts production from cleaved embryos cultured with tissue culture medium and co-culture was higher when oocytes matured in the presence of EGF [29]. EGF positively influences oocyte maturation in a number of species, including cattle [35]. Its addition to serum free TCM199 is sufficient to support high frequencies of bovine oocyte maturation and subsequent development to the blastocyst stage. Moreover, the addition of co-culture in tissue culture increase the cell count of blastocyst [36].

Vitrification requires high concentrations of cryoprotectants to achieve glass transition *In vitro* [37]. A successful vitrification procedure requires optimization of cryoprotectant concentration. In the present work, 25% EG +25% DMSO cryoprotectant gave significant higher cryosurvival rate, post thawing buffalo embryo when compared with 25% G +25% EG (50 vs 21 % respectively). EG has minimal toxicity and is more permeable to most embryos than glycerol, DMSO and propylene glycol [38-40]. EG has however been successfully used as the lone cryoprotectant in vitrification solutions used for various other species (mice, rabbits, horse, cattle, marsupials [41] and buffaloes [20]. A cryoprotectant such as glycerol with less permeability has a primary protective action on cytoplasmic membranes, whereas a more permeable agent like EG protects the intracellular structures [42]. Similarly in the present study, vitrification of blastocysts in 25% G + 25% EG with an exposure time of 4 min showed an acceptable hatching rate on post-thaw *In vitro* culture. The ability of embryos to hatch after 48 h post-thaw culture, improved when two (EG, DMSO) or three (EG, or saccharides) is among the factors influencing the cryosurvival of embryos. The incorporation of DMSO into an EG containing medium has at least two advantages: firstly, vitrification is facilitated because of the greater glass-forming characteristics of DMSO [43] and, secondly, the permeability of each cryoprotectant is enhanced in the presence of the other [44]. In the present study, vitrification of blastocysts in 25% EG + 25% DMSO with an exposure time of 4 min, showed better hatching rates on post-thaw *In vitro* culture than other vitrification solutions.

CONCLUSION

The addition of epidermal growth factor to *In vitro* maturation mediums improved embryo viability with increasing transferable embryos rate and blastocyst cell number. The using of EG + DMSO cryoprotectant in vitrification of buffalo oocytes showed high cryosurvival post thawing buffalo embryo development. Therefore, *In vitro* produced buffalo embryo and vitrification techniques were suitable for future embryo transfer.

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