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Concentration Dependent of Lactoferrin can Improve *In vitro* Maturation, Fertilization and Embryonic Development of Buffalo Oocytes.

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ABSTRACT

Lactoferrin (LF, 80kDa) is secreted by epithelial cells into milk which has the ability to hold iron that gives LF special bioactivities as antipathogenic, immune modulation, anti-oxidant, growth factor and cell proliferation stimulating glycoprotein. The current study aims to investigate the effect of different concentrations of bovine lactoferrin (bLF) on *in vitro* maturation, *in vitro* fertilization and embryo development of Egyptian buffalo oocytes. Each of *in vitro* maturation, *in vitro* fertilization (IVF) and embryonic development medium was supplemented with bLF at concentrations of zero (control), 0.01, 0.03, 0.05 and 0.1 mg/ml for each trail. Lactoferrin insignificantly enhanced maturation rates compared with control group. The highest rates ($p<0.001$) of IVF (52.73 and 43.33%) were observed for both 0.03 and 0.05 mg/ml supplementations to IVF medium. All bLF levels increased ($p<0.0001$) cleavage rate and proportion of embryos at both morula and blastocyst ($p<0.05$) stages than the control medium with advantage to 0.03 mg/ml concentration along examined developmental stages. The current study revealed that low levels of bovine bLF has a supportive effect (especially 0.03 mg/ml) on each of IVF media and embryo development of the Egyptian buffalo oocytes.

Keywords: *Egyptian buffalo; Lactoferrin; oocyte; IVM; IVF; embryo.*

INTRODUCTION

Lactoferrin (LF, 80 kDa and ~700AA glycoprotein), formerly known as lactotransferrin, is a member of a transferrin family originally isolated from milk (Levay and Viljoen, 1995). It is secreted by epithelial cells into most mucosal secretions and body fluids. Also, LF is a major component of the secondary granules of neutrophils released on activation. Three different isoforms (α, β and γ) were isolated of LF. Regulation of LF synthesis depends on the type of cells producing this protein (Adlerova *et al.,* 2008). LF has the ability to retain iron until a pH of about 3.0, a positively-charged surface at physiological pH and other surface features that give LF additional functional peculiarities (Rosa *et al.*, 2017).

This protein has multiple biological functions, including antibacterial (García-Montoya *et al.*, 2012), antiviral (Wakabayashi *et al.*, 2014), antiparasitic (Giansanti *et al*., 2016), immune modulation activities (Siqueiros-Cendón *et al.,* 2014) and even antineoplastic (Zhang *et al.,* 2015) properties. Iron chelation by LF is generally believed to be responsible for these functions, especially for the antibacterial effect, however, all of these functions can be dependent or independent of Lf-iron-binding ability (Rosa *et al.*, 2017).

Lactoferrin acts as an anti-oxidant by reducing oxidative stress-induced apoptosis via diminishing the intracellular levels of reactive oxygen species (ROS) induced by glucose oxidation and preventing lipid peroxidation occurred due to the Haber-Weiss reaction (Actor *et al.,* 2009).

Lactoferrin was known as an *in vitro* growth factor of many cell types; such as preosteoblastic cells (Amini and Nair, 2011), corneal epithelial (Ashby *et al.*, 2011), intestinal (Nguyen *et al.,* 2016) and mammary epithelial cell lines (Pecorini *et al.*, 2009), as well as its ability to increase both cell proliferation and differentiation rates of neuroblastoma (Sriramoju *et al.,* 2015) and endometrial stroma cells (Yanaihara *et al.,* 2000).

Hence; Givens *et al.,* (2005) studied the effects of lactoferrin (2.5-10mg/ml) as an anti-viral agent against bovine herpesvirus-I and found that bLF allows normal development for *in vitro*-produced bovine embryos. Later, Marley *et al.,* (2009) revealed that bLF suppressed the development of *in vitro*-produced bovine embryos with a recommendation for additional research to identify the association of lactoferrin with *in vitro*-produced embryos and embryonic development. Therefore, the current study aims to investigate the effect of different lower concentrations of bLF on *in vitro* maturation, *in vitro* fertilization and embryo development of oocytes recovered from Egyptian buffaloes.

MATERIALS AND METHODS

Current study was carried out at Animal Reproduction Research Institute, Egypt. Meanwhile, frozen semen was obtained from The International Livestock Management Training Center, Sakha, belonging to Animal Production Research Institute. Agriculture Research Center, Ministry of Agriculture. Bovine milk lactoferrin (bLf, L9507) and 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.

Cumulus-oocyte complexes retrieval:

Ovaries were collected from slaughtered buffaloes and placed in NaCl solution (9 mg/ml) containing antibiotics (penicillin, 100UI/ml and streptomycin sulphate, 100 µg/ml) and maintained at 25-30˚C until oocyte recovery. The collected ovaries were washed twice in freshly prepared saline and rinsed in 70% ethyl alcohol for a few minutes to eliminate surface organisms. Compact cumulus-oocytes complexes (COC's) were collected from ovarian follicles (2.0-8.0 mm diameter) by aspiration technique in 5ml Dulbecco's phosphate buffer solution (DPBS)

supplemented with 3% bovine serum albumin (BSA) and 50 µg/ml gentamicin using 18-gauage needles. The oocytes enclosed in a compact, more than three layers, of cumulus cells, evenly granulated cytoplasm were selected under stereomicroscope for the current study.

Three separate experiments were held at current study to investigate the impact of different concentration of bLF on each of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC) of buffalo oocytes as follows:

Experiment 1: investigating the supplementation with different levels of bLF to IVM medium of buffalo oocytes. The maturation medium was comprised of TCM-199 containing 20 mmol pyruvate and 50 µg/ml gentamicin supplemented with different concentrations of bLf at zero (control), 0.01, 0.03, 0.05 and 0.1 mg/ml medium. Prepared IVM medium was placed into four-well dishes and covered by sterile mineral oil. Oocytes were washed three times in washing medium and once in maturation medium; then placed into previously incubated IVM media for 24 h at 39°C, 5% CO2 and 95% relative humidity. Maturation of the oocytes was monitored through the dispersion of cumulus cells surrounding the oocytes and maturation rate was evaluated according to Dhali *et al.,* (2000).

Experiment 2: carried out the impact of bLF on IVF route of previously matured buffalo oocytes, in control IVM medium, using frozen-thawed semen treated by swim-up procedure in Sp-TALP medium for one hour. The uppermost layer of the medium containing the most motile spermatozoa was collected and washed twice by centrifugation. The pellet obtained after centrifugation was resuspended in IVF-TALP supplemented with 10 µg/ml heparin for *in vitro* sperm capacitation. Final concentration of 20×106 sperm/ml were used to IVF. Insemination of previously matured buffalo oocytes was performed in 50 µl drops of IVF-TALP supplemented with different concentrations of bLf (zero, 0.01, 0.03, 0.05 and 0.1 mg/ml) medium; under mineral oil at 39˚C and 5%CO2 and 95% relative humidity. About 20 h after insemination, number of putative zygotes was recorded.

Experiment 3: addition of bLF to *in vitro* culture (IVC) of buffalo embryos was undertaken using zygotes obtained from matured and fertilized oocytes in control IVM and IVF media. Zygotes (18 h of insemination) were stripped of loosely bound spermatozoa and cumulus cells using glass micropipette and washed three times in IVC medium (TCM-199, 20 mMol Na-pyruvate, 6 mg/ml BSA and 50µg/ml Gentamicin sulphate). Putative zygotes immediately transferred IVC medium containing zero, 0.01, 0.03, 0.05 or 0.1 mg/ml of bLF in 4 well Petri dishes; overlaid with sterile mineral oil, incubated at 39˚C and 5%CO2 humidified air. The proportional of cleaved oocytes to 2-4 cells embryos was calculated 48 h after insemination. The embryo culture medium was replaced every 48h for 7-days. After 6-7 days from the onset of IVF, the cleavage rate and the frequency of morula and blastocyst were recorded.

Statistical analysis:

The experiment was replicated three times for both maturation and development rates. Data were statistically analyzed using Statistical Package for Social Sciences (SPSS V.20) using one-way (ANOVA) after arcsine transformation of data. Duncan's Multiple Range Test was followed for test the significant differences among treatments (Duncan, 1955).

RESULTS AND DISCUSSION

***1- In vitro* maturation of buffalo oocytes.**

Results presented in figure 1 shows the effect of supplementation of maturation media with or without bLF. The treatment has insignificant effect on COC's maturation, however, high maturation rate was recorded for 0.03 mg/ml of bLF. In agreement with Ishikawa *et al.,* (2014) who declared that addition of LF (0.01 and 0.1µg/ml) improved maturation of mono-cultured porcine oocytes working as an antioxidant.

***2-In vitro* fertilization of buffalo oocytes.**

Addition of bLF to *in vitro* fertilization medium, enhanced fertilization rates ($p<0.001$). However, the highest rates of IVF (52.73 and 43.33%) were observed for both 0.03 and 0.05 mg/ml supplementations to IVF medium, respectively when compared to other groups; as presented in figure 2. Current results confirmed earlier findings of Zumoffen *et al.,* (2013) and Zumoffen *et al.,* (2015) who established that human LF (hLF) expressed from oviductal fluid; binds to zona pellucida (ZP) of human oocyte and spermatozoa which modulated their interaction in a dose-dependent manner. Increasing concentration of oviductal hLF (0.1–100 mg/ml) can inhibits *in vitro* sperm–ZP binding.

3-Developmental competence of buffalo embryos.

Stages of buffalo embryo's development treated with different levels without or with bLF are shown in table 1. The treatment increased ($p<0.0001$) cleavage rate to 2-4cells and proportion of embryos at both morula ($p<0.001$) and blastocyst ($p<0.05$) stages relative to the total number of fertilized oocytes than the control group. Moreover, the concentration of 0.03 mg/ml bLF has the best performance along examined developmental stages of buffalo embryos. Current results confirmed the importance of LF to murine embryos starting from 2-4 cell embryo until the blastocyst stage of development (Ward *et al.,* 1999).

Present findings coincide with those found by Yanaihara *et al.,* (2007) who found that in human, hLF in the follicular fluid (500.2±35.5 ng/mL) correlated with embryo's quality during IVF cycles, which may stimulate granulosa cells to regulate the release of cytokines such as IL-8 and TNF-α.

Generally, current results evidenced that bLF, at low concentrations, able to improve the proportion of fertilized buffalo oocytes in vitro that might be its ability to reduce the chance of polyspermic fertilization (Coy and Yanagimachi, 2015). In addition, supplementation bLF to IVC of buffalo embryos showed a promising results, supporting the fact that low doses of bLF up-regulate 11 proteins associated with glycolysis, energy metabolism and protein synthesis,

including the important enzymes such as; pyruvate kinase, pyruvate carboxylase and pyruvate dehydrogenase. These enzymes, support and facilitate energy production of cell survival and proliferation. Moreover, bLF up-regulate three aminoacyl-tRNA ligases (glycine, isoleucine and tryptophan-tRNA ligases) catalyzing the ligation between amino acids and their cognate tRNA during the initial step of protein synthesis. Additionally, bLF up-regulate DNA lyase which stimulate DNA repair and binding activity of numerous transcription factors, thereby playing important roles in protein synthesis (Nguyen *et al*., 2016).

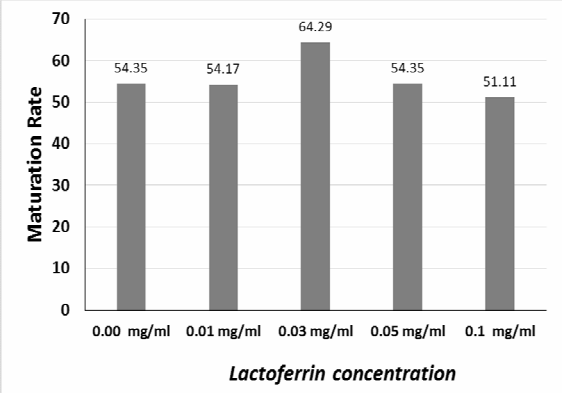

Fig. 1. Impact of supplementation with different concentrations of bLF to IVM medium of Egyptian buffalo oocytes.

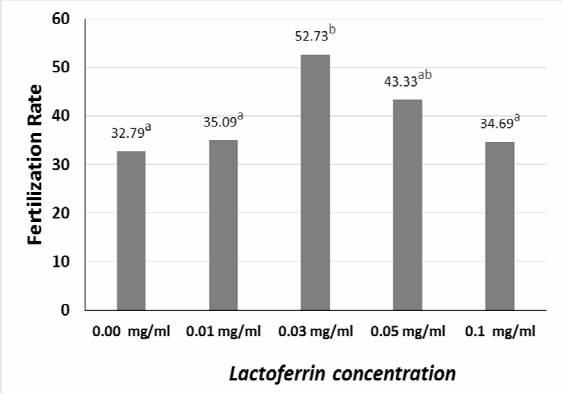

Fig. 2. Effect of bLF levels added to IVF medium of Egyptian buffalo oocytes.

a, b and c means with different superscripts are significantly different at p<0.05.

Table 1. Comparable development of *in vitro* fertilized buffalo's oocytes; as affected by different concentrations of lactoferrin.

Lactoferrin Concentration	N	2-4 Cells Stage		Morula Stage		Blastocyst stage	
		n	%*	n	%*	n	%*
0.00 mg/ml	56	16	28.57^{a}	4^{a}	7.14^{a}	2^{a}	3.57^{a}
0.01 mg/ml	49	15	30.61^{ab}	5^{a}	10.20^{ab}	3^{a}	6.12^{ab}
0.03 mg/ml	52	21	40.38^{c}	15^{b}	28.85^{c}	9^{b}	17.31^{b}
0.05 mg/ml	58	18	31.03^{b}	9^{a}	15.52^{ab}	7^{ab}	12.07^{b}
0.1 mg/ml	57	15	26.32^{a}	4^{a}	7.02^{a}	2^{a}	3.51^{a}

a, b and c means donated with the same column with different superscripts are significantly different at p<0.05

N: the total number of oocytes *As a percentage relative to total fertilized oocytes.

CONCLUSION

Our results revealed that bovine Lactoferrin (bLF) could be advantageous at low doses (especially 0.03mg/ml) to each of IVM, IVF medium and embryo development of Egyptian buffalo oocytes. However, more experimental studies are required to investigate its stimulatory effect/s at the molecular level. First, the modulating effect of bLF to buffalo's oocytes ZP and gametes interaction. Second, investigating the stimulatory growth and proliferative support on in vitro-produced embryos.

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تركيز اللاكتوفيرين يتوقف علية تحسين الأنضاج والأخصاب المعملي والتطور الجنيني لبويضات الجاموس

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تهدف الدراسة الحالية لمعرفة تأثير اضافة تركيزات مختلفة من الاكتوفيرين المستخلص من العائلة البقرية الى بيئات الأنضاج والاخصاب المعملى وكذلك التطور الجنيني لبويضات الجاموس المصري. تم القيام بكل من تجارب الأنضاج والأخصاب المعملي والتطور الجنيني على حدى باضافة تركيزات من اللاكتوفيرين صفر (مجموعة مقارنة) و 0.01 و 0.03 و 0.05 و 0.1 ملجم/مل من البيئات سابقة الذكر. ادت اضافة اللاكتوفيرين الى بيئة الأنضاج الى حسن غير معنوي في معدل انضاج البويضات. في حين انة كان اعلى معدل ($p > 0.05$) للأخصاب المعملي (52.7 و 43.33 %) للتركيزين 0.03 و 0.05 ملجم/مل على التوالي. عموما ادت اضافة اللاكتوفيرين في بيئة التطور الجنيني الى تحسن في معدل تكشف الأجنة والوصول لمرحلتي المريولا والبلاستوسيست ($p > 0.05$) في مقابل بيئة النمو المقارنة مع ملاحظة الافضلية للتركيز 0.03 ملجم/مل من الاكتوفيرين على طول فترة دراسة التطور الجنيني. اوضحت الدراسة الحالية ان التركيزات المنخفضة من اللاكتوفيرين (وبتركيز 0.03 ملجم/مل بصفة خاصة) لها تأثير مدعم لكلاً من عمليتي الأخصاب المعملي للبويضات والتطور الجنيني في الجاموس المصري.