EFFECT OF BOVINE SERUM ADDED TO MATURATION MEDIUM ON IN VITRO-MATURATION AND SOME GENES EXPRESSIONS OF BUFFALO OOCYTES

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ABSTRACT

The effect of supplementing TCM-199 culture medium with bovine sera on buffalo's oocytes maturation in vitro and expression of some related genes were studied. A total 1149 immature buffalo oocytes were randomly divided into three groups, G1(n=315) were cultured in medium with no serum supplement(control), G2(n=531) were cultured in medium supplemented with 10% Fetal calf serum (FCS) and G3(n= 303) were cultured in medium supplemented with 10% Estrous cow serum (ECS). After maturation, oocytes in each group were classified according to its shape and expansion into 2 subgroups, good (GO) and fair (FO) expanded oocytes. Expression of GAPDH, CX43, IGF II and β- Actin genes were studied in each subgroup. Results revealed that G3 and G2 showed high maturation rate (P<0.01 & 0.05 respectively) compared to G1(control) and ECS was superior (P<0.05) than FCS. The qualitative expression of genes under study showed no difference among the studied groups, except CX43 gene expression was missed in FO in G1 concluding that addition of bovine sera especially estrus cow serum enhance in vitro maturation of the buffalo oocytes and CX4 gene expression and further studies is needed to enhance the expression of CX43 gene which might help in increasing maturation rate.

Keywords: buffalo oocytes, IVM, gene expression, type of serum

INTRODUCTION

Cumulus cells support and regulate oocyte development and maturation ***(Vozzi et al., 2001; Ward et al., 2002 and Xiang et al., 2010)***. Oocytes with many layers and compact cumulus oocyte complexes showed better *in vitro* maturation rate and blastocyst formation ***(Vanderhyden et al., 1992)*** compared to denuded ones. Under *in vitro* fertilization circumstances not all cultured oocytes undergo maturation and fertilization (IVM-IVF) due to incomplete maturation of oocytes during folliculogenesis ***(Sirard, 2001)***. The composition of maturation medium of *in vitro* maturation plays a significant role in the achievement of full developmental competence by oocytes ***(Kim et al., 2005 and Song et al., 2010)***. Maturation media are supplemented habitually with different types of blood serum like, bovine fetal serum; serum of cow in estrus; or albumin fraction V of bovine serum ***(Blanco and Simonetti, 2002)***. Estrus cow serum improves rate of maturation and the blastocyst formation compared to the use of fetal bovine serum ***(Hannelore et al., 2008)***, Moreover, presence of serum during culture period resulted in a significant increase in the level of gene expression of oxidative stress (MnSOD and SOX), apoptosis (Bax), differentiation (LIF) and implantation (RL-ß) ***(Rizos et al., 2003)***.

Oocyte growth is accompanied by cytoplasmic changes including mRNA transcription, protein translation and post translational modification ***(Sirard et al., 1989 and Kastrop et al.,1991)***. Gap junctions are thought to play a crucial role in regulating cell growth and development in various tissues, including ovaries ***(Herve et al., 2008 and Laws et al., 2008)***. One particular gap junction protein, which identified as connexin 43 (Cx43) is found in granulosa cells. This protein

contributes in embryonic and fetal development ***(Gershon et al., 2008)***. Expression of all mRNA transcript genes (Cx43, GDF 9, FGF-4 and Fibronectin) is positively correlated with cumulus expansion and polar body extrusion. Mitogenic lectin supplemented maturation media improves oocyte quality for *in vitro* embryo production ***(Pandey et al., 2009).***

IGF-II is found to mediate growth in early mouse embryos and it forms a pathway in which imprinted genes affect development during pre-implantation stages ***(Rappolee et al., 1992)***. The transcript level of the insulin-like growth factor genes is significantly altered by *in vitro* culture condition ***(Pandey et al., 2010)***.

The aim of the present study is to clarify the effect of supplementing TCM-199 culture medium with bovine serum on buffalo's oocytes maturation and their relationship to some genes expressions that may affect the maturation process.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management and Training Center, Animal Production Research Institute, Agricµltural Research Center, Ministry of Agricµlture, Egypt. All chemicals used in this study were purchased from Sigma (Sant Luis, MO, USA), unless otherwise indicated.

Oocyte recovery:

Collected ovaries from slaughtered buffalo cows were placed in NaCl solution (0.85 mg/ml) containing antibiotics (penicillin, 100 iu/ml and streptomycin sulphate,100 µg/ml) and maintained at 25-30°C until oocyte recovery. The collected ovaries were washed twice in sterile

freshly prepared saline. Oocytes were recovered from follicles with 2-8 mm in diameter by aspiration technique, using 10 ml disposal plastic syringe attached to 18-gauge needle. The contents of syringe were placed slowly into 60 mm sterile Petri dish and examined under stereomicroscope for oocytes harvesting and evaluation. Oocytes having compact cumulus complex (COC) surrouding evenly granulated cytoplasm (more than three layers) were cultured after washing three times in sterile Dulbecco's phosphate buffer saline (DPBS) medium and finally with the maturation medium.

Oocyte maturation:

TCM-199 medium supplemented with 10 iu/ml PMSG (Gonaser, Laboratory Hipra, S.A.17170 Amer, spain), 20 iu/ml LH as Chrionic gonadotropin (Pregnyl, The Nile company for pharmaceuticals and chemical industries, Cairo-ARE.R.C.C.115668), 1.0 µg/ml estradiol β_{17} and 50 µg/ml Gentamycin sulfate with pH value of 7.3-7.4 and osmolarity of 280-300 mOsmol/kg was used for maturation of buffalo oocytes. COCs were divided into three groups. G1(n=315) oocytes were cultured in TCM-199 without serum supplementation as(control). G2(n= 531) oocytes were cultured in TCM-199 with 10 %fetal calf serum; FCS, while G3(n= 303) were cultured in TCM-199 with 10% estrus cow serum; ECS supplement. The three media were filtrated by 0.22-µm millipore filter, A 500 µl from each prepared maturation medium was placed into four well dishes and covered by 500 µl sterile mineral oil and equilibriated in CO_2 incubator (5% CO_2 at 38.5°C and high humidity) for 60 minutes. Thereafter, oocytes were transferred from washing medium to maturation medium. Then oocytes of the three groups were incubated for 24 hrs (maturation period) in 5% $CO_2$5 at 38.5°C and high humidity.

After the maturation period, oocytes were classified under stereo microscope into good (GO) and fair (FO) according to cumulus expansion and darkness of their ooplasm. GO had good expanded cumulus cells and even ooplasm while FO had weak cumulus expansion, naked and/or had uneven dark ooplasm ,Shamia (2004). All oocytes were washed using PBS containing 1 mg /ml hyaluranidase to remove the cumulus cells, then, washed twice in PBS supplemented with 3% BSA. Oocytes then either preserved in PBS at – 70 $\overset{o}{C}$ for RNA isolation (72 oocytes from all subgroups as 21 from G1, 33 from G2 and 18 from G3) or loaded on clean slide for fixation in a fixative solution (3 ethanol: 1 glacial acetic acid) overnight and stained with 1 % orcein in 45% acetic acid. Stained oocytes

Gene expression evaluation:

RNA isolation was executed from each subgroup using easy-RED total RNA extraction kit (according to the procedure described by the manufacturer Cat.No.17063, iTRON Biotechnology Company, Korea) and immediately used for reverse transcription (RT) using the PCR Sigma (Thermo Fisher Scientific Inc.): Revert Aid First Strand cDNA Synthesis Kit (Cat. No. K1691, USA).

Semi-quantitative RT .PCR:

The 1st strand cDNA (2 ug) from different subgroups were used as template for PCR with a pair of specific primers. The sequences of specific primers and product sizes are listed in Table (1). PCR amplification was performed in a total reaction volume of 25 ml containing 1 µl of cDNA template, 10 µ mol/ l forward and reverses primers, 10 m mol/l of dNTP mix, 10x PCR buffer and 2 units of Taq

Gold polymerase. PCR conditions were: 1 cycle (94 °C for 4 min), 35 cycles (94 °C for 45s, 56 °C for 45s, 72 °C for 90s) for GAPDH and 1 cycle (95 C for 4 min), 35 cycles (95 °C for 30s, 55 °C for 30s, 72 °C for 45s) for Cx43, 1 cycle (95 °C for 4 min), 35 cycles (95 °C for 30s, 55 °C for 30s, 72 °C for 45s) for β-actin, 1 cycle (95 °C for 4 min), 35 cycles (95 °C for 30s, 55 °C for 30s, 72 °C for 45s) for IGF2, and 72 °C for 7 min (Metabion international AG Lena-Christ-Stresse 44/I A-8215 Martinsried/ Deuschland). All PCR products (8 µl of 25 µl total reaction volume) were electrophoresed in agarose gels (1.5%, w/v) containing ethidium bromide and visualized over UV light.

Statistical Analysis:

It was carried out using SPSS version 17. Results were expressed as mean ± SEM. Analysis of variance (one way ANOVA) followed by Duncan' test were used to determine whether there were significant differences among the groups. Differences were considered significant when P values were less than 0.05.

Table (1): Primers used for Real-Time PCR experiments to amplify studied genes

Genes and sequence reference (GenBank accession no.)	Primer sequences	Size of PCR product (bp)	Annealing temperature
GAPDH (NM_001034034)	F- TGA CCC CTT CAT RGA CCT TC R- TAC TCA GCA CCA GCA TCA CC	172	56
Cx43 (J 05535)	F -GGG AAA GAG CGA TCC TTA CCA CAC TAC CAC R -CCA CCT CCA ATG AAA CAA AAT GAA CAC CTA	164	55
β-actin (NM_001101)	F- CGT GAG AAG ATG ACC CAG ATC A R -GGG ACA GCA CAG CCT GGA T	162	55
IGF2 (X53553)	F -TCT ACT GCC GAC CAT CCA R -TTC GGA AGC AAC ACT CTT CCA	204	55

RESULTS

The highest response (76.84%) in term of the percentage the good oocytes was recorded in G3 suggesting the beneficial effect of ECS on maturation of buffalo oocytes. However the percentages of the good oocytes were 53.61% and 56.12% in G2 and G1 respectively.

Addition of serum to TCM-199 maturation medium significantly improved the nuclear maturation rate (MII stage) of G2 at $P<0.05$ and G3 at $P<0.01$ compared with G1(control). Moreover, the type of serum affected nuclear maturation rate as indicated by significant ($P<0.05$) increase in G3 compared with G2.Also, serum addition significantly decreased the percentages of intermediate stages in G3 at $P<0.01$ and in G2 at $P<0.05$ compared with G1 as well as the degenerated oocytes was high($P<0.05$) in G1 compared with either G3 and G2(Table,2).

Expression of GAPDH, CX43, IGF II and β- Actin genes were not differed among the three studied groups except CX43 is not expressed in FO subgroup in G1(Figure 1).

Table (2): Effect of serum addition to maturation medium on both oocyte expansion quality and nuclear maturation of IVM of buffalo oocytes

Maturation indices	G1 (X±SE) %			G2 (X±SE) %			G3 (X±SE) %		
	GO n= 165	FO n=129	Total N= 294	GO n=267	FO n=231	Total N= 498	GO n=219	FO n=66	Total N=285
DO	6.6±5.87	22.4±6.78*	13.4±4.69^{a}	8.5±4.80	26.4±5.25*	16.6±5.01^{a}	0.0±0.00	22.5±8.31*	7.5±4.79^{b}
GV	11.1±3.72	10.3±4.30	4.4±2.56	5.9±3.04	6.0±3.33	6.0±2.41	8.8±3.72	8.3±5.27	8.6±2.97
GVBD	7.2±3.96	14.0±4.58	10.1±4.01	7.3±3.24	2.5±3.54	5.1±2.07	3.7±3.96	5.0±5.60	4.1±2.05
Inter.S	29.1±5.13	38.2±5.13*	33.0±4.77^{a}	18.1±4.18	29.9±4.58*	23.5±3.43^{b}	8.6±5.13	22.5±7.25*	13.2±3.76^{c}
MII	57.2±7.07**	15.1±8.16	39.1±9.36^{c}	60.2±5.77**	35.2±6.32	48.8±6.35^{b}	78.9±7.07**	41.7±9.99	66.5±8.35^{a}

DO= Degenerated ooctes GO= good expanded oocyte FO= fair expanded oocyte GV= Germinal vesicle stageGVBD= Germinal vesicle breakdown stage Inter.S= Intermediate stages MII= Metaphase-II stage

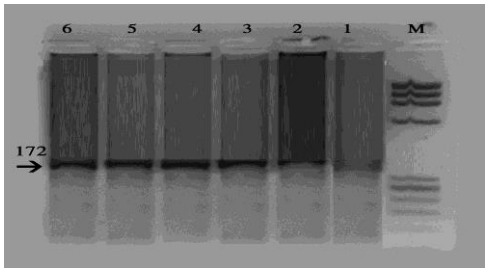
Figures carry superscript a & b or b & c within the same row are significantly different at $P<0.05$

Figures carry superscript a & c within the same row are significantly different at $P<0.01$

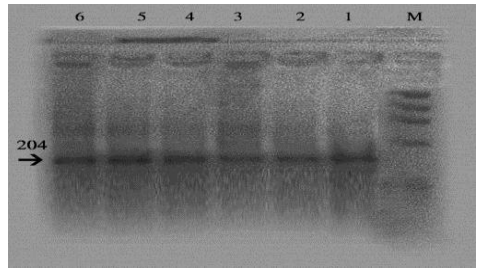
Figures carry superscript (**) within the same row and the same group are significantly different at $P<0.01$

Figures carry superscript (*) within the same row and the same group are significantly different at $P<0.05$

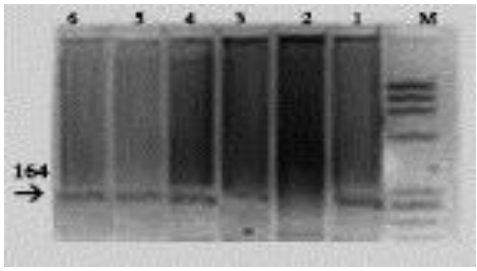
Fig. (1): Genes expressions associated with different treatments:



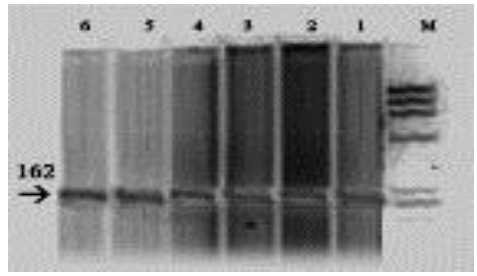
GAPDH



IGF II



CX 43



B-actin

M= Marker 1- Good expanded oocytes in serum free group, G1 2- Fair expanded oocytes in serum free group, G1 3- Good expanded oocytes in FCS subgroup,G2 4- Fair expanded oocytes in FCS subgroup, G2 5- Good expanded oocytes in ECS subgroup,G3 6- Fair expanded oocytes in ECS subgroup, G3

DISCUSSION

Supplementing maturation medium TCM-199 with blood sera in both G3 and G2 improved the maturation rate of buffalo oocytes compared to G1. This result agrees with the finding of ***Sagirkaya et al. 2004*** and ***Hannelore et al., 2008*** who found that Estrus cow serum improves rate of maturation and the blastocyst formation compared to the use of fetal bovine serum. Addition of serum to culture medium may affect not only the oocyte maturation and developmental potential of embryos but also, the gene expression pattern and apoptotic index ***(Rizos***

et al.,2003). Manipulation of the culture medium composition may modulate global gene expression and improve the overall efficiency of this technique ***(Watson et al., 2000; Lonergan et al., 2003 and María et al., 2013).*** Oocytes that had good cumulus expansion was accompanied with high nuclear maturation rate, M II ($P<0.01$) than fair ones. This could be explained in the light of the fact that the cumulus cells play an important role in regulating oocyte maturation and meiotic progression ***(Vozzi et al., 2001; Ward et al., 2002; Kakkassery et al,.2010 and Xiang et al., 2010)***. So, Co-culturing COCs and denuded oocytes (Dos) may be an effective culture system for both intact COCs and immature Dos ***(Dey et al,.2012)***. Qualitative expression of studied genes (GAPDH, CX43, IGF II and β- Actin) was not differed except CX43 gene, which was not expressed in FO when serum was absent during IVM. ***Herve et al., 2008 and Laws et al., 2008*** concluded that gap junctions are thought to play a crucial role in regulating cell growth and development in various tissues, including ovaries. The improved maturation rate as indicated by significant increase in MII and decrease in both degenerated and intermediate stages in oocytes cultured on serum supplemented TCM-199 may be attributed to the fact that serum induced expression of CX43 gene. ***Pandey et al., 2009*** found that expressions of genes (Cx43, GDF 9, FGF-4 and Fibronectin) is positively correlated with cumulus expansion and polar body extrusion and presence of serum in the culture medium alters the expression of genes ***(Wrenzycki et al., 1999)***, particularly genes involved in lipid metabolism ***(Plourdea et al., 2012)***. Connexin 43 (Cx43) absence disrupts progression of follicles beyond primary stages in transgenic mouse ovaries ***(Bolamba et al., 2002)*** and it expressed in immature and *in vitro* matured oocytes but in

embryos it was detected up to morula stage only ***(Mishra et al., 2010)***, the relative abundance of Cx43 could be used as a marker of developmental potential for embryos derived from oocytes. Level of Cx43 transcript was reported to be greater in embryos derived from oocytes with greater developmental competence compared with those derived from oocytes with less developmental competence ***(Nemcova et al., 2006)***.

Variation in GAPDH expression at the maturation stage of *in vitro* produced oocytes may be attributed to the type of maturation media which affecting the stability and turn over rate of mRNA ***(Feuerstein et al.,2007)***.

It could be concluded that addition of bovine sera especially estrus cow serum enhance in vitro maturation of the buffalo oocytes and genes expression (GAPDH, IGF2, β-actin and CX43) and Further studies is needed to enhance the expression of CX43 gene which might help in increasing maturation rate.

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تأثير إضافة السيرم البقرى الى بيئة انضاج البويضات الجاموسى معمليا على كل من الإنضاج وبعض التعبيرات الجينية المصاحبة

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لاستبيان تأثير اضافة السيرم البقرى الى بيئة إنضاج بويضات الجاموس معمليا على معدل الإنضاج و كذلك على تعبير بعض الجينات ذات الصلة فقد جمعت 1149 بويضة جاموسى غير ناضجة جيدة المظهر (بويضات محاطة بخلايا وسادية ومتجانسة البلازما الخلوية) و زراعتها عشوائيا على حسب نوع السيرم المضاف الى بيئة الزراعة الخاصة بنمو البويضات معمليا :المجموعة الأولى (عدد 315) و التى كانت فيها بيئة الانضاج خالية من السيرم, المجموعة الثانية (عدد 531) و التى أضيف إليها سيرم العجول الجنينى بواقع 10% بويضة والمجموعة الثالثة (عدد 303) و التى أضيف إليها سيرم البقر الشبقى بواقع 10%. قسمت البويضات بعد انضاجها فى كل مجموعة على حسب مظهرها من تمدد الخلايا الوسادية وتجانس السيتوبلازم الى بويضات جيدة التمدد وبويضات ضعيفة التمدد. وقد أوضحت النتائج أن إضافة السيرم كانت محددة لعملية النضج حيث انخفضت نسبة الانضاج فى البيئة التى لم يضف اليها السيرم بشكل معنوى وكذلك أوضحت النتائج ان التعبيرات الجينية المختلفة (GAPDH, CX43, IGF II and β- Actin) كانت واضحة فى جميع المجموعات فيما عدا مجموعة واحدة (البويضات ضعيفة التمدد فى غياب السيرم) حيث لم تظهر تعبير جين CX43 وأن البويضات المحاطة بالخلايا الوسادية المتمددة أظهرت نضجا عاليا بمعنوية احصائية ($P<0.01$) خلافا عن البويضات التى فقدت خلاياها الوسادية او انكمشت فيها البلازما الخلوية. وبناء على النتائج يمكن ان نخلص بأن اضافة السيرم الى بيئة النمو وخاصة سيرم دم البقر الشبقى يزيد كل من نسبة انضاج البويضات الجاموسى وكذلك التعبيرات الجينية المصاحبة وأن اختيار البويضات على أساس الخلايا الوسادية المحيطة فقط يعد غير كاف ولكن ضبط بيئة الانضاج وإضافة المواد التى تحفز وتسرع من أداء التعبيرات الجينية وخاصة جين CX43 تعد هى الامل فى زيادة نسب الانضاج.