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EFFECTS OF FETAL CALF SERUM (FCS) WITH ESTRADIOL 17B (E2) ON EMBRYO PRODUCTION IN LOCAL BUFFALOS(*Bubalus bubalis)*

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ABSTRACT

The study was conducted to investigate the effect of add supplement (FCS and E2) on culture media (Ham's F-10 and DMEM) on in vitro maturation , in vitro fertilization(IVF) and embryo development. This study was conducted at the laboratories of Theriogenology, Department of Surgery and Obstetrics, College of Veterinary Medicine, Basrah University, during the period extended from January 2017 to the end of April 2018. The samples of study were female reproductive system and male testis (150 Ovaries and 30 testes) collected from (Al-Basrah abattoir house) after slaughter at fifteen minutes. All samples were transported in sterilize and clean cool boxes at (4-8ºC) within 1-2hrs to the center research unit. Oocytes were collected by aspiration method. Only grad A and B quality oocytes were selected and incubated in an appropriate maturation medium (Ham's F-10 and DMEM) at (38.5 C), 5% CO2 and 95% relative humidity for 24-28 hrs. Spermatozoa were obtained by slicing of caudal epididymal of buffalo's bull. Sperms with matured oocytes were incubated in an appropriate maturation medium at (38.5 C), 5% CO2 and 95% relative humidity for 16 -20 hrs. then the fertilized ova were re-incubated in fresh media with changes 50% of media every day and examined every 24hrs for(4) days to follow embryonic development The results showed:

There was high significant ($P<0.01$) difference in the percentage of Oocytes maturation in Ham's F-10 and DMEM with supplement (FCS and E2) media groups compared with control media groups. The results also showed high significant($P<0.01$) difference in the percentage of Oocytes fertilized in Ham's F-10

and DMEM supplement (FCS and E2) media groups compared with control media groups.

INTRODUCTION

Buffaloes represent an integral part of the agricultural economy in world this important animal species has provided draft power,milk, meat and hide to millions of people. However, buffaloes have low reproductive potential which could be related to the low total number of follicles in the ovary; poor super ovulatory response and high percentage of atretic follicles (1and 2). Efforts have been initiated in recent years to augment the reproductive potential of these animals using biotechnology (3). There are new techniques for improving the reproductive efficiency and enhancing the production of genetically superior animals. *In vitro* fertilization (IVF) technology provides an opportunity to produce embryos for genetic manipulation and embryo transfer (4). Producing embryos by IVF can be done based on three subsequent techniques: *in vitro* maturation (IVM) of Oocytes, IVF of *in vitro* matured oocytes and then *in vitro* culture (IVC) of fertilized oocytes for cleavage up to blastocyst stage (5and 6). (7) refer that the FCS serves as a protein source and may have hormones bound to it. (8) who suggest that BSA contains a number of known growth factors playing an important role in the regulation of oocyte maturation. And also prevents the hardening of zona pellucida. Moreover, the beneficial action of FCS may be related to its anti-oxidant properties, which favors the increased maturation rate. (9) found the percentage of buffalo Oocyte maturation with supplement FCS 67.52 compared with control 40.78 this result refer that supplement of FCS had an important role on *in vitro* maturation of buffalo oocytes. (10) was believed that the beneficial effects of BSA are due to its content of cyclic adenosine monophosphate, catecolamines, vitamins, putative growth factors, lipids and albumin and it has been also demonstrated that the beneficial effect of FCS supplementation is due to the presence of a relatively high molecular weight protein which contributes to maturation of oocytes.

MATERIALS AND METHODS

Collection of Ovaries. Buffalo ovaries (n=150) were collected from local Basrah abattoir were collected 15 min after slaughter according to (11) and transported

immediately to the laboratory in a cold box containing sterile normal saline with antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin sulphate) at 4-8°C. Extraneous tissue was removed to clean the ovaries. Prior to Oocyte collection the ovaries were rinsed in 70% ethanol to minimize the risk of contamination followed by three rinses with sterile normal saline to remove the traces of ethanol.

Recovery of oocytes. Follicular Oocytes (n=320) Oocytes were recovered by **Aspiration method**:

Oocytes were aspirated from 10-20 mm visible follicles using an 18- gauge needle attached to a 10-ml disposable syringe containing 1 ml of media. The recovered compact Oocyte complexes (COCs) were examined and classified according to their quality (12) into:

Grade 1: Good Oocytes had a homogenous evenly granular Ooplasm and were surrounded by compact and dense cumulus cell layers.

Grade 2: Fair Oocytes were surrounded by 1-3 layers of cumulus cells.

Grade 3: Denuded Oocytes had uneven Ooplasm and were completely devoid of cumulus cells around them.

Experiment 1 **:-The Oocytes were divided into four groups according to the type of maturation medium with supplemented:**

Group 1: oocytes (n = 80) matured using Ham's F-10 (control group) (Hycolne, Logan ,Austria) without supplemented.

Group 2: oocytes (n = 80) matured using Ham's F-10 Hycolne, Logan Austria) supplemented with either 10% FCS with 1 mg/ml estradiol 17β (E2).

Group 3: oocytes (n = 80) matured using DMEM (control group) (Sigma) supplemented without supplemented .

Group 4: oocytes (n = 80) matured using DMEM (Sigma) supplemented with either 10% FCS with 1 mg/ml estradiol 17β (E2).

Only grade 1 and grade 2 were used supplemented with (100 IU/mL penicillin, 100 µg/mL streptomycin sulphate). The culture dishes were placed in a CO2 incubator (95% relative humidity, 5% CO2 at 38.5ºC) for 24 h. Maturation oocyte was assessed according to polar body (13) and determined by the degree of cumulus cells expansion to excellent, described by (14).

Experiment 2**:-Spermatozoa maturation and Capacitation:**

After collection of sperm ,the spermatozoa samples transported from petri dish to 10ml test tube containing 2ml maturation media (TCM-199) with Nystatin and Penicillin , the test tube was incubated at 37 ºC for 4 hrs , the loose of cytoplasmic droplet from the tail of spermatozoa was the criteria of sperm maturation (15). To complete the next step (sperm capacitation) 50 IU/ml heparin were added at the last 45minutes of the time of maturation to complete the step of sperm maturation and sperm Capacitation (16).

***Experiment 3*:-*In vitro* fertilization**

Matured Oocytes were washed twice with medium supplied with antibiotics and antifungal before transferred to a glass Petri-dishes containing IVF medium which is supplemented with E2, FCS, pencilline- streptomycin and antifungal preparation (17). Capacitated spermatozoa sample was prepared and diluted to yield 1-2× 106 sperms needed for fertilization (17). After that, Petri- dishes which containing mixture Gametes was incubated at (95% relative humidity, 5% CO2 at 38.5ºC) for 14- 18 hrs (18 and 19). Approximately every 24 h for up to 5 days was checked in order to confirm the occurrence of fertilization. The cleavage rate was recorded according to (20) as the total number of cleaved Oocytes/total number of matured cultured Oocytes multiplied by 100.

Statistical analysis

Chi-square test was used to compare maturation and fertilization rates among different groups (21). A 1% significance level was used.

RESULTS

1- Effect FCS with Estradiol 17β (E2) on *In vitro* maturation (IVM) of local iraq buffalos Oocyte:-

The results indicate that Oocytes maturation percentage increased significantly value ($P< 0.01$) in Ham's F-10 media with 10% FCS and (E2) supplemented

compared group with Control group 50% (80/40), 13.75% (80/11) respectively. On the other hand the percentage of oocytes maturation in DMEM with 10% FCS and (E2)supplemented group increased significantly value ($P< 0.01$) than the control group 61.25% (80/49) , 16.25% (80/13) respectively . While there was non-significantly value ($P> 0.01$) between control group and treated group as shown in table (1).

Table (1): Effect of FCS with E2 on *In vitro* maturation (IVM) of local iraq buffalos oocyte:-

Parameter / Media	No-oocytes culture	No-oocytes maturated	Percentage oocytes maturated	Percentage oocytes non-maturated
Ham's F-10 (Control)	80	11 b	(13.75%) b	(86.25%) b
Ham's F-10 with FCS and E2	80	40 a	(50%) a	(50%) a
DMEM (Control)	80	13 b	(16.25%) b	(83.75%) b
DMEM with FCS and E	80	49 a	(61.25%) a	(38.75%) a

Different small letters vertically denote significant ($P<0.01$) different between parameters.

2-Effect FCS with Estradiol 17β (E2) on *In vitro* fertilization (IVF) of local Iraq buffalos oocytes:-

The results illustrated that the percentage oocytes fertilization increased significantly value ($P< 0.01$) in Ham's F-10 media with 10% FCS and (E2) supplemented group compared with Control group 40% (40/16), 18.18% (11/2) respectively. On the other hand the percentage of embryo development (2, 4, 8 and 16 cell) at 24,48.72 and 96 hrs high significantly value ($P< 0.01$) in Ham's F-10 media

with 10% FCS and (E2) supplemented group compared with Control group. The results showed that the percentage oocytes fertilization increased significantly value (P< 0.01) in DMEM with 10% FCS and (E2) supplemented group compared with Control group 38.77% (49/19), 23.07% (13/3) respectively. On the other hand the percentage of embryo development (2 , 4 ,8,16 cell) at 24,48.72 and 96 hrs high significantly value (P< 0.01) in DMEM with 10% FCS and (E2)supplemented group compared with Control group . While there was non-significantly value (*P*> 0.01) between control group and treated group as shown in (table 2).

Table (2): Effect of FCS with E2 on In vitro Fertilization (IVF) of local iraq buffalos oocyte:-

Parameter Media	No-oocytes maturated	No. and percentage zygotes	No. and percentage two cells At 24 hrs.	No. and percentage Four cells At 48 hrs.	No. and percentage Eight cells At 72 hrs.	No. and percentage sixteen cells At 96 hrs.
Ham's F-١٠ (Control)	11 b	2 (18.18%) b	١ (٥٠%) b	٠ (٠.٠%) b	٠ (٠.٠%) b	٠ (٠.٠%) b
Ham's F-10 with 10% FCS and (E2)	40 a	16 (40%) a	9 (56.25%) a	6 (37.5%) a	4 (25%) a	2 (12.5%) a
DMEM (Control)	13 b	3 (23.07%) b	1 (33.33%) b	0 (0.0%) b	0 (0.0%) b	0 (0.0%) b
DMEM with 10% FCS and (E)	49 a	19 (38.77%) a	7 (36.84%) a	5 (26.31%) a	4 (21.05%) a	4 (21.05%) a

Different small letters vertically denote significant (P<0.01) different between parameters

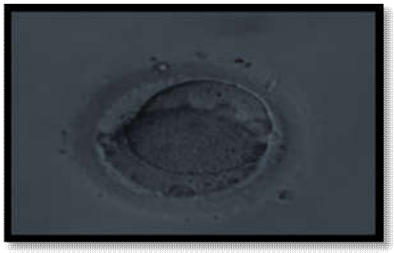

Fig .1 Two cells

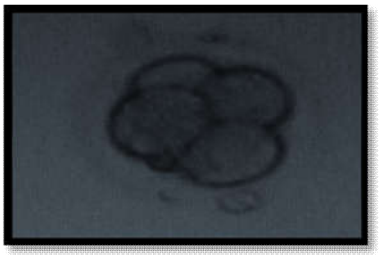

Fig .2 four cells

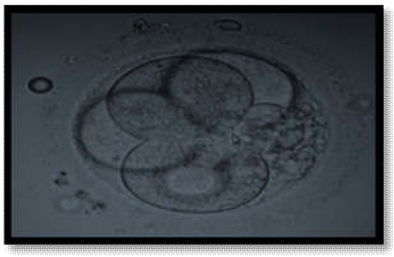

Fig .3 Eight cells **Fig .4 Sixteen cells**

DISCUSSION

Many factors affect *in vitro* maturation of buffalo oocytes. These factors are either the selection of proper maturation medium, the quality of Oocytes , the hormones added and serum supplemented (22).

The present study revealed that supplementation of maturation media with FCS with E2 led to higher maturation and fertilization rates compared with free medium these results coincide with those of (23) and (18) who observed difference between serum free group and serum supplemented group for IVM. Moreover, these results are in agreement with those obtained by (24), (25), (26) and. (27) who recorded beneficial effect of serum supplementation on *in vitro* maturation rate of buffalo oocytes. This might be attributed to the action of serum that promotes cumulus cells-oocyte uncoupling by retaining the hyaluronic acid within the COCs in a manner that results in cumulus mucification. This uncoupling could be responsible for stopping the transfer of oocyte maturation inhibition factor via gap junction (28).

The results of this study revealed that the addition of serum to the TCM-199 medium enhanced the maturation rate of follicular oocytes. Similar findings have been reported by (29) in cattle. (30) found a favourable effect of oestrus cow serum when added to the maturation medium. These authors have postulated that the addition of sera to the culture medium during the maturation of oocytes promotes the rupture of germinal vesicle and induces oocyte maturation. Hence, supplementation of the media with serum had a biphasic favourable effect on maturation. The beneficial effects of serum for oocyte maturation may also act via cumulus cells or directly on the oocytes. (31).

Serum may provide beneficial factors to the culture medium, including energy substrates, amino acids and vitamins. There may be specific effects of serum component possibly growth factors on oocyte maturation That is manifested as

improved embryo development following IVF (32). Also, (33) suggested that it may be important to include serum in the *in vitro* maturation medium to prevent hardening of zona pellucida which could adversely affect fertilization. In the same manner, (34) reported that fetuin, a major glycoprotein constituent of fetal calf serum, can prevent hardening of zona pellucida during *in vitro* maturation as it acts by preventing the action of proteolytic enzymes originating from precociously released cortical granules and improve the fertilization capacity of oocytes. Also, (35) recorded another beneficial action of serum which is its antioxidant properties by reducing superoxide formation. In addition, serum added to the oocyte medium provides a source of albumin that balances the osmolarity (36). There are different sources for supplemented sera such as fetal calf serum (37,38), oestrus buffalo serum (39), steer serum (40) and superovulated buffalo serum (41). Estradiol has been found to improve the completion of maturational changes and also to support the synthesis of presumed male pronuclear growth factor (42, 43).

تأثيرات مصل العجول البقري (FCS) مع هرمون الاستردايول (E2) على انتاج اجنة الجاموس العراقي المحلي

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الخلاصه

هدفت الدراسة الى تأثير مصل العجول البقري وهرمون الاستوجين (FCS and E2) المضاف الى الاوساط الزرعيه (Ham's F-10 and DMEM) على نسبة انضاج واخصاب وانتاج اجنة الجاموس المحلي. أجريت الدراسه في مختبرات كليه الطب البيطري(فرع الجراحه والتوليد وحدة الابحاث المركزيه) جامعه البصرة للفتره من شهر كانون الثاني لنهاية شهر ميسان ٢٠١٨ جمعت نماذج الدراسه الاجهزه التناسليه الانثويه للجاموس وخصى ذكور الجاموس (عدد المبايض التي تم الحصول عليها ١٥٠ مبيض وعدد الخص ٣٠خصيه) بعد الذبح مباشرة بخمسة عشر دقيقة من مجزره البصره العصريه. ، حيث نقلت نماذج الدراسه في حاويات خاصه نضيفه ومعقمه تحتوي على محلول فسلجي مبرد وبدرجه حرارة(٤-٨) درجه مئويه خلال مدة (١-٢) ساعة الى وحدة الابحاث المركزيه . تم أستحصال البيوض من المبايض بطريقة السحب Aspiration. أختيرت البيوض من صنف A,B فقط لغرض الأنضاج. حضنت البيوض المختارة في الاوساط الزرعيه (Ham's F-10 and DMEM) في حاضنه ثنائي اوكسيد الكاربون بنسبه ٥% والرطوبه

النسبية ٩٥% وبدرجة حراره ٣٨.5 درجه مئويه لمدة ٢٤-٢٨ ساعة. اما بالنسبه للحصول على النطف عن طريق اسئصال ذيل البربخ للثيران. حضنت البيوض والنطف الناضجه في الاوساط الزرعيه (Ham's F-10 and DMEM) في حاضنه ثنائي اوكسيد الكاربون بنسبه ٥% والرطوبه النسبية ٩٥% وبدرجة حراره ٣٨.5 درجه مئويه لمدة ١٦-٢٠ ساعة . تم عزل البيوض المخصبه واعادتها الى الوسط الزرعي ، تم متابعه تطور الاجنه كل ٢٤ ساعه مع تغير ٥٠% من حجم الوسط الزرعي يوميا بوسط زرعي طازج ولغاية(٤) أيام الى حين تكون التويثه ، وقد اظهرت النتائج التاليه.

وجود فرق معنوي ($P<0.01$) في نسبه البيوض الناضجه في الوسط (Ham's F-10 and DMEM) المعامل بمصل العجول البقري وهرمون الاستروجين مقارنة مع نفس الاوساط الزرعيه الغير معاملة بمصل العجول البقري وهرمون الاستروجين ، كما تبين من الدراسه وجود فرق معنوي ($P<0.01$) في نسبه البيوض الخصاب في الوسط (Ham's F-10 and DMEM) المعامل بمصل العجول البقري وهرمون الاستروجين مقارنة مع نفس الاوساط الزرعيه الغير معاملة بمصل العجول البقري وهرمون الاستروجين

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