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IN VITRO BUFFALO EMBRYO DEVELOPMENT IN DEFINED MEDIA SUPPLEMENTED WITH TRI-SODIUM CITRATE AND MYO-INOSITOL

(With 4 Tables and 2 Figures)

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نمو أجنة الجاموس معملياً في أوساط (ميديا) محددة مدعمه بسترات صوديوم الثلاثى والميوانستول

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صممت هذه الدراسة للتعرف على تأثير نضوج وإخصاب ونمو أجنة الجاموس معملياً في أوساط (ميديا) خالية من السيرم وكذلك تأثير سترات صوديوم الثلاثى والميوانستول على نمو أجنة الجاموس معملياً . البويضات الحويصلية الغير ناضجة تم تجميعها من المبايض بطريقة الشفط وتم زراعتها لمدة ٢٤ ساعة في وسط TCM-199 في حضانة ثانـــي أكسيد الكربون (٥% ك أم ، ٣٨,٥ درجة مئوية ودرجة رطوبة عالية) لإتمام عملية النضوج . تم إجراء إخصاب البويضات معملياً باستخدام سائل منوي طازج من طلوقه جاموس بعد تجهيزه وتهيئيه في وسط (ميديا) تالب (S.TALP media) يحتوى على الهيبارين والكافيين لمدة أربع ساعات في حضانة ثانى أكسيد الكربون . ثم بعد إحداث عملية الإخصاب ثم استكمال عملية الزراعة لمدة ٥ – ٧ أيام في أوساط m SOF ثم إجراء الدراسة في أربع تجارب هي كالتالى: **التجربة الأولى** : دراسة تأثير الأنواع المختلفة من السيرم ، ١٠% مستخلص من الأجنة (FCS) أو ١٠% سيرم الألبومين البقرى أو باستخدام ٣ مجم/مللي من مركب بولي فنيل الكحول. **التجربة الثانية**: دراسة تأثير نضوج وإخصاب بويضات الجاموس في أوساط خالية من السيرم، على معدل الإخصاب. **التجربة الثالثة**: دراسة نمو بويضات الجاموس المخصبة في أوساط خالية من السيرم حتى المراحل المتقدمة. **التجربة الرابعة**: دراسة تأثير إضافة مادتى سترات الصوديوم الثلاثى وكذلك ميوانستول إلى أوساط خالية من السيرم ومدى تأثيرها على درجة نمو أجنة الجاموس معملياً . وأشارت نتائج هذه التجارب إلى أن معدل النضوج معملياً بلغ ٦٥,٢٨ ، ٥٥,٨٨ ، ٥٣,٩٥ % في الأوساط التي تحتوى على سيرم أجنة (FCS) ، الألبومين سيرم البقري وكذلك بولي فنيل الكحول على التوالي (التجربة الأولى) وهو معدل لايختلف إحصائياً اختلافاً معنوياً بينما لوحظ انخفاض كبير في معدل الإخصاب المعملى بالنسبة للبويضات التي نضجت وأخصبت في أوساط خالية من السيرم (تحتوى على بولى فنيل الكحول) ٢٩,١٧ % مقارنة بالبويضات التي نضجت وأخصبت في أوساط تحتوى على سيرم الأجنة (FCS) ٥٧,٥٨% (التجربة الثانية) كذلك كانت معدلات

الإنقسام والنمو إلى الطور التوتى وطور البلاستيولا منخفضاً بالنسبة للبويضات التي نضجت وأخصبت وتمت في وسط يحتوى على بولى فنيل الكحول ٢٩,٦٨ ، ٧,٨١ ، ٣,١٣ % على التوإلى مقارنة بتلك التي عوملت في أوساط تحتوى على السيرم في كل مراحل الزراعة ٤٨,٠٨ ، ٢٣,٠٨ ، ١٧,٣١ % على التوالي (التجربة الثالثة) ولكن عندما احتوى الوسط (الميديا) الخإلى من السيرم على سترات الصوديوم الثلاثى والميوانستول (التجربة الرابعة) تحسن معدل الإنقسام والنمو إلى الطور التوتى وطور البلاستيولا (٣٩,٠٦ ، ٢٠,٣١ و ١٢,٥٠ على التوإلى) إلى درجة لا تختلف إحصائياً عن تلك التي زرعت في أوساط تحتوى على السيرم في كل مراحل الزراعة (٥٢,٩٤ ، ٢٦,٤٧ ، ١٦,١٨ % على التوالي . ومن نتائج هذه الدراسة يتبين أن إضافة سترات الصوديوم الثلاثى والميوانستول إلى الأوساط (الميديا) الخالية من السيرم يحسن كثيراً معدلات نمو بويضات الجاموس إلى الطور التوتى وطور البلاستيولا وبالتإلى تثبت هذه الدراسة قدرة بويضات الجاموس على النمو إلى أطوار متقدمة معملياً عند زراعتها في أوساط خالية من السيرم .

SUMMARY

This study is concerned with the effect of serum supplementation at the different steps of in vitro production (IVP) of buffalo embryos and to establish an in vitro system in which buffalo oocytes can be matured, fertilized and cultured up to the blastocyst stage without serum support. The basic maturation medium was TCM-199 that is supplemented with hormones and either fetal calf serum (FCS), bovine serum albumin (BSA) or polyvinyl alcohol (PVA). In vitro fertilization (IVF) was carried out using fresh ejaculated buffalo bull semen in F-TALP media containing heparin, and either 6 mg/ml BSA or 3mg/ml PVA. In vitro culture (IVC) up to the blastocyst stage was performed in synthetic oviduct fluid media (SOF) supplemented with either FCS or PVA. The first experiment investigated the effect of different proteins supplementation (FCS or BSA) or macromolecules (PVA) on the rate of nuclear maturation. It revealed no significant differences among treatment groups (65.28%, 53.95% and 55.88%, respectively). The effect of the different protein supplementation on IVF rate was investigated in Experiment 2, in which medium was supplemented with either FCS/BSA, PVA/PVA, BSA/BSA and PVA/BSA during maturation and fertilization. Penetration rate was not significantly different (53.23%, 42.86%, 45.45% and 46.55 %, respectively), however, formation of pronuclei was reduced significantly ($P<0.05$) for oocytes matured and fertilization under defined media (29.17) compared to those matured and fertilized with FCS/BSA (7.58%). In Experiment 3, four treatment groups were performed (1) FCS during maturation, and embryo culture and BSA during fertilization (standard undefined conditions); (2) FCS

during maturation and BSA during fertilization and PVA during embryo culture; (3) PVA during maturation, fertilization and culture (standard defined conditions) and (4) PVA during maturation and culture and BSA during fertilization . Under completely defined IVP conditions the rates of cleavage and the development to the morula or blastocyte (29.68%, 7.81% and 3.13%, respectively) were reduced significantly ($P<0.05$) compared to those cultured in standard undefined media (48.08%, 23.08% and 17.31%, respectively). Experiment 4, investigated the influence of addition of tri-sodium citrate and myo-inositol to the culture media on the buffalo embryo development in vitro. The results revealed that oocytes matured, fertilized and cultured under completely defined conditions with tri-sodium citrate and myo-inositol enhanced the rates of cleavage, morula and blastocyst development (39.06%, 20.31% and 12.5%, respectively), comparable to those cultured in undefined media (52.94%, 26.47% and 16.18, respectively). It is concluded that, the cleavage rate, morula and blastocyst development were decreased significantly under defined media, the addition of tri-sodium citrate and myo-inositol to the defined IVC media improved morula and blastocyst development to rates similar to those obtained with the undefined media.

Key words: *Buffalo, embryo development, in vitro fertilization.*

INTRODUCTION

In buffalo, embryo production has faithfully followed cattle (Boni *et al.*, 1994), numerous approaches have been utilized to enhance in vitro embryo development in the buffalo including oviductal (Totey *et al.*, 1992 and Madan *et al.*,1994), cumulus (Badr, 2001 and Ocampo *et al.*, 2001) , granulosa (Suzuki *et al.* , 1992) or BRL cells co-culture (Boni *et al.*, 1994). However, to date, the efficiency of in vitro buffalo embryo production is lower than that obtained in cattle (Boni *et al.*, 1999). Up to now, most culture systems have relied on macro molecules such as BSA or complex biological fluid such as serum in the culture medium for provision of components essential for development, however , too often these macro molecules are complex and contain undefined mixtures of different proteins, growth factors , vitamins , peptides, and array of defined and undefined molecules (Gardner and Lane,1993). Serum is generally included as the fixed nitrogen source for the pre implantation embryo (Gardner, 1994). Factors leading to improved embryo development in culture system using serum are not

understood, but include embryotrophic factor(s) (Gardner, 1994; Pinyopummintr and Bavister, 1994 and Holm *et al.*, 1995) and/or inactivation of embryo toxic agents (e.g free radicals / heavy metals; Eckert and Niemann, 1995). Different batches of BSA possibly contaminated with various effects on embryonic development ranging from markedly stimulatory to inhibitory (Pnyopumminter and Bavister, 1994). In BSA, citrate has been identified as a positive embryo trophic factor (Gray *et al.*, 1992), but BSA may also stimulate embryo development through amelioration of embryo toxic substances. Therefore, supplementation of culture media with serum undoubtedly conflicts with proper quality control (Eckert and Niemann , 1995) and represent sources of variation between laboratories (Boni *et al.*, 1999) .In order to improve the quality control , it is imperative to develop a more defined culture system (Abeydeera *et al.*, 1998). So far, no information are available on embryo metabolism in buffalo. Therefore, development of a defined culture system may help to advance understanding of the basic molecular mechanisms responsible for stimulatory or inhibitory cytoplasmic maturation and development in vitro (Boni *et al.*, 1999). Bovine oocytes have been shown matured (Saeki et al., 1991) and fertilized (Tajik *et al.*, 1994; Keskintepe *et al.*, 1995 and Saeki *et al.*, 1995) in vitro in the absence of serum without compromising subsequent embryo development in vitro. However, when zygotes were cultured up to the blastocyst stage in medium lacking protein supplementation, they developed with various rates of success (Pinyopumminter and Bavister, 1991). Recently, bovine embryo development has been significantly improved by the inclusion of growth or embryo trophic factors such as citrate (Keskintepe *et al.*, 1995) and myo-inositol (Holm *et al.*, 1999). Therefore, the present study was designed to define conditions for IVM, IVF and IVC without compromising the quantity and quality of buffalo embryo development in vitro via the entire IVMFC system and to study the influence of addition of tri-sodium citrate and myo-inositol to the defined culture media on the rate of embryo development in vitro.

MATERIAL and METHODS

Oocyte recovery, evaluation and maturation:

Ovaries were collected from slaughtered buffaloes at a local slaughter house and were transported to the laboratory within 2 to 4 h in warm 0.9% Nacl solution containing 100 µg/ml penicillin G and 50 µg/ml streptomycin sulfate, maintained at 25–30 C^{o}. Cumulus oocytes

were aspirated from medium-sized follicles 2- 6 mm in diameter with a 10-cc disposable syringe fitted with 18 gauge needle using a low constant vacuum. Oocytes selection and all manipulations were carried out at 39C° *(Younis et al., 1989)*. The cumulus-oocyte complexes (COCs) surrounded by a compact multilayers of cumulus cells and having evenly granulated, homogenous ooplasm were selected for in vitro culture. The selected COCs were washed three times in the respective oocyte maturation media (TCM-199) without hormonal supplements. TCM-199 with L-glutamine and without sodium bicarbonate (Sigma, M. 3769), was the basic medium used for IVM. The medium was supplemented with 50 µg/ml sodium pyruvate, 2.6 mg/ ml sodium bicarbonate and 1% antibiotic antimycotic. The medium was freshly prepared weekly, filtered sterile through a 0.22-µm filter and stored at 4C° until use. In vitro maturation lasted for 24 h and took place in 50 µl drops under silicone oil at 39 C°, 5% Co2 in a humidified atmosphere. The basic culture medium was supplemented with 10% µg/ml LH, 5 µg/ml FSH and 1 µg/ml estradiol - 17 β and either 10% FCS or 10 mg/ml BSA (free fatty acids, fraction-V, Sigma) or 3 mg/ml PVA(defined media) on the day of experiment . Each experiment (treatment groups) was repeated four times .

Evaluation of cumulus expansion:

At the end of IVM culture, the degree of cumulus expansion was assessed subjectively under a stereomicroscope and classified into five categories (Abeydeera *et al.,* 1998), category 0, no expansion; category 1, very slight expansion, observed in 2-3 layers from the periphery; category 3, moderate expansion, observed in about 50% of the cumulus cell layers; and category 4, full expansion except in the corona radiata layer.

In vitro Fertilization (IVF):

The matured oocytes were inseminated in vitro with fresh ejaculated spermatozoa from one fertile buffalo bull. Semen was swim-up separated in S-TALP medium containing either 6 mg/ml BSA or modified with addition of 3 mg/ml PVA, for 1 hour (Parrish *et al.,* 1988). The uppermost layer of spermatozoa was selected and washed twice by centrifugation at 2000 rpm for 10 minuts. At the end of the washing procedure, the resulting sperm pellet was resuspended in F-TALP media containing 100 µg/ml heparin (Sigma, H.3149) and either 6mg/ml BSA or 3 mg/ml PVA. After appropriate dilution, 2µl of sperm -

suspension was added per fertilization drop to obtain a final concentration of $2X10^6$ sperm cell/ml, sperm and oocytes were co-incubated in fertilization drops under silicon oil 18 to 24 h at 39 C° in 5% Co2 in humidified air.

Assessment of in vitro nuclear maturation and fertilization parameters:

Random samples of oocytes were freed from cumulus cells by vortexing . Oocytes were mounted on glass slides and fixed for 48 – 72 h in acetic acid: ethanol (1: 3). They were then stained with 1% (w/v) orcein in 45% acetic acid (v : v) and examined under a phase – contrast microscope at x200 and x400 magnification, The miotic stage was assessed according to Hunter and Polge (1966). Oocytes were considered penetrated when they had one or more swollen sperm heads and/or male pronuclei and their corresponding sperm tails.

In vitro culture (IVC):

The surrounding cumulus-cells of inseminated oocytes were removed by gentle pipetting and in vitro cultured over 7 to 9 d in 50 µl drops under silicon oil in SOF media (Keskintepe *et al.*, 1995) Proportions of cleaved oocytes were recorded 48 h post insemination and that developed to morulae or blastoocysts were recorded from day 5 onwards. At least 4 replicates were conducted per each treatment group.

Experimental design:

Experiment 1:

This experiment was designed to develop a well defined medium for the IVM of COCs . Oocytes were matured in TCM-199 containing three protein supplementations FCS (10%) , BSA (10 mg/ml) and synthetic macromolecles, PVA (3 mg/ml). Nuclear maturation was evaluated as the rate of oocytes reaching metaphse II (MII) in each system.

Experiment 2:

This experiment was designed to achieve in vitro fertilization in defined and undefined media. Oocytes either matured in undefined media (FCS), fertilized in F-TALP supplemented with BSA (6 mg/ml) or matured and fertilized in defined media, PVA (3 mg/ml) or matured and fertilized in media supplemented with BSA.

Experiment 3:

This experiment investigated the developmental capacity of COCs up to the morula and blastocyte stages in media supplemented

with FCS, BSA or under defined conditions PVA. The oocytes were matured, fertilized and cultured as follow :1) FCS (10 %), BSA(6mg/ml) and FCS (10%), 2) FCS (10%), BSA(6mg/ml) and PVA(3mg/ml), 3) PVA(3mg/ml), PVA(3mg/ml) and PVA(3mg/ml) and 4) PVA(3mg/ml) , BSA(6mg/ml) and PVA(3mg/ml), respectively.

Experiment 4:

This experiment was carried out to study the effect of addition tri sodium citrate 5mM and myo-inositol 5mg/ml to the defined culture media on the developmental capacity of COCs up to morula and blastocyst stages.

Statistical analysis:

Maturation and fertilization rates were based on the number of fixed oocytes , cleavage rates, morula and blastocytes rates were based on the number of cultured oocytes , the experiments were repeated 4 times. The data were analyzed by Chi-square (X^2) analysis.

RESULTS

Experiment 1:

Rates of nuclear maturation in all treatment groups did not differ significantly (P>0.05; 65.28, 55.88% and 53.95; Table 1). The results revealed that, the presence or absence of serum during IVM did not have an influence on nuclear maturation. Oocytes matured under defined condition (TCM-199-PVA), cumulus expansion was reduced compared with maturation under undefined conditions .

Table 1: In vitro maturation rates for buffalo oocytes matured in defined and undefined media.

Treatment Groups	No. of oocytes	Degree of Expansion	Metaphase II MII (%)
FCS (10%)	72	Full expansion	47(65.28)a
BSA (10mg/ml)	68	Moderate expansion	38(55.88)a
PVA (3 mg/ml)	76	Slight expansion	41(53.95)a

No statistical difference between the treated groups .

Experiment 2:

No significant difference between treated groups (Table 2) with regard to the sperm penetration rates. The penetration rates were 53.23, 46.55, 45.45 and 42.86% for FCS/BSA, PVA/BSA, BSA/BSA and PVA/PVA, respectively. However, with regard to the formation of male and female pronuclei, oocytes that matured and fertilized under defined media (PVA/PVA) was significantly reduced (29.17 %) compared to those matured and fertilized under undefined media (FCS/BSA, 57.58 %, $P<0.05$), although no significant differences were found between FCS/BSA (57.58%), BSA/BSA (46.67%) or PVA/BSA (48.15%) in the formation of pronuclei.

Table 2: In vitro fertilization rates for buffalo oocytes under defined and undefined media

Treatment Groups	No. of oocytes	Penetration rate (%)	Fertilization rate (%)	Polyspermy	abnormal Fertilization
FCS/BSA	62	33(53.23)a	19(57.58)a	5(15.15)a	9(27.27)a
PVA /BSA	58	27 (46.55)a	13(48.15)ab	2(7.41)a	12(44.44)a
BSA/ BSA	66	30(45.45)a	14(46.67)ab	4(13.33)a	12 40.00)a
PVA/ PVA	56	24(42.86)a	7(29.17)b	2(8.33)a	15(62.50)b

Dissimilar superscripts within the same columns indicate significantly different values at $P< 0.05$.

Experiment 3:

Results of this experiment are shown in Table 3, oocytes that matured, fertilized and cultured in defined media reduced significantly ($P<0.05$) the cleavage rate (29.68%), morula (7.81%) and blastocyst (3.13%) compared to oocytes that matured, fertilized and cultured under undefined conditions (48.08, 23.08 and 17.31%), respectively. No significant differences were found in the cleavage, morula or blastocyst rates between presumptive zygotes cultured with serum or that cultured in PVA supplemented medium,when serum was present during IVM-IVF procedures.

Table 3: Comparison of buffalo embryo development following maturation , fertilization and culture in undefined or in defined media.

Treatment Groups	No.of oocytes	Cleavage Rate (%)	Morula (%)	Blastocyst (%)
FCS/BSA/FCS	52	25(48.08)a	12(23.08)a	9(17.31)a
FCS/BSA/PVA	65	31(47.69)a	13(20.00)a	8(12.31)a
PVA/BSA/PVA	72	34(47.22)a	14(19.44)a	9(12.5)a
PVA/PVA/PVA	64	19(29.68)b	5(7.81)b	2(3.13)b

Dissimilar superscripts within the same columns indicate statistical difference ($P<0.05$).

Experiment 4:

Data showing the effect of addition of tri-sodium citrate and myo-inosital to the defined culture media are shown in Table 4. Oocytes that matured, fertilized and cultured in defined media supplemented with sodium citrate and myo-inosital, (SOF- PVA CI) or in undefined media (SOF – FCS) with cumulus monolyer, were not significantly different with respect to cleavage rates, morula and blastocyst rates. Addition of tri sodium citrate and myo-inositol to the defined culture media increased embryo development to the morula (Fig. 1) and blastocyst stage (Fig. 2) comparable to the levels recorded in the undefined culture media.

Table 4: Effect of tri sodium citrate and myo inositol added to the defined media on in vitro buffalo embryo development

Treatment Groups	No. of Oocytes	Cleavage rate (%)	Morula (%)	Blastocyst (%)
PVA/PVA/PVA	66	21(31.82)a	5(7.58)a	2(3.03)a
PVA/PVA/PVA Ci *	64	25(39.06)ab	13(20.31)b	8(12.5)b
PVA/BSA/ PVA Ci*	62	32(51.61)b	15(24.19)b	9(14.52)b
FCS/BSA/ FCS Ci*	68	36(52.94)b	18(26.47)b	11(16.18)b

Dissimilar superscripts within the same columns indicate statistical difference ($P < 0.05$).
Ci* , tri sodium citrate and myoinositol

DISCUSSION

The results of the present study demonstrate that immature buffalo oocytes are able to mature, fertilize and develop to blastocyst stage in defined media without serum or BSA, which is confirmed by the findings of (Darwish, 1993; Eckert and Niemann, 1995; Keskintepe and Brackett, 1996 and Holm *et al.*,1999) in the cattle and (Boni *et al.*,1999) in the buffalo . Omission of serum from IVM-medium did not affect nuclear maturation, which is in agreement with previous reports (Saeki *et al.*, 1991 and Eckert and Niemann, 1995). Despite, no significant difference between different protein supplementation regarding in vitro maturation, oocytes matured in the defined media, showed decreased cumulus expansion compared to those matured in undefined media. Similar observation have also been reported in cattle oocytes (Harper and Brackett, 1993). This observation suggests that cumulus expansion and nuclear maturation are not necessarily dependent on each other. IVF data provide evidence that penetration rate for oocytes matured in protein supplemented media and oocytes that matured under protein-free conditions are not significantly different. In contrast reduction in the fertilization rate (expressed as ratio of oocyte with 2 normal pronuclei) was observed, when serum or BSA were absent from both maturation and fertilization media. This confirmed by the finding of (Eckert and Niemann 1995) who concluded in a study on defined IVP of bovine embryo that serum-proteins were required for normal pronuclei formation not for cleavage and subsequent embryo development. Moreover (Holm *et al.*, 1999) indicated that exposure of the matured oocytes and/or 1-cell embryo to component(s) in serum is of utmost importance for both optimal fertilization and further embryo development. However (Tajik *et al.*, 1994) postulated that, penetrated oocytes that matured in protein supplemented media and those in protein-free have an equal ability to transform a sperm nucleus into a male pronucleus. This emphasizes that the first critical step in which problems start to arise when proteins are to be replaced seems to be in vitro fertilization and that normal fertilization requires support of macromolecular component at insemination. The present study demonstrate that, omission of serum or BSA from both IVM and IVF media and continued culture under defined condition resulted in a significant reduction in the cleavage rate and embryo development to morula and blastocyst stage compared to those matured, fertilized and

cultured under undefined media. These findings are in accordance with the findings of (Holm *et al.*, 1999). Contrary to these results, other investigators obtained similar cleavage and developmental rates to the blastocytst stage when oocytes were fertilized in simple defined media (Bavister *et al.*, 1992; Kim *et al.*, 1993 and Keskintepe *et al.*, 1995) or in semi-defined media (Rorie *et al.*, 1991) .The reduced development to morulae or blastocysts under defined media could be attributed to the poor expansion of cumulus cell (Eckert and Niemann, 1995). Moreover, the kinetics of embryo development was affected by serum during IVM/IVF and culture, the first and fourth cell cycles were prolonged by 4-5 h in the absence of serum during IVM/IVF whereas the presence of serum during culture decreased the duration of the fourth cell cycle and triggered premature blastulation (Holm *et al.*, 2002). Serum might changes the pattern of blastulation by inducing blastocoel formation (Pinyopummintr and Bavister, 1994; Vanlangendonckt *et al.*, 1997 and Yoshioka *et al.*, 1997). Furthermore, the extremely low embryo development matured and fertilized in defined media in bovine (Rose and Bavister, 1992) and in pig oocytes (Abeydeera *et al.*, 1998) could be related to a low intracellular glutathione level. Oocytes glutathione content after IVM is correlated with in vitro developmental ability of bovine embryos to the blastocyst stage (De-Matos *et al.*, 1995). The present study revealed that, exclusion of serum from the maturation had a lesser influence on the subsequent fertilization and cleavage of embryos, as serum was present during fertilization. Furthermore, addition of tri sodium citrate and myo-inositol to the defined culture medium, improved morula and blastocyst to a degree that did not differ significantly from that cultured under undefined conditions. The embryotrophic effect of sodium citrate has been reported by (Gray *et al.*, 1992 and Kestintepe *et al.*, 1995). Citrate stimulates fatty acids synthesis (Goodridge, 1973) and is a chelator of metal ions (Ca++) a feature that may be of importance for maintaining junctional integrity and thus of importance for compaction and blastocoel formation (Gray *et al.*, 1992). The embryotrophic affect of BSA may be related to a significant citrate contamination (Kane, 1987 and Holm *et al.*, 1995).

Moreover, myo-inositol or its metabolites are essential components in cellular signaling (Downes and Macphee, 1990). Myo-inositol is associated with intracellular calcium release at fertilization (Kline, 1996) and it act as an osmolyte together with glycine, providing substantial protection of rabbit embryos cultured in medium with high osmolarity (Li and Foote, 1995). Furthermore, embryotrophic properties

of human serum have been correlated with high concentration of myo-inositol (Chiu and Tam, 1992).

This study is the first report in Egypt to achieve completely defined conditions for buffalo oocytes maturation, sperm treatment and in vitro culture. It demonstrates that defined media supplemented with tri sodium citrate and myo inositol seem to be supportive for buffalo embryo production in vitro.

In conclusion, the present results indicate that tri-sodium citrate and myo inositol enhance in vitro buffalo embryo production to the blastocyst stage under completely defined conditions. However serum supplementation during in vitro fertilization seems to play an essential role for optimal fertilization rates and the subsequent high blastocyst development.

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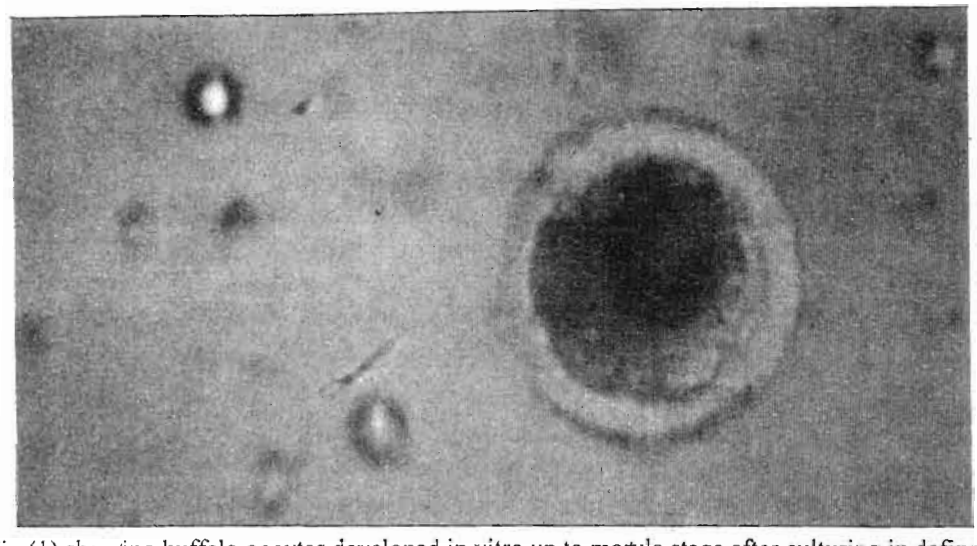

Fig (1) showing buffalo oocytes developed in vitro up to morula stage after culturing in defined media enriched with sodium citrate and myo inositol (X 400)

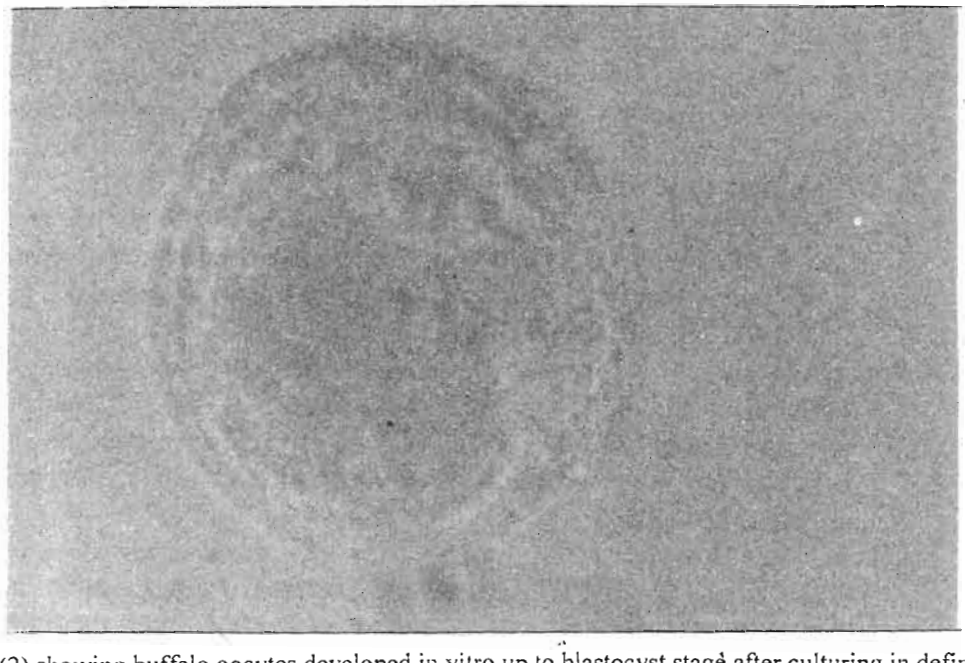

Fig (2) showing buffalo oocytes developed in vitro up to blastocyst stage after culturing in defined media enriched with sodium citrate and myo inositol (X 400)