IN VITRO MATURATION, FERTILIZATION AND DEVELOPMENT OF PREPUBERTAL AND MATURE BUFFALO OOCYTES

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SUMMARY

The competence of pre-pubertal buffalo heifers' oocytes for in vitro maturation, fertilization and development were studied and compared with that of the adult buffalo oocytes. Ovaries were collected from local abattoir. The oocytes were recovered from antral follicles (3-8 mm) in diameter by aspiration technique and cultured in TCM-199 medium supplemented with 10% fetal calf serum(FCS), 10 IU/ml PMSG , 20 IU/ml LH , 1 µg/ml Estradiol 17 β and 50 µg/ml Gentamycin sulfate and incubated in 5% CO_2 and high humidity for 24 h at 38.5°C.

Number of antral follicles per ovary were insignificantly higher on heifer's ovary (7.00 ± 0.74) comparing with adult buffalo cow's (6.45 ± 0.34). The recovered immature oocytes per ovary were higher, (P<0.001) in buffalo heifers than that in the adult ones (4.35 ± 0.28 and 2.99 ± 0.14, respectively). The competence of immature recovered oocytes to resume meiosis and reach metaphase II were lower (P<0.01) in heifer's ovaries (28.3%) compared to that of the adult cows (59.6%). The fertilized and developed oocytes reached the transferable stages were nearly the same rate in both adult buffalo cow and heifers (26.3 and 27.4%, respectively). Cleaved oocytes were insignificantly low in the heifer group (55.3%) from the initial cultured oocytes number compared with that of the adult group (64.5%). It can be concluded that the pre-pubertal heifers could be a good source for oocytes needed for IVF techniques.

Keywords: Buffaloes, heifers, IVF, Pre-pubertal, antral follicles

INTRODUCTION

Pre-pubertal heifers could be used as oocyte donors in breeding programs to decrease the generation interval and to increase the genetic rate of gain (Duby *et al*., 1996). Wave-like patterns of follicular development have been observed in heifer calves as early as two weeks of age (Evans *et al.*, 1994). The waves observed at eight months of age continued to first ovulation at 12 months (Adams *et al*., 1994). The diameters of dominant follicles of heifers show exponential increase with puberty hood (Bergfeld *et al.*, 1994 and Evans *et al*., 1994), meanwhile quality of the follicles were similar to those of adult cattle. Follicles failed to ovulate were explained by alterations in the patterns of secretion of estradiol, inhibin, or IGF-I (David *et al*., 2005).

Precocious puberty does occur in developing beef heifers; as many as 25% of heifers have transient luteal function before 300 d of age. However, exposure to a bull has no effect on the incidence of precocious puberty (Wehrman, 1996). Also, Gasser *et al*. (2006) found that early weaning and feeding on high energy concentrates, Precocious puberty (<300 d of age) occurred in 100, 56, and 50% of heifers in the EWH, EWC, and NW treatment crossbred, Angus and Simmental heifer.

It has been well established that bovine embryos can be produced successfully in vitro using oocytes from sexually immature animals (Kajihara *et al.*, 1991 and Armstrong *et al.*, 1992). This is important in genetic improvement programs in milk (Brem *et al*., 1995 and Lohuis, 1995) and beef (Brem, 1993) production systems. However, the potential use of calf ovaries collected from the abattoir for *in vitro* fertilization has not been thoroughly investigated.

The average age of puberty in buffalo heifers is about 37 months of age (Bashir, 2006), while, Ingole *et al.* (2001) reported that LH started to increase at the age of 15-18 months and FSH increased around 18-21 months of age suggesting that this increase leads to puberty. Schilloa *et al.* (1982) found that E_2 inhibited LH release in a dose dependent by influencing pulsatile mode of secretion, and that the threshold to negative feedback increased as heifers approach puberty. Ovarian maturation was accelerated in heifers weaned early and fed a high-concentrated diet (Gasser, 2006). Heifers were considered to have exhibited a precocious puberty when the onset of luteal function was before 300 d of age (Wehrman *et al.*, 1996). Number and characteristics of follicles in pre-puberty phase are varied in due to the variation in size of the ovarian reserve (Erickson, 1966). The maturation, fertilization and cleavage rates did not differ significantly between oocytes obtained from adult and pre-pubertal goats (Izquierde *et al*., 2002).

A great number of pre-pubertal buffalo heifers have been slaughtered in the Egyptian rural areas can play a good source for immature buffalo oocytes.

Therefore, comparing maturation, fertilization and development rate under *in vitro* condition, with adult ones is the objective of the current study.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center, Sakha, Khafer El-Sheikh belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. All chemicals used in this study were purchased from Sigma (Sant Luis, MO, USA), unless otherwise indicated. Incubation was run in CO_2 incubator (5% CO_2 at 38.5 °C and high humidity).

Ovaries were collected from slaughtered buffaloes of age between >2 and < 15 months (heifers) and polyparous cows (adult). Collected ovaries were placed in NaCl solution (9 mg/ml) containing 100 IU/ml penicillin and 100 µg/ml streptomycin sulphate) with tepmerature of 25-30 °C. Ovaries transported to the lab within 4 ± 0.5. hrs. The collected ovaries were washed twice in distilled water and once in freshly prepared saline.

Oocytes were recovered from follicles with >3-8 mm in diameter by aspiration technique, using 10 ml sterile disposal plastic syringe attached to 18-gauges needle. The contents of syringe were put slowly into 60 mm sterile plastic petri dish, then oocytes were examined under stereomicroscope. Both oocytes enclosed in a compact cumulus cells (COCs) and partially denuded oocytes(PD) with evenly granulated cytoplasm were selected and washed three times in Dulbecco's phosphate buffer solution (DPBS) medium. The total and aspirated antral follicles were counted and recorded for both buffalo heifers and cows. TCM-199 medium was supplemented with 10% Fetal calf serum(FCS), 10 IU/ml medium as PMSG (Gonaser, Laboratory Hipra, S.A.17170 Amer, Spain), 20 IU/ml medium LH (Chrionic gonadotropin produced by the nile company for pharmaceuticals and chemical industries, Cairo-ARE.R.C.C.115668), 1 µg/ml estradiol 17β and 50 µg/ml Gentamycin sulfate were used as maturation medium. The medium pH was adjusted at 7.3-7.4 and osmolarity at 280-300 mOsmol/kg. The medium was filtrated by 0.22-µm millipore filter. Each of 500 µl from prepared maturation medium was placed into each well of a four well dish and covered by sterile mineral oil. Before placing oocytes into culture dish, the medium was incubated for equilibration for 60 minutes. After evaluation, oocytes were washed three times in each of Phosphate buffer saline (PBS) plus 10% FCS and once in maturation medium. Thereafter, oocytes were placed in the maturation medium and incubated for 24 hrs.

After 24 hrs of maturation period, one quarter of matured oocytes were washed using PBS containing 1 mg /ml hyaluranidase to remove the cumulus cells. Then, oocytes were washed twice in PBS supplemented with 3% BSA and loaded on clean slide. Slides were placed into fixation solution (3 ethanol: 1 glacial acetic acid) overnight. Thereafter, oocytes were stained with 1 % orcein in 45% acetic acid and examined for nuclear maturation under phase-contrast microscopy using the criteria of Shamiah (2004) to determine stages of metaphase II (MII), oocytes with germinal vesicle (GV) and oocytes with germinal vesicle breakdown (GVBD) and oocytes at metaphase 1 (M1) and addition to degenerated oocytes

Fresh semen collected by artificial vagina from a buffalo bull were swim-up separated as described by Parrish *et al.* (1986) and diluted to 25×10^6 sperm / ml. Swim-up separated sperm diluted with IVF-TALP medium were incubated with 20 µg/ml heparin (heparin sodium) and 0.6% Bovine seum albumin(BSA) for 30 minutes in CO_2 incubator.

Matured oocytes were washed three times in TL-HEPES medium (Parrish *et al.,* 1989) and twice in fertilization medium (IVF-TALP). Oocytes were inseminated with capacitated semen (1.5×10^6 sperm/ml) and incubated for 22 hrs in CO2 incubator.

After co-incubation, presumptive zygotes were stripped of cumulus cells, washed three times in embryo culture medium, (TCM–199 medium supplemented with 20 mMol Na- pyruvate, 6 mg/ml BSA, and 50 µg/ml Gentamycin sulphate) and cultured in pre-equilibrated embryo culture medium in four well petri dishes and overlaid with sterile mineral oil, then incubated at 38.5 °C, 5% CO2 with 95% humidity (Eyestone and First, 1989). Half of the medium was replaced every 48 hour with fresh medium. Development of the fertilized oocytes and cleavage rates were recorded on day seven after fertilization. The data were statistically analyzed by one way ANOVA and t-test using SPSS Statistics 17.0.

RESULTS AND DISCUSSION

Number of antral follicles per ovary was insignificantly higher in the pre-pubertal heifer's ovaries compared to the adult buffalo cow's ovary. While, the recovered immature oocytes number were higher ($P<0.001$) in the pre-pubertal heifers than in the adult cows (Table 1). Similar results were found by Gasparrini *et al.* (2000) who reported that controlled follicular aspiration of abattoir-collected buffalo ovaries allows the retrieval of 2.4 good quality oocytes per ovary on average, in comparison with 10 good quality oocytes recovered in cattle (Gordon, 1994). Samad *et al.* (1998) found that incision technique for Nili buffalo ovaries crop was 3.85 oocytes /ovary compared with 1.76 for aspiration technique with no sig. difference in the maturation rate when the serum added was different; it ranged from 66.23 to 80%. Khalil *et al.* (2010) reported lower number of recovered oocytes / ovary (2.73) compared to the present results. The competence of immature oocytes to resume meiosis and reach metaphase II were lower ($P<0.01$) for oocytes recovered from ovaries of pre-pubertal

heifers compared to that of the adult (Table 2). Intermediate stages and the degenerated oocytes were higher (P<0.01) in the pre-pubertal heifers group indicating that more attention is needed for adjustment of the incubation time and hormones concentrations added to the culture medium. Khalil *et al.* (2010) found that buffalo oocytes maturation rate increased from 46% when incubated at 22 hrs to 81.4% when incubation time was increased to 26 hrs incubation. Culture medium, sera and hormones have effects on the buffalo oocytes maturation range, (Jamil *et al.*, 2007). Previous studies showed that follicles' growth could be stimulated with exogenous gonadotrophins. IVF rates (sperm penetration) are similar to those of oocytes recovered from adult cattle, cleavage and development rates are low suggesting that maturation of the ooplasm and/or nucleus is incomplete (Armstrong *et al.*, 1992 & 1993, Damiani *et al.*, 1995 and Duby *et al.*, 1996).

Table 1. Characteristics of ovarian follicles and oocytes collected from pre-pubertal heifer and adult buffalo cow's ovaries

Parameters	Heifers	Adult cows
Number of ovaries (n)	60	102
Total follicles counted (n)	420	658
Follicles/Ovary(X±SE)	7.0 ± 0.7	6.5 ± 0.3
Total recovered oocytes (n)	261	306
Recovered oocytes/ ovary(X±SE)	4.4 ± 0.3***	3.0 ± 0.1
Compact oocytes complex (N & %)	137 (52.5)	161 (52.6)
Partial denuded Oocytes (N & %)	75 (28.7)	62 (20.3)
Denuded oocytes (N & %)	49 (18.8)	83 (27.1)

*** Significant difference at P<0.001

Table 2. Maturation stages (number and %) of both pre-pubertal heifers and adult buffalo cows

Group	No of oocytes	GV n (%)	GVBD n (%)	MS n (%)	M11 n (%)	DG n (%)
Pre-pubertal heifers	60	3 (5.0)	3 (5.0)	20 (33.3)**	17 (28.3)	17 (28.3)**
Adult cows	99	6 (6.0)	10 (10.1)	17 (17.2)	59 (59.6)**	7 (7.1)

GV= Oocytes in germinal vesicle stage GVBD=Oocytes in germinal vesicle breakdown stage
MS=Oocytes in intermediate stages (From prophase to Anaphase-1) M11= Oocytes reached metaphase 11
DG= Degenerated oocytes ** significant difference at P<0.01

Table-3. Cleavage and different developmental stages of prepubetal heifers and adult buffalo cows

Group	Total Cultured oocytes	Cleaved Oocytes N %	2-cells n %	4-cells n %	8-16 cells n %	Morula n %	Blastocyst n %	Transferable Embryos n (%)
Prepubertal Heifers	152	84(55.26)	12(7.90)	16(10.52)	16(10.52)	20(13.16)	20(13.16)	40 (26.32)
Adult Cows	124	80(64.52)	22(17.74)*	16(12.90)	8(6.45)	16(12.90)	18(14.52)	34 (27.42)

*significant difference at p<0.05

Maturation rate of the pre-pubertal heifers' oocytes is lower but insignificantly than that of adult buffalo cow oocytes (Table 3). Abdoon *et al.* (2001) found that the cleavage rate was close to that obtained in the first (63.0± 0.5%). In cattle, Rizos *et al.* (2005) noticed higher proportion (P < 0.01) of cow oocytes than heifer oocytes reached the blastocyst stage (46.5% *versus* 33.4%). Brevini *et al* (2007) indicated the low rate of maturation and development competence to Cytoskeleton dynamics.

CONCLUSIONS

Results of the present study showed that, pre-pubertal heifers yielded around one third more of immature oocytes than the adult buffalo cows with a good potentiality to reach the transferable stages with insignificant difference from that recovered from the adult cows.

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الإنضاج والإخصاب والتطور الخارجى لبويضات عجلات الجاموس المُستخلصة من مبايض عجلات جاموسي فى مرحلة ما قبل البلوغ الجنسي ومن الجاموس البالغ

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تم دراسة مقدرة البويضات الجاموسى للعجلات ما قبل البلوغ للنضج والإخصاب والتطور ومقارنتها بمثيلاتها من الجاموس الناضج. جمعت البويضات بطريقة البذل من الحويصلات المبيضة ذات التجاويف مابين 3-8 مم قطر وزرعت فى بيئة زراعة الانسجة (TCM 199) المزودة 10 % من سيرم الاجنة البقرى, 10 وحدة دولية هرمون سيرم الفرس الحامل , 20 وحدة دولية من هرمون LH, 1 ميكروجرام من هرمون الإستراديول 17 بيتا , 50 ميكروجرام جنتاميسين لكل ملليلتر من البيئة المستخدمة وحضنت عند درجة حرارة 38.5 °C و 5% ثانى أكسيد الكربون ودرجة رطوبة عالية. وقد أوضحت النتائج ارتفاع غير معنوى إحصائيا لعدد الحويصلات المعدودة على مبيض العجلات الجاموسى قبل البلوغ مقارنة بمثيلاتها على مبيض الجاموس الناضج (0.74 ± 7.00 و 0.34 ± 6.45) بالتتابع. فى حين أن عدد البوضات المتحصل كان أكثر بمعنوية إحصائية ($p< 0.001$) فى العجلات ما قبل البلوغ عنه فى الجاموس الناضج (0.28 ± 4.35 و 0.14 ± 2.99 بالتتابع) لكل مبيض إلا ان مقدرة البويضات الغير ناضجة لبدأ الانقسام الميوزى والوصول إلى الطور الاستوائى الثانى للعجلات (28.3%)ما قبل البلوغ كان أقل بمعنوية إحصائية ($p<0.01$) عنه للجاموس الناضج (59.6%) وكذلك الأطوار الوسطية والبويضات المحطمة كانت أعلى بمعنوية إحصائية ($p<0.01$) فى المجموعة ما قبل البلوغ. على الرغم من انخفاض معدل انضاج البويضات فى المجموعة ما قبل البلوغ إلا ان إخصابها وتطورها ووصولها الى أطوار النقل كانت بنفس المعدل من مثيلاتها فى المجموعة الناضجة (26.32 و 27.42 % بالتتابع) , أيضا معدل الانقسام للجرثومة المفترضة كان منخفض بغير معنوية فى المجوعة ما قبل البلوغ عنه فى المجموعة الناضجة من اجمالى البويضات المزروعة من البداية للإنضاج (55.26 و 64.52 % بالتتابع) عنه فى المجموعة الناضجة. من هنا يمكن ان نخلص أن العجلات ما قبل البلوغ يمكن ان تكون مصدرا جيدا للبويضات غير الناضجة التى يمكن الاستفادة منها فى تقنية الاخصاب الخارجى.