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EFFECTS OF OOCYTE QUALITY, MATURATION MEDIA AND GONADOTROPINS ON INVITRO MATURATION OF BUFFALO OOCYTE

(With 3 Plates and 8 Figures)

By

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تأثير جودة البويضات، وأوساط النضج، والهرمونات المحفزة للمناسل على النضج الخارجى لبويضات الجاموس

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أجريت هذه الدراسة فى قسم الفسيولوجيا – كلية الطب البيطرى – جامعة قناة السويس وقسم التكاثر الحيوانى والتلقيح الاصطناعى – المركز القومى للبحوث بالقاهرة ، وذلك خلال المدة من أغسطس ٢٠٠٥ إلى يناير ٢٠٠٧ م. وكان الهدف من هذه الدراسة هو معرفة تأثير جودة البويضات وبيئات النضج المختلفة وكذلك تأثير الهرمون المحفز لنمو حويصلات جراف ، هرمون المشيمة الآدمى (منفردين أو مجتمعين) على نضج بويضات الجاموس معمليا. ولقد تم تجميع ١١٩٧ بويضة من ٥٩١ مبيض جمسى من مجزر المنيب بالجيزة. وتم عد الحويصلات على سطح المبيض وحساب عدد الحويصلات للمبيض الواحد ، وكذلك تم تقسيم البويضات على حسب ظهور خلايا التراكم المبيضى حولها إلى ممتازة ، جيدة ، مقبولة ، ورديئة (معراة) الجودة. وتم تحضين البويضات فى حضانة مزودة بـ ثانى أكسيد الكربون لمدة ٢٤ ساعة عند درجة حرارة ٣٨°م سواء فى بيئة mSOF أو TCM-199 مزودة بالمعاملات الهرمونية التالية: الهرمون المحفز لنمو حويصلات جراف ، هرمون المشيمة الآدمى ، وكذلك الهرمون المحفز لنمو حويصلات جراف + هرمون المشيمة الآدمى. تم تقييم نضج البويضات عن طريق تمدد خلايا التراكم المبيضى ونضوج الأنوية. وأظهرت النتائج ما يلى: ١٧٤ بويضة من اجمالى ٢٥٨ ممتازة الجودة وصلت الدرجة الثالثة من تمدد خلايا التراكم المبيضى بنسبة ٦٧,٤% ، ١٤٣ بويضة من اجمالى ٢٤٩ جيدة الجودة وصلت الدرجة الثالثة من تمدد خلايا التراكم المبيضى بنسبة ٥٧,٤% ، ١٤٦ بويضة من اجمالى ٣٠٩ مقبولة الجودة وصلت الدرجة الثالثة من تمدد خلايا التراكم المبيضى بنسبة ٤٧,٢%. أما ٢٨٠ بويضة رديئة الجودة فلم يحدث بها أى نوع من التمدد. ولقد الفروق معنوية جداً. كانت نسبة نضج الأنوية فى البويضات ممتازة وجيدة الجودة ٨٣,٩% و٧٥,٦% على التوالى ، فى حين أنها كانت ٧١,٢% و ٥٩,٥% فى الويضات مقبولة ورديئة الجودة على التوالى ، وكانت الفروق معنوية جداً. أوضحت النتائج وجود فروق

معنوية عالية لتمدد خلايا التراكم المبيضى من الدرجة الثالثة باستخدام بيئة mSOF عنها فى بيئة TCM-199 بنسبة (٥٥,٧% و ٣٩% على التوالى) فى حالة اضافة هرمون FSH ، أما فى حالة هرمون FSH+hCG فقد كانت النسبة ٥٧,٨% و ٢٦% على التوالى. لقد أعطت بيئة TCM-199 فروق معنوية عالية لنضج الأنوية عنها فى بيئة mSOF بنسبة (٧٤,٢% و ٦٧,٥% على التوالى) عند استخدام FSH. ولم يلاحظ أى فروق معنوية بين البيئتين عند استخدام FSH+hCG. وبالنسبة لتأثير الهرمونات فلقد أظهرت الدراسة أن هرمون FSH أعطى أعلى نسبة من تمدد خلايا المبيض التراكمية (٣٩% للدرجة الثالثة) فى حين كانت النسبة ٣٢,٤% لـ hCG ، ٢٦% لـ FSH+hCG وذلك باستخدام بيئة TCM-199. أما باستخدام بيئة mSOF فلم تلاحظ أى فروق معنوية بين المعاملات الهرمونية الثلاثة. وبالنسبة لنضج تالأنوية فلقد أظهرت الدراسة أن هرمون FSH+hCG أعطى أعلى نسبة (٧٩,٥%) عن هرمون FSH(٧٤,٢%) وهرمون hCG (٦٢,٩%) عند استخدام بيئة TCM-199 ، وبالنسبة لبيئة mSOF فلقد كانت النتائج متشابهة إلى حد كبير جداً. ومما سبق نخلص الى: ١- يتأثر الانضاج المعملى لبويضات الجاموس بجودة البويضات وبيئة النضج واضافة الجونادوتروبين. وساعدت بيئة mSOF على تمدد خلايا التراكم المبيضى أكثر من بيئة TCM-199 بينما ساعدت بيئة TCM-199 على نضج الأنوية أكثر من بيئة mSOF. ٢- أعطى الاتحاد الهرمونى FSH+hCG أفضل نتائج لتمدد خلايا التراكم المبيضى ونضج الأنوية. وعموما يعتبر استخدام FSH+hCG مع بيئة TCM-199 هوالأفضل للحصول على أعلى معدل لنضج البويضات معمليا.

SUMMARY

This study was carried out to investigate the effect of oocyte quality and different culture media as well as hormones on in vitro maturation of buffalo oocytes. A total of 1197 oocytes were collected from 591 ovaries obtained from a slaughterhouse. The number of follicles on ovarian surface was counted and the number of follicles / ovary was calculated. Oocytes were aspirated and the number of recovered oocytes per ovary was calculated. The recovery rate was determined. Oocytes were classified according to the appearance of cumulus cells into excellent, good, fair and poor. They were cultured either in TCM-199 or mSOF with different hormonal supplements (FSH, hCG or combination of both) for 24 h in CO_2 incubator at 38 ^{o}C. Assessment of maturation by cumulus cell expansion and nuclear maturation was carried out. The results revealed that 174 out of 258 excellent oocytes reached G3 with a percentage of 67.4%, 143 out of 249 good oocytes reached G3 with 57.4%. and 146 out of 309 fair oocytes reached G3 with 47.2% while all 280 poor (denuded) oocytes did not reach G3. Excellent oocytes exhibited higher significant ($P<0.01$) value than the other qualities. The percentage of nuclear maturation of excellent and good oocytes were

83.9% and 75.6%, respectively while that of fair and poor oocytes were 71.8% and 59.5%, respectively. The maturation rate for excellent quality oocytes is significantly (P<0.01) higher than the other qualities. Buffalo oocytes that reached G3 of cumulus cells expansion in case of FSH addition were 55.7% and 39% for mSOF and TCM-199, respectively while in case of FSH + hCG addition the ratios were 57.8% and 26.7%, respectively. The differences were highly significant (P<0.01). Concerning nuclear maturation, TCM-199 exhibited a higher significant (P<0.01) maturation rate than mSOF medium using FSH hormone (74.2% and 67.5%, respectively). There was no significant difference of maturation rate between TCM-199 and mSOF media using FSH plus hCG. Concerning the effect of hormones on buffalo oocytes, the study revealed that FSH gave higher (P<0.05) G3 cumulus cell expansion (39%) than hCG (32.4%) and FSH + hCG (26%) in case of TCM-199 medium. No significant differences were observed between hormonal treatments in case of mSOF medium. FSH + hCG treatment gave higher (P<0.01) nuclear maturation rate (79.5%) than FSH (74.2%) and hCG (62.9%) in TCM-199 medium. Parrallel results were observed in case of mSOF medium. We conclude that: 1- mSOF enhanced cumulus expansion more than TCM-199, while TCM-199 enhanced nuclear maturation of buffalo oocytes than mSOF. 2- FSH + hCG is the best combination of gonadotropins that enhanced both cumulus expansion and nuclear maturation rate of buffalo oocytes.

Key words: *Buffalo, oocytes, maturation, cumulus cells, FSH, hCG, TCM-199 and mSOF.*

INTRODUCTION

Buffaloes are known to suffer from many inheritant problems, as delayed maturity, silent heat particularly during summer season, long post partum period before return of estrus and low conception rates. The low reproductive efficiency could be related to the low number of follicles in the ovary; poor superovulatory response and high percentage of atretic follicles (Misra *et al.*, 1991). Efforts have been initiated to augment the reproductive potential of these animals using biotechnology (Madan *et al.*, 1994). Producing embryos by in vitro fertilization can be done based on three subsequent techniques: *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) of matured oocytes and then in vitro culture (IVC) of fertilized oocytes for cleavage up to blastocyst stage (Goswami *et al.*, 2004). The culture medium and selection of protein supplements and hormones for IVM play an important role in the

subsequent maturation rate, and embryonic development following IVF (Bavister *et al.*, 1992). Several factors such as addition of FSH, LH and their combination to culture media have to be considered for maximizing success (Fuki and Ono, 1989).

Oocyte maturation is defined as reinitiation of the first meiotic division, progression to metaphase II, and the accompanying cytoplasmic processes occurring within the oocyte that are essential for fertilization supporting early embryo development (Gary, 2001). Maturation rate of the oocytes is assessed by various methods (e.g. staining method (MII stage), degree of expansion of cumulus cells, and identification of extruded first polar body in the perivitteline space (Totey *et al.*, 1992 and Das *et al.*, 1996). The criteria used for selecting ideal IVM oocytes was: (1) the surrounding cumulus cells were fully expanded without evidence of degeneration of ooplasm, (2) the dimension of perivitelline space was increased, and (3) the first polar body extruded into that space (Nandi *et al.*, 2002).

Expansion of cumulus cells depends largely on the media used for maturation of the oocytes (Nandi *et al.*, 2002). As a basic medium, tissue culture medium (TCM-199) has been used for maturation of buffalo oocytes either exclusively or in a modified way through chemical and biological supplementation (Hammam *et al.*, 1997). TCM-199, commonly used for bovine IVM, contains both glutamine and glucose (Michele *et al.*, 2003). Therefore in the absence of serum, adequate concentrations of glucose and glutamine might be necessary for FSH to induce cumulus expansion in vitro. Raza *et al.* (2001) revealed that TCM-199 resulted in a significantly high maturation rate (73.3%). Ali (2004) reported that the maturation percentage of buffalo oocytes using mSOF was (87.6 ± 4.17) which was lower than that obtained by using TCM-199 medium with growth factors or hormones (96.1 ± 1.97% and 93.9 ± 2.29%, respectively). Similar results were reported by Barakat (2005) where TCM-199 medium exhibited higher maturation rate (77.6 ± 0.9) than mSOF medium (40.9 ±0.7).

Gonadotropins, either administrated to animals prior to harvesting of oocytes or added in vitro to maturation media, enhanced bovine oocyte quality as shown by improved completion of nuclear maturation (95-100% matured oocytes), fertilizability, and developmental ability (Eppig 1991). The role of FSH may be to enhance the expansion of cumulus cells surrounding the oocyte which in turn enhances sperm capacitation and the fertilization process (Totey *et al.*, 1993). IVM of bovine cumulus oophorus cells in serum free medium

supplemented with 0.05 IU/ml hCG or 0.05 IU/ml hFSH, showed a beneficial effect of FSH on oocyte maturation and blastocyst formation, while no effect of hCG could be observed (Bevers *et al.*, 1997).

The addition of FSH to IVM medium (TCM-199) in buffalo, stimulated nuclear maturation (79% mature oocytes compared to control 47%) (Palta and Chauhan, 2003). The effect of FSH on nuclear maturation and cumulus expansion of bovine oocyte was dependent on substrates present on IVM medium (Ali and Sirard, 2002). The aim of the study was directed to:

1- Demonstrate the effect of oocyte quality on in vitro maturation of buffalo oocytes.
2- Clarify the effect of culture media (TCM-199 or mSOF) on in vitro maturation of buffalo oocytes.
3- Investigate the effect of addition of gonadotropins (FSH, hCG or combination of both) on in vitro maturation of buffalo oocytes.

MATERIALS and METHODS

The present study was carried out in the Department of Physiology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia and the Department of Animal Reproduction and Artificial Insemination, Veterinary Division, National Research Center, Cairo. It was conducted during the period from August 2005 till January 2007. All chemicals used in the present study were purchased from Sigma Company (Saint Louis, MO, USA), unless otherwise mentioned.

Ovaries collection

Ovaries from adult Egyptian buffaloes were collected once a week from El-Moneib slaughterhouse at Giza and transported in an insulated thermos (at 32-35°C) containing sterile normal saline solution (NSS, 0.9% NaCl) supplemented with 100 I.U/ml penicillin and 100 µg/ml streptomycin. The ovaries were transported to the laboratory within 3 hours of slaughtering. Upon arrival, they were washed several times in warm NSS at 37°C until obtaining clear saline free from blood and then kept in water bath at 37°C during oocyte collection.

Aspiration media:

HEPES buffered tissue culture medium (HTCM-199, catalogue number M-2520) supplemented with 6 mg/ml bovine serum albumin (BSA) and 50 µg/ml gentamycin was used for aspiration of oocytes.

Basic maturation media

Tissue culture medium 199 (Cat. 2415) and Modified synthetic oviductal fluid (mSOF) were used in maturation of buffalo oocytes.

MSOF was prepared according to Holm *et al.* (1994). All media were filtered using 0.2 μm (Milipore, USA) syringe filter and incubated for at least 2 hrs in a humidified atmosphere (95%) under 5% CO_2 at 38°C before culturing of the oocytes.

Oocyte yield and recovery rate of buffalo ovaries

The number of follicles was counted on the surface of each ovary, and then the number of follicles/ovary was calculated. Cumulus oocyte complexes (COCs) were aspirated by using 18-gauge needle attached to 10 ml disposable syringe containing 1 ml aspiration medium. The follicular fluid containing oocytes were pooled into a sterile 15 ml plastic Falcon tube (Falcon, USA) and allowed to settle for 15 min in water bath at 37°C. After settling, the sediment was aspirated and placed in 10 cm diameter sterile polysterene Petri dish (Nunclon, Denmark) containing 5 ml aspiration medium. The number of recovered oocytes was counted under stereomicroscope (Nakamura, Japan) at X 32. The number of oocytes / ovary was calculated. The recovery rate was determined by dividing the number of recovered oocytes over the number of follicles counted in buffalo ovaries.

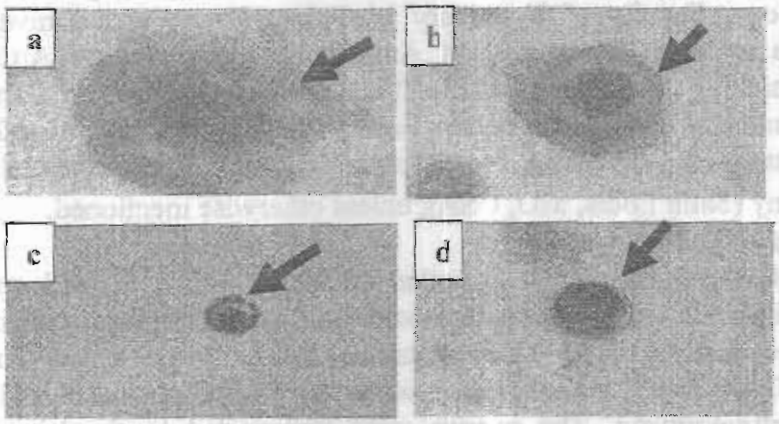

Plate 1: Showing buffalo oocyte quality.
(a): Excellent quality (cumulus cell layers more than 4), **(b):** Good quality (cumulus cell layers 2-3), **(c):**Fair quality (one cumulus cell layer), and **(d):** Denuded or poor quality devoid of cumulus cells.

Oocyte quality from buffalo ovaries

Quality of the recovered COCs (Plate 1) was determined according to (Furnus *et al.*, 1997), under stereomicroscope at 32 X into:

a- Excellent oocytes which were surrounded by compact and dense cumulus cell layers (≥ 4 layers) and homogenous evenly granular ooplasm (No. = 258).

b- Good oocytes which were surrounded by two- three layers of cumulus cells and homogenous evenly granular ooplasm (No. = 249).

c- Fair oocytes which were surrounded by one layer of cumulus cells (No. = 309).

d- Denuded or poor oocytes which were completely devoid of cumulus cells around them and uneven ooplasm.

$$\text{Oocyte quality yield} = \frac{\text{No. of recovered oocytes for this grade}}{\text{Total number of oocytes recovered in the trial}} \times 100$$

Effect of oocyte quality on buffalo oocyte maturation

COCs were washed two times by HEPES buffer TCM-199, 6% BSA and gentamycin 50 µg/ml. They were washed again by maturation medium (pH 7.2-7.4) and finally each class of COCs was cultured in a separate well of a four-well culture plate (Nunclon, Denmark) containing maturation medium for 24 h at 38°C under 5% CO2. After 24 hrs, maturation rate was assessed on the basis of the following:

1. Cumulus cells expansion

The criteria used for assessing the degree of cumulus cells expansion (Plate 2) as described by Abd El- Kader (2005) under stereomicroscope were: Grade 0 (Go): no expansion, Grade 1 (G1): few expansions of cumulus layers, Grade 2 (G2): moderate expansions of cumulus layers and Grade 3(G3): full expansion of cumulus layers.

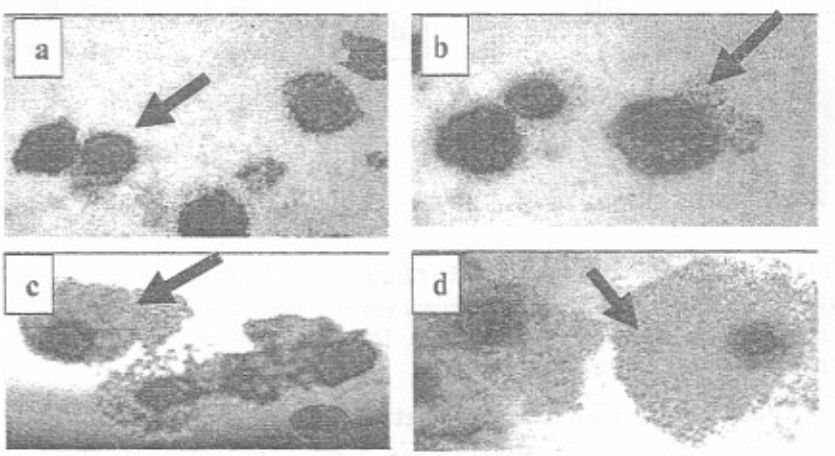

Plate 2: Showing the degree of cumulus cells expansion
(a): G0 showing no expansion. **(b):** G1 showing few expansions.
(c): G2 showing moderate expansions. **(d):**G3 COC showing full expansion.

2. Nuclear maturation

After assessment of cumulus cells expansion, the first polar body was determined in the perivetteline space when the oocytes were denuded after maturation. The cumulus cells of oocytes were removed with repeated pipetting using 100 µl pipette. The oocytes were fixed for 48 hrs with acetic acid and ethanol (1: 3) (Totey *et al.*, 1993) and stained with aceto-orcein stain 1% (Merck, CAS-NO.1400-62-0) according to Uguz *et al.* (1994). The numbers of excellent, good, fair and poor oocytes were 174, 186. 202 and 210 were, respectively. These oocytes were evaluated (Plate 3) according to Abd El- Kader (2005) under microscope into: a- Immature oocytes: at either germinal vesicle stage (GV) or germinal vesicle break down stage (GVBD). b- Mature oocytes: Anaphase (An), Telophase (T) and Metaphase II (MII with polar body) stage. c- Unidentified or degenerated. Nuclear maturation rate (NMR) = No. of mature oocytes / total No. of oocytes x 100.

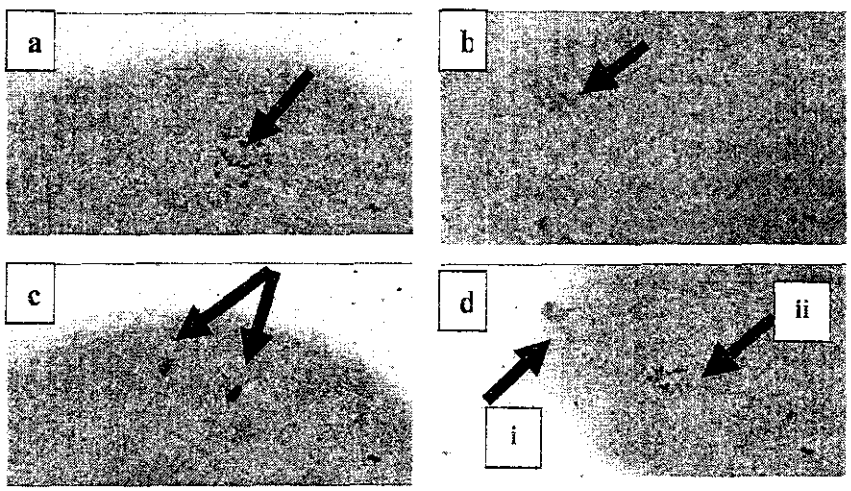

Plate 3: Showing buffalo oocyte nuclear maturation
(a): Germinal vesicle stage. **(b):** Germinal vesicle breakdown stage. **(c):** Telophase stage. **(d):** Metaphase II stage: **i**-First polar body chromosomes. **ii**-Oocyte chromosomes.

Effect of media on buffalo oocytes maturation

A total number of 466 COCs were matured in TCM-199 medium supplemented either with FSH or FSH plus hCG. A total of 442 COCs were matured in mSOF medium supplemented either with FSH or FSH plus hCG. After 24 h from incubation, assessment of maturation was carried out by cumulus cells expansion. A total 315 oocytes for each media were evaluated for nuclear maturation.

Effect of hormones on buffalo oocytes maturation

A total of 654 COCs were maturated using TCM-199 medium with the following hormonal treatments:

- 231 COCs were matured in TCM-199 supplemented with 10 µg/ ml FSH, 10% Fetal calf serum (FCS) and 50 µg/ ml gentamycin.
- 188 COCs were matured in TCM-199 supplemented with 10 IU hCG (Pregnyl, El Nile Co, Egypt) + 10% FCS + 50 µg/ ml gentamycin.
- 235 COCs were matured in TCM-199 supplemented with 10 µg/ml FSH + 10 I.U/ml hCG + 10% FCS and 50 µg/ ml gentamycin.

A total of 442 COCs were matured using mSOF medium supplemented with the following hormonal treatment:

- 219 COCs were matured in mSOF medium supplemented with 10 µg/ml FSH + 10% FCS and 50 µg/ ml gentamycin.
- 223 COCs were matured in mSOF medium supplemented with combination of FSH and hCG in the same previous doses +10% FCS and 50 µg/ml gentamycin.

The obtained data were analyzed statistically by Chi-square test using Minitab statistical program release 8, (1991).

RESULTS

Oocyte yield and recovery rate from buffalo ovaries:

A total of 1872 follicles were collected from 591 buffalo ovaries with an average of 3.3±0.21 follicle / ovary. The recovery rate was 64.6±2.21. A total of (1197) oocytes were recovered from 591 buffalo ovaries giving an average of 2.1±0.14 oocyte / ovary.

Oocyte quality from buffalo ovaries:

The buffalo oocyte quality was classified into excellent, good, fair and poor according to the number of layers of COCs. Excellent and good were 25.16±1.51% and 25.28±1.94% respectively. The other low quality grades (fair and poor) were 26.05±1.6% and 23.56±2.42% respectively. The number of oocytes for excellent and good qualities were 306 and 290 with a mean of 16.11±1.83 and 15.26±1.25 (per ovary), respectively. On the other hand the number of oocytes for fair and poor quality were 321 and 280 with a mean of 16.89±1.73 and 14.74±1.98 respectively.

Effect of oocyte quality on cumulus cells expansion of buffalo oocytes:

Figure 1 demonstrates that 174 (out of 258) excellent oocytes reached G3 with a percentage of 67.4%, 143 (out of 249) good oocytes with a percentage 57.4%, 146 (out of 309) fair oocytes with a percentage

of 47.2% and 0 (out of 280) poor oocytes with a percentage of 0.0%. The excellent oocytes exhibited a higher significance (P<0.01) value than the other qualities.

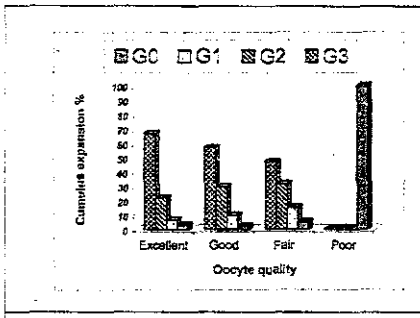

Fig. 1: Effect of buffalo oocyte quality on cumulus cells expansion of buffalo oocytes

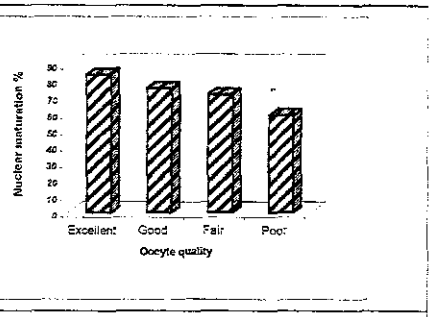

Fig. 2: Effect of buffalo oocyte quality on nuclear maturation

Effect of oocyte quality on nuclear maturation of buffalo oocytes:

Figure 2 demonstrates that the numbers of stained excellent and good oocytes were 174 and 168 from which 146 and 127 were matured with maturation rate 83.9% and 75.6%, respectively. The numbers of fair and poor oocytes were 202 and 210 from which 145 and 125 were matured with maturation rate 71.8% and 59.5%, respectively. The nuclear maturation rate of excellent quality oocytes was significant (P<0.01) higher than the other qualities.

Effect of maturation media on cumulus cell expansion of buffalo oocytes:

Figure 3 demonstrates that using TCM-199 and mSOF media resulted in 39% and 55.7%, respectively maturation of oocytes (G3 cumulus cell expansion) in case of FSH hormone supplementation. The difference was highly significant (P<0.01). In case of using FSH+hCG hormone supplementation the expansion (G3) ratios were 26.0% and 57.8% for TCM-199 and mSOF media, respectively (Figure 4). The difference was highly significant (P<0.01).

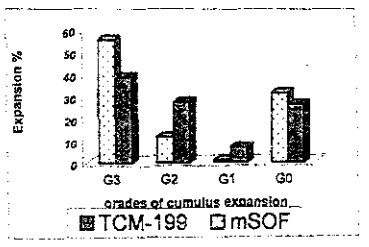

Fig. 3: Effect of media on cumulus expansion of buffalo oocytes using FSH

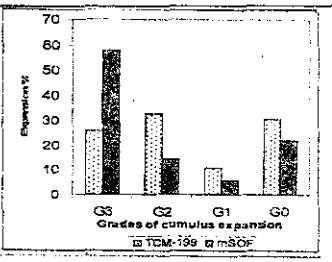

Fig. 4: Effect of media on cumulus expansion of buffalo oocytes using FSH plus hCG hormones

Effect of media on nuclear maturation of buffalo oocytes:

Nuclear maturation rates were 74.2% and 67.5% for both TCM-199 and mSOF media, respectively in case of using FSH (Figure 5). The difference was highly significant (P<0.01). The maturation rates were 79.5% and 74.7% for TCM-199 and mSOF media, respectively in case of using FSH + hCG (Figure 6) without significant differences.

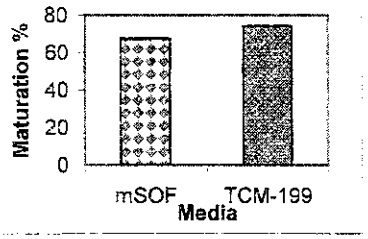

Fig. 5: Effect of maturation media on nuclear maturation of buffalo oocytes using FSH

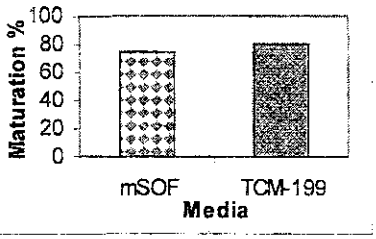

Fig. 6: Effect of maturation media on nuclear maturation of buffalo oocytes using FSH+hCG

Effect of hormones on cumulus cells expansion of buffalo oocytes:

Figure 7 demonstrates that the percentages of cumulus cells expansion (G3) of *in vitro* matured buffalo oocytes were 39%, 32.4% and 26.0% for FSH, hCG and FSH + hCG, respectively in case of TCM-199 medium. The results of FSH were significantly (P<0.01) higher than the others. In case of mSOF, the percentages were 55.7% and 57.8% for FSH and FSH + hCG, respectively without significant differences.

Effect of hormones on nuclear maturation of buffalo oocytes:

Nuclear maturation rates were 74.2%, 62.9% and 79.5% for FSH, hCG and FSH+hCG, respectively in case of TCM-199 (figure 8). The differences were highly significant (P<0.01). In case of mSOF, the rates were 67.5% and 74.7% for FSH and FSH + hCG, respectively. The difference was significant (P<0.05).

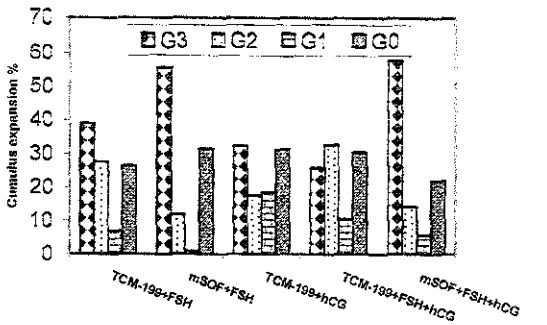

Fig. 7: Effect of hormones on buffalo oocytes cumulus cells expansion

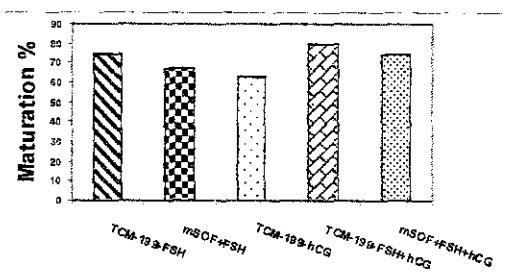

Fig. 8: Effect of hormones on nuclear maturation of buffalo oocytes

DISCUSSION

Many factors affect in vitro maturation of buffalo oocytes to produce offsprings in vitro after transfering them from the slaughterhouse. These factors are either the selection of proper maturation medium, the quality of oocytes or the hormones added (Bavister, *et al.,* 1992). The present study dealt with in vitro maturation technique using two media (TCM-199 and mSOF) supplemented with three hormone treatments (FSH, hCG and their combination).

Effect of oocyte quality on invitro maturation of buffalo oocytes:

The present study revealed that the percentage of oocytes that reached full expansion was 67.4%, 57.4, 47.2 and 0.0% for excellent, good, fair and poor quality oocytes, respectively (Figure 1). The highest rate of cumulus cell expansion with the excellent quality oocytes is due to the number of layers of cumulus cells surrounding the oocytes. The poor quality oocytes that were devoid of cumulus cells showed no expansion at all. These results are in agreement with those of Abd El-Kader, (2005), Barakat, (2005) and Zoheir, (2005) who reported

a significant positive correlation between the number of excellent and good quality oocytes and the number of oocytes that showed full expansion (G3). The explanation of our results may be due to the participation of oocytes for cumulus cell to synthesize hyaluronic acid and undergo cumulus expansion *in vitro* (Buccino *et al.*, 1990). The role of cumulus cells in providing nutrients to the oocyte during its growth, to participate in the zona formation and following the LH surge to synthesize the matrix composed of proteins and hyaluronic acid was reported by Bedford and Kim, (1993).

The present study revealed that nuclear maturation rate of *in vitro* matured buffalo oocytes was higher in excellent quality (83.9%) oocytes followed by good (75.6%) when compared with fair and poor oocytes (71.8% and 59.5%, respectively) (figure 2). These results are similar to those of Leibfried-Rutledge, *et al.* (1989) who found that only 44% of denuded oocytes matured *in vitro* compared with 71% of oocytes with cumulus cells. The study of Warriach and Chohan (2004) revealed that buffalo oocytes with homogenous cytoplasm and surrounded by compact layers of cumulus cells had a significantly higher maturation rate than oocytes with partial remnants or no cumulus cells. These findings indicate that the presence of cumulus cells is necessary for *in vitro* nuclear maturation and helps oocytes to reach metaphase II. Physical contact between oocyte and cumulus cells is necessary for the transfer of nutrients and other factors essential for oocyte development (Albertini *et al.*, 2001). Cumulus cells enclosing the oocyte are responsible for the recognition and utilization of oocyte to the added compounds such as glucose and glutamine (Downs and Verhoeven, 2003).

Effect of media on invitro maturation of buffalo oocytes:

Expansion of cumulus cells depends largely on the media used for maturation of the oocytes (Nandi *et al.*, 2002). The present study revealed that the percentage of oocytes that reached full cumulus cell expansion was 39.0% and 55.7% for TCM-199 and mSOF media, respectively when using FSH (Figure 3). On using FSH plus hCG the percentages were 26.0% and 57.8% for TCM-199 and mSOF, respectively (Figure 4). These results disagree with those of Ali (2004) and Barakat (2005) who reported that TCM-199 medium was effective in cumulus expansion than mSOF medium. The study of Zhang and Armstrong (1990) revealed that supplementation of amino acids to the maturation medium (as in mSOF) may increase the pool size of endogenous amino acids and denovo protein synthesis.

The present study revealed that the maturation rate using FSH hormone was 74.2% and 67.5% for TCM-199 and mSOF media, respectively (Figure 5), while with FSH + hCG, it was 79.5% and 74.7% for TCM-199 and mSOF, respectively (Figure 6). These findings are parallel with those reported by Ali, (2004) and Barakat, (2005). The difference in maturation percentage between TCM-199 and mSOF may be attributed to the composition of the media (Nandi *et al.*, 2002). TCM-199 contains both glutamine and glucose (Michele *et al.*, 2003). Presence of glucose is essential to generate ATP via glycolytic metabolism, while glutamine can feed into tricarboxylic acid cycle and serves as a potential energy source (Downs and Verhoeven, 2003).

Effect of hormones on invitro maturation of buffalo oocytes:

The present study revealed that the percentage of oocytes showing full cumulus cell expansion was 39.0%, 32.4% and 26.0% for FSH, hCG and FSH + hCG, respectively on using TCM-199 medium (Figure 7), while was 55.7% and 57.8% for FSH and FSH + hCG hormones, respectively for mSOF medium (Figure 7). These results agree with those of Totey *et al.*, (1992). On the other hand, they are lower than those obtained by Nandi *et al.* (2003) and Ali *et al.* (2004). FSH and hCG increase the use of cAMP system as an intracellular second messenger (Bevers *et al.*, 1997) and thus increase the level of enzyme activity for hyaluronic acid sythesis and induce cumulus expansion in intact complexes (Buccino *et al.*, 1990).

The present study revealed that the maturation rate using TCM-199 medium was 74.2%, 62.9% and 79.5%, for FSH, hCG and FSH + hCG, respectively (Figure 8). In case of mSOF medium it was 67.5% and 74.7% for FSH and FSH + hCG, respectively. These results agree with those reported by (Ali, 2004 and Abd El- Kader, 2005). It was observed that the addition of FSH to in vitro maturation medium stimulated cumulus cells of oocyte-cumulus complexes to secrete a positive factor that could override arrest due to hypoxanthine and could trigger meiotic resumption. (Choi *et al.*, 2001). The authors added that LH increased glycolysis and glucose oxidation in oocyte-cumulus complexes and glutamine oxidation in mature oocytes denuded after LH exposure.

In conclusion:

1- In vitro maturation of buffalo oocytes was affected by oocyte quality, maturation media and addition of gonadotropins. mSOF enhanced cumulus expansion more than TCM-199, while TCM-199 enhanced nuclear maturation of buffalo oocytes than mSOF.

2- FSH + hCG is the best combination of gonadotropins that enhanced both cumulus expansion and nuclear maturation rate of buffalo oocytes.

So collectively the best combination to be used to improve buffalo oocyte *in vitro* maturation is TCM-199+FSH +hCG.

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