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# MORPHOLOGICAL AND HISTOLOGICAL CLASSIFICATIONS OF BUFFALO OVARIAN FOLLICLES AND THE ABILITY OF THEIR ENCLOSING OOCYTES TO MATURE AND DEVELOPE IN VITRO.

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## SUMMARY

The developmental competence of buffalo oocytes in vitro is much lower than that of cattle. This could be due to factors related to the quality of the oocytes. Two experiments were conducted to evaluate (1) the relationship between macroscopic, microscopic characteristics of buffalo ovarian follicles and the quality of cumulus-oocytes complexes (COCs), and (2) to study the ability COCs retrieved from these follicles to mature and develop in vitro to the blastocyst stage. In experiment 1: follicles (3 to 8 mm in diameter) were dissected from buffalo ovaries and classified according to their macroscopic appearance into 3 groups; clear vesicular, intermediate and opaque. Under stereomicroscope, follicles from the 3 groups were punctured using fine needle and COCs were collected and classified into 3 categories (good, fair and poor quality). Follicular wall was fixed in formol saline (10%) for histological examination and COCs were fixed in 2.5% glutaraldehyde and processed for semi-thin section to evaluate the oocyte quality In experiment 2, good and fair quality oocytes were collected as in experiment 1 and in vitro matured in TCM medium supplemented with 10% fetal calf serum (FCS) + 10 µg/ml FSH + 10 IU/ml hCG + 50 µg/ml gentamycin. Maturation was performed for 24 h under 5% $CO_2$ in humidified air. After maturation, 50 oocytes from each group were fixed in 1:3 acetic:ethanol and stained with 1% orcein to evaluate the nuclear maturation (metaphase II stage). The rest of matured oocytes were fertilized using frozen-thawed buffalo

spermatozoa and cultured for 8 days for subsequent development to the blastocyst stage. Results indicated that clear vesicular and intermediate follicles produced a significantly higher percentage of good quality oocytes compared with opaque follicles (P<0.01). Clear vesicular follicles were surrounded with ≥ 7 layer of granulose cells, while opaque follicles possesses more than 90% apoptotic cells and produced higher percentage (P<0.01) of poor quality oocytes. The highest percentage of COCs reaching M II was achieved when oocytes were collected from clear vesicular and intermediate follicles. Also, significantly higher (P<0.01) percentage of oocytes was developed to the morula and blastocyst stages for oocytes recovered from clear vesicular and intermediate follicles. In conclusion, macroscopic evaluation of ovarian follicles in buffalo is a simple and non invasive technique that might assist the in vitro production of buffalo embryos.

## INTRODUCTION

In vivo, the oocytes to be fertilized are donated from healthy follicle during estrus phase of the cycle. While, the COCs collected for in vitro embryo production are obtained from follicles regardless follicular phase or stage of the estrous cycle. The ability to identify good-quality follicles and oocytes for in vitro embryo production is important particularly in buffaloes. Macroscopic appearance of bovine follicles dissected free of ovarian tissue and transilluminated, was found to be correlated with the histological indications of atresia in cattle (Kruip & Dielman, 1985). Moreover, there was a high correlation between the follicle quality and the distribution of the COC quality (de Wit et al., 2000). Follicles with a low degree of atresia contained a relatively high percentage of COC-A (good quality). While, oocytes recovered from opaque follicles are more likely to be strongly atretic than those from clear or intermediate follicles (Grimes et al., 1987; Wurth & Kruip, 1992). In buffaloes, oocytes surrounded by multi-layers of cumulus-cells had a significantly higher in vitro maturation, fertilization and developmental rates than those partially surrounded or denuded oocytes (Abdoon et al., 2001; Nandi et al., 1998).

To our knowledge there are no available literatures on the relationship between follicle quality and the developmental competence of COCs in buffaloes. Therefore, the present work was conducted to evaluate; (1) the relationship between macroscopic, microscopic characteristics of buffalo ovarian follicles and the quality of cumulus-oocytes complexes (COCs), and (2) to study the ability of COCs

retrieved from those follicles to mature and develop in vitro to the blastocyst stage.

## MATERIALS AND METHODS

All chemicals used in the present study are Sigma grade and purchased from Sigma-Aldrich (Sant. Louis, Mo, USA).

### Ovary collection

Ovaries were collected from non-pregnant buffaloes at a local abattoir and placed in phosphate buffered saline (PBS) within 30 min of slaughter, and transported to the laboratory within 2 h of slaughter. All ovaries were processed within 3 to 4 h of collection.

### Experimental design

Experiment 1: Relationship between macroscopic, microscopic characteristics of buffalo ovarian follicles and the quality of cumulus-oocytes complexes (COCs)

#### 1.1. Macroscopic and microscopic evaluation of buffalo ovarian follicles

Follicles 3 to 8 mm in diameter (n= 647) were dissected free from the ovarian stroma in PBS+ 4 mg/ml BSA under a stereomicroscope using fine forceps. Dissected follicles were examined under the stereomicroscope and their appearance was evaluated as describe by Grimes & Ireland (1986). Follicles were divided into 3 groups; (1) clear non atretic follicles (Group 1, n= 169) of more than 70% of its surface was clear vesicular; (2) heavy atretic follicles (Group 2, n=356), at least 70% of its exposed surface was cloudy and (3) early atretic follicles (Group 3, n=122). Follicle wall was fixed in 10% formol saline solution, processed for paraffin sections and stained with hematoxilin and eosin. Based on their histological structure follicles were assigned to be one of 3 stages of atresia (1) clear non atretic follicle, in which the granulosa cells had at least 7 layers of cells of which only relatively few appear fragmented or apoptotic cells, (2) early atretic follicles, granulosa cells were reduced to 2 to 6 layers and may be disorganized, apoptotic or fragmented granulosa cells are abundant and (3) Heavy atretic follicle, granulosal layers were sparse or almost absent or mostly composed of apoptotic cells (Grimes & Ireland, 1986).

#### 1.2. Relationship between follicle quality and COCs quality

Follicles were dissected and classified as previously mentioned. They were punctured with a fine needle and COCs were recovered and classified according to the compactness and clarity of the cumulus investment into 3 categories; Category 1, had compact and bright cumulus–cells; Category 2 had less compact and obviously darker cumulus-cells, and category 3 had expanded cumulus-cell

investment or completely denuded (de Wit et al., 2000).

### 1.3. Histological examination of COCs

Twenty five COCs from each group were fixed for 2 h in 2.5% glutaraldehyde in 0.1 PBS (pH 7.4) at 4 °C. After fixation, COCs were washed 3 times in PBS and then post fixed with 1% osmium tetraoxide in PBS at 4 °C. Subsequently, COCs were dehydrated in ascending concentrations of ethanol and embedded in epoxy resin. Semi-thin sections were cut with a glass knife, placed on glass slides, stained with 1% toluidine blue and examined using a light microscope (Hyttel et al., 1987).

## Experiment 2: Relationship between follicle quality and maturation rate and developmental competence of COCs.

### In vitro maturation

In this experiment, COCs were aspirated from clear, intermediate and opaque follicles using 18-gauge needle attached to 10-ml sterile syringe. A total of 659 COCs of category 1 and 2 were selected from the 3 types of follicles and used for in vitro maturation. COCs were cultured in TCM 199 medium (Cat. 2145, bicarbonate buffered) supplemented with 10% fetal calf serum (FCS) + 10 µg/ml follicle stimulating hormone (FSH) + 50 µg/ml gentamycin. Maturation was performed for 24 h at 38.5°C under 5% $CO_2$ in humidified air.

### 2.1. Assessment of nuclear maturation

At the end of maturation time, 50 oocytes from each group were vortex for removal of cumulus-cells and then fixed in acetic ethanol (1:3) for 48 h and stained with 1% orcein dye for determination of the stage of nuclear maturation.

### 2.2. Developmental competence

#### 2.2.1. In vitro fertilization

Mature oocytes were fertilized using frozen-thawed buffalo spermatozoa (separated by Percoll density gradient). Fertilization was done in Fert-TALP medium supplemented with 4 mg/ml BSA + 2.5 mM caffeine + 10 µg/ml/heparin. In vitro fertilization was performed for 20 h at 38.5°C under 5% $CO_2$ in humidified air.

#### 2.2.2. Embryo culture

Presumptive zygotes were washed 3 times in in vitro culture (IVC) medium which consists of TCM 199 + 5% FCS + 50 µg/ml gentamycin. Culture was done for 8 days at 38.5°C under 5% $CO_2$ in humidified air. Cleavage rate, morula and blastocyst rates were checked on Days 2, 5 and 8, respectively.

### 3. Statistical analysis

Data were statistically analyzed using Student Chi squire "$X^{2}$" analysis according to Snedecor & Cochran (1980).

## RESULTS

**Experiment 1: Macroscopic and microscopic evaluation of follicles and their enclosing COCs.**

Macroscopic evaluation of buffalo ovarian follicles is presented in Table 1. Data showed that a significantly higher (P<0.05) percentage of opaque follicles was present in buffalo ovaries compared to clear vesicular or intermediate follicles.

**Table 1: Macroscopic evaluation of dissected buffalo ovarian follicles**

| Follicles quality | Follicles quality (%) |
|---|---|
| Clear vesicular | 169/647 (26.1)[b] |
| Intermediate | 122/647 (18.9)[b] |
| Opaque | 356/647 (55.0)[a] |

a,b differed at P<0.05

The highest percentage (P<0.05) of good quality COCs (Fig. 1, b,e) were recovered from clear vesicular and intermediate follicles compared to that from opaque follicles.

Significantly higher (P<0.05) percentages of poor quality COCs with expanded cumulus-cells or denuded oocytes (Fig. 1, h) were recovered from opaque follicles

**Table 2. Relationship between follicle and COC qualities in buffalo ovaries**

| Follicles quality | No. COCs | COCs quality (%) | | |
|---|---|---|---|---|
| | | Good | Fair | Poor |
| Clear vesicular | 148 | 118 (79.7)[a] | 17 (11.5) | 13 (8.8)[a] |
| Intermediate | 113 | 86 (76.1)[a] | 15 (13.3) | 12 (10.6)[a] |
| Opaque | 334 | 89( 26.6)[b] | 46 (13.8) | 199 (59.6)[b] |

a,b differed at P<0.05

Microscopic examination of follicular wall of clear vesicular, intermediate and opaque follicles and the semi-thin sections of COCs were presented in Fig. 1. Microscopic examination revealed that clear vesicular follicles possess more than 7 layers of healthy granulosa cells and very few apoptotic cells (Fig. 1, A). However, opaque follicle was composed mainly of apoptotic granulosa cells (Fig.1, D). Microscopic examination of semi-thin sections revealed that COCs recovered from clear vesicular follicles had healthy cumulus investment, and the cumulus cells process endings penetrated into the ooplasm. Lipid droplets and small vesicles were centrally located in the ooplasm (Fig. 1, C). COCs recovered from intermediate follicles showed a higher number of lipid droplets and few vesicles but no cumulus investment (Fig.1, F). COCs from opaque follicles showed degenerative changes in their ooplasm and the presence of large black spots (Fig 1, I).

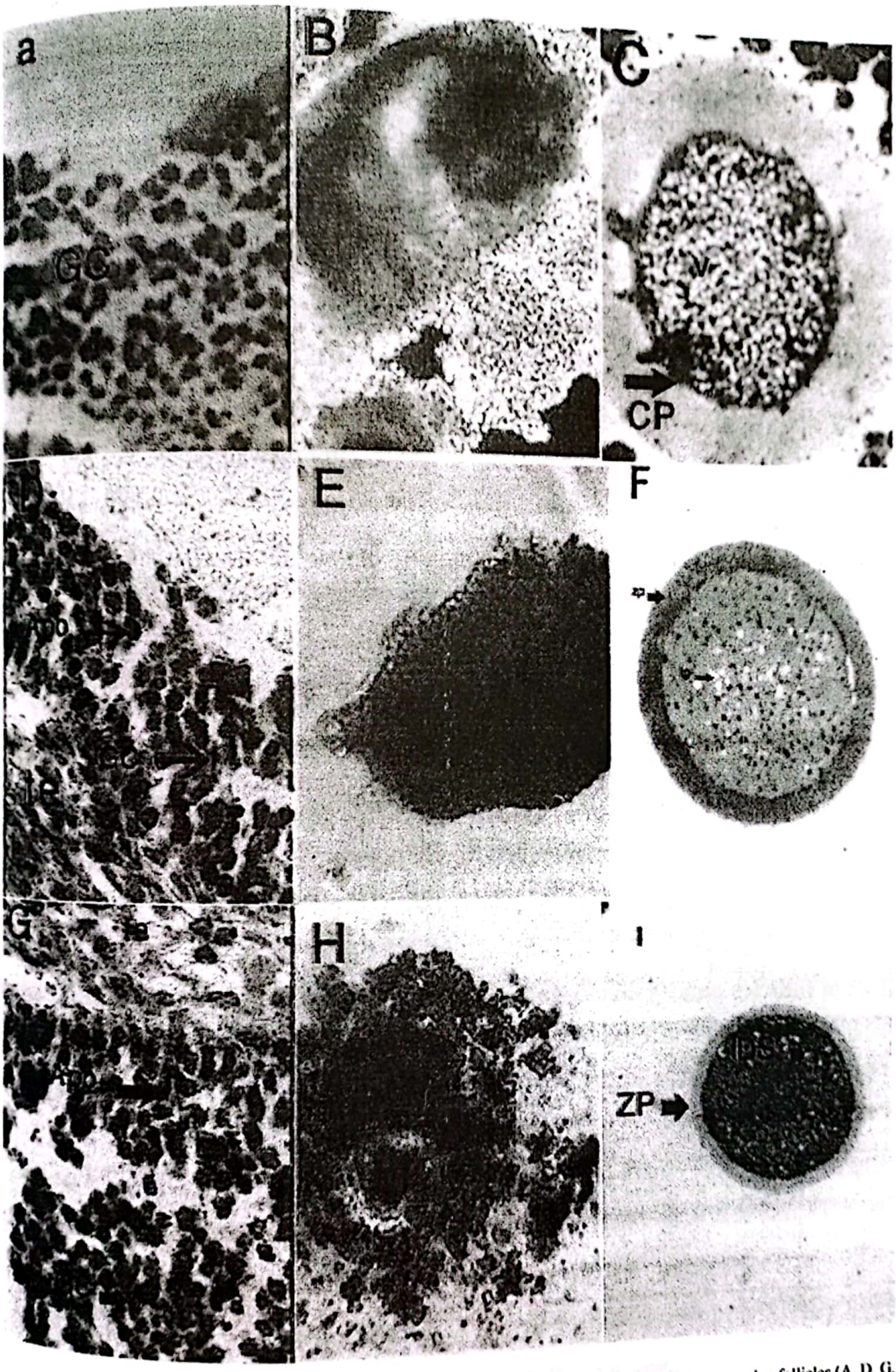


Fig. 1: Micrograph showing microscopic structure of follicular wall of clear, intermediate and opaque ovarian follicles (A, D, G, 400 X), COCs quality (B, E, H, 100 X) and microscopic structure of oocytes (C, F, I, 200 X). Apo (apoptotic granulose cells), GC (granulose cells), te (theca interna), ZP (zona pellucida), LD (lipid granules), CP (cytoplasmic process), and V (vesicles).

Maturation rate, as indicated by the percentage of oocytes from clear vesicular, intermediate and opaque follicles reaching the MII stage was illustrated in Table (3). The data demonstrated that significantly higher (P<0.05) percentage of oocytes reached the MII stage when oocytes were recovered from clear vesicular or intermediate follicles compared with that recovered from opaque ones.

**Table 3: Effect of follicles quality on the percentage of oocytes reaching metaphase II stage**

| Follicles quality | Matured oocytes at M II (%) |
|---|---|
| Clear vesicular | 38/50 (76)[a] |
| Intermediate | 35/50 (70)[a] |
| Opaque | 6/50 (12)[b] |

a,b differed at P<0.05

The ability of oocytes from clear vesicular, intermediate and opaque follicles to develop in vitro was presented in Table (4). Results showed that cleavage rate and embryo development were significantly higher (P<0.05) for oocytes recovered from clear vesicular and intermediate follicles compared to opaque follicles.

**Table 4. Effect of follicles quality on cleavage and developmental rates of IVF buffalo oocytes.**

| Follicles quality | No. of fertilized oocytes | Cleavage rate (%) | Embryo development (%) | | | |
|---|---|---|---|---|---|---|
| | | | 2-4-cell stage | 8-16-cell stage | Morulae stage | Blastocyst stage |
| Clear vesicular | 142 | 112 (78.9)[a] | 27 (21)[a] | 13 (12)[a] | 48 (43)[a] | 27 (24)[a] |
| Intermediate | 132 | 94 (71.2)[a] | 11 (11.7)[a] | 23(24.5)[a] | 39 (41.5)[a] | 21 (22.3)[a] |
| opaque | 147 | 20 (14)[b] | 8 (40)[b] | 10 (50)[b] | 2 (10)[b] | 0.00[b] |

a,b differed at P<0.05

## DISCUSSION

It is possible that intrafollicular environment to which oocytes are exposed is a major cause of the variability in developmental competence (Callesen et al., 1986). So, the study presented here examined the developmental capacity of buffalo COCs in relation to morphological and histological indices of follicle health. The results demonstrated that macroscopic classification of buffalo ovarian follicles according to their morphology into clear vesicular, intermediate and opaque revealed that significantly higher ($P<0.05$) percentage of opaque follicles was present in buffalo ovaries. The histological examination, also, showed that clear vesicular follicles were lined with more than 7 layers of healthy granulosa cells and they had very few number of apoptotic cells. The intermediate follicle, the wall was lined by less than 5 layers of granulosa cells and nearly 50% of them were apoptotic. In opaque follicles the wall was formed mainly of apoptotic granulosa cells and apoptosis was extended to the theca interna.

Moreover, a high ($P<0.05$) percentage of good quality COCs was recovered from clear vesicular and intermediate follicles, and a significantly higher ($P<0.05$) percentage of poor quality oocytes was recovered from opaque follicles. These findings are in concomitant with that previously recorded in cattle (Blondin & Sirard, 1995; de Wit et al., 2000). These authors found a high correlation between the follicles quality and the distribution of COCs quality.

Histological examination of semi-thin sections of COCs indicated that COCs recovered from clear vesicular follicles were surrounded by multi-layers of cumulus cells and the ooplasm was filled with fine lipid droplets and vesicles and cytoplasmic processes were seen penetrating the zona pellucida from the cumulus cells. COCs from intermediate follicles showed absence of cumulus investment and present of higher numbers of lipid droplets. In COCs recovered from opaque follicles, the ooplasm was pale and contained dark black spots. These findings completely agreed with observations of Moor et al. (1984), De loos et al. (1991), Hazelegr et al. (1992), , and Nagano et al. (2006). Those authors found a higher percentage of COCs showing degenerative changes when the degree of follicular atresia was more sever.

Furthermore, maturation rate, indicated that a significantly higher ($P<0.05$) percentage of

oocytes collected from clear vesicular and intermediate follicles reached the M II stage compared with oocytes recovered from opaque follicles. This could be primarily related to the quality of oocytes. Similarly, Grimes & Ireland (1986) found that nuclear maturation of bovine oocytes in vitro was markedly greater for oocytes collected from clear non atretic than from late atretic follicles.

Cleavage rate and embryo development to the morula and blastocyst stages were significantly higher ($P<0.05$) in oocytes recovered from clear vesicular follicles and intermediate follicles compared with that from opaque ones. These results indicated that oocytes competence only decreases at a high level of follicular atresia, while it appears to be improved by a low level of atresia (Jewgenow et al., 1999). Healthy cumulus cells around the oocytes play a pivotal role in glucose utilization by the COCs, probably by producing pyruvate and lactate, the oocyte's preferred substrate (Sutton et al., 2003), and poor quality oocytes rely on glutamine and pyruvate for ATP synthesis (Zuelke & Brackett, 1992).

Degeneration of follicles leads to a gradual decrease of estradiol-17B and testosterone concentration and an increase of progesterone levels, and decreased desmolase and aromatase activities (Kruip & Dieleman, 1989). Other reports recorded that COCs from early atretic follicles showed higher developmental rates than COCs from nonatretic follicles (Wurth & Kruip, 1992; Assey et al., 1994; Machatkova et al., 1996; de Wit et al., 2000). However, Blondin & Sirard (1995) did not detect any difference in the developmental competence from nonatretic, early atretic or heavy atretic follicles. This discrepancy difference could be due to species difference or could be related to the source of oocytes.

In conclusion, macroscopic selection of ovarian follicles could be beneficial for in vitro production of buffalo embryos. Oocytes recovered from opaque follicles showed degenerative changes and poor developmental rate compared with that from clear or intermediate follicles.

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# التقسيم المرفولوجى و الهستولوجى للجريبات المبيضية فى الجاموس ومقدرة البويضات المجمعة منها على النضج و التطور معمليا

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ان مقدرة بويضات الجاموس على الانقسام والتطور معمليا أقل بكثير عنها فى الأبقار. وقد يرجع ذلك لاسباب تتعلق بنوعية هذه البويضات. أجريت تجربتين لدراسة (1) تحديد العلاقة بين الحالة المرفولوجية و الهستولوجية للجريبات المبيضية و نوعية البويضات المجمعة منها. (2) دراسة مقدرة البويضات المجمعة من الجريبات المبيضية على النضج و الأنقسام والتطور معمليا حتى مرحلة الموريولا و البلاستوسيست. التجربة الأولى تم عزل الجريبات المبيضية من مبايض الجاموس و قسمت حسب الشكل الظاهرى الى ثلاثة أنواع هى الشفافة و المتوسطة و القاتمة. جمعت البويضات من الجريبات المعزولة بطريقة الوخز ثم قسمت الى ثلاثة نوعيات الجيدة و المقبولة و الرديئة بينما تم حفظ جدار الحويصلات المبيضية فى الفورمالين و جهزت للفحص الهستولوجى. أيضا تم تجهيز البويضات المجمعة للفحص الهستولوجى لتقييم نوعيتها. فى التجربة الثانية تم تجميع البويضات تحت نفس الظروف التجربة الأولى و زرعت فى أوساط لأتمام النضج ثم خصبت بالحيامن معمليا و زرعت لاتمام عملية الأنقسام والنضج حتى مرحلة الموريولا و البلاستوسيست.

اوضحت النتائج أن نسبة البويضات المجمعة من الجريبات الشفافة و المتوسطة أعطت نسبة عالية من البويضات الجيدة بينما أعطت الجريبات القاتمة نسبة أعلى من البويضات الرديئة. بين الفحص الهستولوجى أن جدار الجريبات الشفافة تميز بوجود عدد كبير من الخلايا التراكمية السليمة بينما كان معظم هذه الخلايا ميتة فى حالة الجريبات القاتمة كذلك اوضح الفحص الهستولوجى للبويضات أن البويضات المجمعة من الجريبات الشفافة و المتوسطة كانت أفضل فى التركيب عها فى البويضات المجمعة من الجريبات القاتمة. كانت نسبة البويضات الناضجة فى مرحلة الميتافيز 2 أعلى عند تجميعها من الجريبات الشفافة و المتوسطة كذلك كانت معدلات الانقسام و التطور أعلى فى بويضات الجريبات الشفافة و المتوسطة مقارنة بالبويضات المجمعة من الجريبات القاتمة.