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## Evaluation of Some Factors Affecting Quantity, Quality and *in vitro* Maturation of Buffalo Oocytes

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### ABSTRACT

This study aimed to evaluate the influence of collection techniques (dissection, aspiration, slicing and aspiration plus slicing) on yield and quality of buffalo oocytes *in vitro* matured (IVM) in TCM-199 or Ham's F12 with or without hormones (follicle-stimulating hormone, FSH and estradiol, E<sub>2</sub>). Recovery Rate (RR) and category of the collected oocytes were determined and only Cumulus Oocyte Complexes (COCs) were matured *in vitro*. Results showed that RR was 84.6, 83.3, 72.7 and 52.0% for aspiration plus slicing, slicing, aspiration and dissection technique, respectively (p<0.05). Percentage of COCs was higher by slicing than aspiration, aspiration plus slicing and dissection (63.2 vs. 51.3, 51.2 and 42.0%, p<0.05). The corresponding percentages of expanded oocytes were 29.9, 30.3, 27.6 and 32.7%, respectively (p<0.05). Percentage of oocytes arrested at metaphase-II (MII) was higher (67.1%, p<0.001) while those at Germinal Vesicle (GV) (8.1%, p<0.001) and Deg. (9.1%, p<0.01) were lower in TCM-199 than in Ham's F12. Hormonal addition increased percentage of oocytes arrested at metaphase-I (MI) and MII (p<0.05 and p<0.001, respectively) and decreased those at GV and Deg (p<0.001). Such results may indicate efficacy of slicing technique as a collection method on quantity and quality of buffalo oocytes. Also, *in vitro* maturation of buffalo oocytes was improved in TCM-199 supplemented with hormones (FSH and E<sub>2</sub>).

**Key words:** Buffalo, harvesting technique, oocyte yield, maturation medium, hormones

### INTRODUCTION

*In vitro* maturation (IVM) is one of assisted reproductive technologies which have been introduced to increase the number of offspring from selected females and to reduce the generation intervals in buffaloes (Suresh *et al.*, 2009). An economical source of oocytes is ovaries of slaughterhouse (Sianturi *et al.*, 2002) but the quality of these oocytes play a key role in the acquisition of oocyte developmental competence *in vitro* (Amer *et al.*, 2008), in particular the appearance of cumulus cell layers of an oocyte (Sianturi *et al.*, 2002). Therefore, the efficacies of different methods of oocyte collection were compared on the basis of Cumulus Oocyte Complexes (COCs) yield (Mehmood *et al.*, 2011).

In buffaloes, the very poor recovery of good quality immature oocytes is a serious problem associated with the production of embryos through IVF (Chauhan *et al.*, 1998). Several methods for collection of oocytes have been described in domestic animals: Aspiration of the oocyte from

follicles (Boediono *et al.*, 1995), slicing the ovaries (Wang *et al.*, 2007a), puncture of visible surface follicles (Shirazi *et al.*, 2005) and post-aspiration slicing (Kumar and Maurya, 2000) of ovarian follicles but these methods were used with varying degrees of success.

The first and most critical step towards successful *in vitro* embryo production is method of oocyte collection for IVM (Hegab *et al.*, 2009) because use of these oocytes makes it feasible to obtain a large number of oocytes from ovaries at relatively low cost (Roushandeh *et al.*, 2007). There are many factors affecting IVM of buffalo oocytes, including the selection of proper maturation medium or the hormones added (De Wit *et al.*, 2000). A variety of culture media are available and the choice of the base medium for IVM is considered particularly important (Kim *et al.*, 2011). Different culture media such as TCM-199 (Kharche *et al.*, 2006), Minimum Essential Medium (MEM) (Ravindranatha *et al.*, 2001), Ham's F-10 (Tamilmani *et al.*, 2005) and Ham's F-12 (Jamil *et al.*, 2007) have been used for IVM mammalian oocytes. Also, hormonal addition such as follicle stimulating hormones (Chauhan *et al.*, 1998) and E<sub>2</sub> (Nandi *et al.*, 2002a) to culture media has been made to improve the developmental competence of IVM oocytes. Therefore, the objectives of this study were to investigate the efficiency of dissection, aspiration, slicing and aspiration plus slicing as harvesting techniques on the availability of quantity and quality of buffalo oocytes collected from slaughtered animals (1st experiment) and to evaluate the effect of maturation medium type (TCM 199 or Ham's F12) with or without hormonal addition (FSH or E<sub>2</sub>) during IVM of buffalo oocytes (2nd experiment).

## **MATERIALS AND METHODS**

This study was carried out at the International Livestock Management Training Center (ILMTC), belonging to the Animal Production Research Institute, Agriculture Research Center, Ministry of Agriculture, in cooperation with Department of Animal Production, Faculty of Agriculture, Tanta University, during the period from June until October 2014.

**Collection of ovaries:** Buffalo ovaries were collected from local slaughter houses (El-Batanoun-Menofeya governorate, Egypt) immediately after slaughter. The collected ovaries were placed into thermos at 27-30°C in normal saline (0.9%) containing gentamicin (50 µg mL<sup>-1</sup>) and transported to the laboratory within three hours post slaughter. In the laboratory, extraneous tissues were removed and the ovaries were washed three times in phosphate buffer saline (PBS, pH 7.3).

### **1st experiment**

**Oocyte collection methods:** In the laboratory, the oocytes were collected aseptically from the ovaries by four methods, including dissection, aspiration, slicing and aspiration plus slicing. In all collection techniques, the visible ovarian follicles (2-8 mm in diameter) were counted before oocyte collection:

- **Dissection technique:** The ovaries were placed in a sterile glass petri dish containing 2 mL of PBS. All the visible follicles were carefully subjected to blunt dissection with the help of forceps and the remaining ovarian tissues were removed after a brief rinsing. The follicles were ruptured and the follicular fluid was allowed to flow into the PBS in petri dish (Singh *et al.*, 2013)
- **Aspiration technique:** The oocytes were aspirated from individual ovaries after carefully removing the extraneous tissues and placed in petri dish containing 1 mL of PBS (Rao and Mahesh, 2012). Oocytes were aspirated from the visible follicles presented on the ovarian

surface. Oocytes were aspirated with 22 gauge needle fixed to 5 mL disposable syringe containing 1-2 mL of PBS. Slicing technique in which the ovaries were held firmly with the help of forceps in a sterile glass petri dish containing 2 mL of PBS. The ovaries were sliced into possible thin sections with a blade fixed to the artery forceps. The oocytes containing PBS medium were placed in petri dish and examined under Stereomicroscope (Mehmood *et al.*, 2011)

- **Aspiration plus slicing technique:** The aspirated ovaries were subjected to further slicing to obtain count of the residual oocytes (post aspiration slicing) according to Kumar and Maurya (2000). Then number of oocytes collected from both techniques was counted

In all the four techniques, the petri dishes were kept undisturbed for 5 min, allowing the oocytes to settle down. The petri dishes were examined under stereomicroscopy and the oocytes were transferred to a searching dish containing PBS for grading. Phosphate Buffer Saline (PBS) medium was prepared according to Gordon (1994).

About 2 mg from bovine serum albumin was added to each one ml PBS. The pH value of the medium was adjusted to 7.2-7.4 using pH-meter and to osmolarity of 280-300 mOsmol kg<sup>-1</sup> using osmometer. The medium was filtered by 0.22 µm millipore filter.

**Oocyte evaluation:** Oocytes were examined under stereomicroscopy and classified according to their compaction, number of cumulus cell layers and homogeneity of ooplasm according to Ravindranatha *et al.* (2002) into 5 categories, (1) Cumulus oocytes-complexes (COCs) with compact cumulus cells (≥3 layers) and homogenous ooplasm, (2) Expanded cumulus cells oocytes, (3) Denuded oocytes with completely devoid cumulus cells and heterogeneity ooplasm, (4) Partial denuded oocytes with cumulus cells present either incompletely surrounding the oocyte and (5) Degenerated (Deg.) oocytes with ooplasm shrunken away from the zona pellucida or not evenly filling the zona pellucida.

**Recovery rate:** Oocyte yield from each method was counted and calculated per ovary. Oocyte Recovery Rate (RR) was determined as the following:

$$RR (\%) = \frac{\text{No. of recovered oocytes}}{\text{Total number of follicles}} \times 100$$

## **2nd experiment**

### ***In vitro* maturation (IVM)**

**Type of maturation medium and hormonal addition:** Only COCs collected by slicing technique were used in this experiment. These oocytes were matured in two maturation media, namely tissue culture medium-199 (TCM-199, Sigma) or Ham's F12 (Egyptian Organization for Biological Product and vaccine, Agoza) supplemented with 0.3% bovine serum albumin (BSA, w/v) and 100 IU sodium penicillin G and 100 µg streptomycin/mL. Each type of media was used with or without hormonal supplementation with 1 µg mL<sup>-1</sup> E<sub>2</sub> and 0.04 IU mL<sup>-1</sup> FSH. The pH value of the medium was adjusted to 7.2-7.4 and to osmolarity of 280-300 mOsmol kg<sup>-1</sup>. The medium was filtered by 0.22-µm millipore filter.

***In vitro* maturation procedures:** An amount of 500 µL from prepared maturation medium was placed into four well dishes and covered by sterile mineral oil. Before placing the oocytes in culture dishes, the medium was incubated in CO<sub>2</sub> incubator (5% CO<sub>2</sub>, at 38°C with saturated humidity) for

at least 60 min to attain equilibrium between the temperature and gases. The COCs were washed three times in washing medium, then washed twice in the intended maturation medium before culturing in that medium. Batches of 8-10 oocytes were placed into maturation medium, then overlaid with liquid paraffin oil (Sigma) and then the petri dishes were transferred into a CO<sub>2</sub> incubator (38.5°C, 5% CO<sub>2</sub> in air, 95% relative humidity) for 24 h as a maturation period.

**Fixation, staining and examination of oocytes:** After 24 h as a maturation period, oocytes were washed using PBS containing 1 mg mL<sup>-1</sup> hyaluronidase to remove the cumulus cells. Then, oocytes were washed two times with PBS supplemented with 2% 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 maturation under phase-contrast microscopy into different stages including germinal vesicle (GV, denoting chromosomes enclosed within a nuclear membrane), Germinal Vesicle Breakdown (GVBD, illustrating an absence of a visible nuclear membrane and chromatin condensation), metaphase<sup>-2</sup> (MI, showing chromosomes condensed in pairs without detected polar body), metaphase-II (MII, denoting one large group of chromosomes formed an equatorial plate and the remaining chromosomes are highly condensed or have extruded 1st polar body, mature oocytes) and degeneration (Deg., illustrating vacuolated oocytes with shrunk cytoplasm or highly condensed chromatin).

**Statistical analysis:** Statistical analysis for the obtained data was analyzed using general linear model of (SAS., 2001) while differences among the treatment means were performed using Duncan's Multiple Range Test (Duncan, 1955). The percentages values were adjusted to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from transformed values to percentages.

## RESULTS AND DISCUSSION

### 1st experiment

#### Effect of collection method on

**Number and rate of recovered oocytes:** Results presented in Table 1 showed that oocyte recovery rate from buffalo ovaries was higher ( $p < 0.05$ ) by aspiration plus slicing and slicing alone than by aspiration alone (84.7 and 83.3 vs. 72.7%, respectively), while dissection technique showed the lowest oocyte recovery rate (52.0%,  $p < 0.05$ ). It is of interest to note that there was slight increase in recovery rate when oocytes were collected by additional post-aspiration slicing, although number of follicles on the ovarian surface and recovered oocytes were different for each collection technique.

Table 1: Effect of collection method on number and rate of recovered buffalo oocytes

Item	Aspiration plus slicing	Slicing	Aspiration	Dissection
Total ovary (n)	206	200	206	262
Total follicles (n)	921	1062	921	1087
Total oocytes (n)	756	831	665	567
Visible follicles/ovary	4.33±0.21 <sup>b</sup>	5.24±0.43 <sup>a</sup>	4.33±0.21 <sup>b</sup>	4.16±0.05 <sup>b</sup>
Recovered oocytes/ovary	3.67±0.20 <sup>ab</sup>	4.10±0.36 <sup>a</sup>	3.14±0.16 <sup>b</sup>	2.16±0.39 <sup>c</sup>
Oocyte recovery rate (%)	84.7±1.72 <sup>a</sup>	83.3±0.95 <sup>a</sup>	72.7±1.59 <sup>b</sup>	52.0±0.76 <sup>c</sup>

<sup>a,b,c</sup>Means denoted within the same row with different superscripts are significantly different at  $p < 0.05$

In accordance with the present results, Rao and Mahesh (2012) showed that among the three harvesting techniques, the slicing method appeared to be superior in terms of both total recovery and number of culture grade oocytes. Slicing yielded significantly ( $p < 0.01$ ) higher number of oocytes ( $7.98 \pm 0.70$ ) per ovary compared to the puncture ( $3.46 \pm 0.31$ ) and aspiration methods ( $2.38 \pm 0.19$ ). Also, Gasparini (2002) reported that the oocytes yield increased by using slicing method for collection of buffalo oocytes. Moreover, Khan *et al.* (1997) reported that oocytes were recovered via aspiration from 55% of follicles, as compared to the slicing method which recovered oocytes from 78% of follicles from buffalo ovaries. In cow, Wang *et al.* (2007b) concluded that the recovery of oocytes using slicing and puncture techniques yielded more oocytes per ovary than other aspiration method.

In the present study, post aspiration slicing of buffalo ovaries provided additional 0.53 oocyte/ovary versus 0.46 oocytes/ovary as reported by Kumar and Maurya (2000). In buffaloes, Das *et al.* (1996) found that number of oocytes per ovary (follicles with 2-6 mm in diameter) recovered by slicing was significantly ( $p < 0.01$ ) higher (5.7/ovary) than that achieved by follicles puncture (2.6/ovary) and aspiration (1.7/ovary). Higher oocyte recovery in ovarian slicing may be due to their release from both surface follicles as well as from deeper cortex (Das *et al.*, 1996). Generally, the lower number of oocytes recovered by the aspiration method may be attributed to the presence of some follicles embedded deeply within the cortex which are released by slicing of the ovary. Some of the oocytes may even be lost during aspiration of follicles which is not possible when using the slicing method.

On the other hand, Shirazi *et al.* (2005) documented that the number of oocytes per ovary for slicing and aspiration didn't differ significantly in ewes. Also, Gupta and Sharma (2001) stated that there was no effect of slicing, aspiration and combined methods on the recovery of buffalo oocytes. The conflicted results in this respect may be attributed to the effect of interaction between collection method and reproductive status.

**Oocyte quality:** Data in Table 2 showed that percentage of COCs was the highest (63.17%,  $p < 0.05$ ) using slicing technique, followed by aspiration or aspiration plus slicing techniques (51.34 and 51.24%, respectively), while dissection method showed the lowest percentage of COCs (42.03%,  $p < 0.05$ ). On the other hand, dissection technique showed the highest percentage of expanded oocytes (32.68%,  $p < 0.05$ ) versus the lowest percentage (27.55%,  $p < 0.05$ ) for aspiration plus slicing method. Also, slicing showed the lowest percentages of denuded, partial denuded and Deg. oocytes (1.89, 3.05 and 1.96%,  $p < 0.05$ ) compared with other techniques, respectively (Table 3). Such differences may be attributed to other causes occurring in oocytes pre-collection rather than collection method.

The present results showed that the COCs recovery rate or even acceptable oocytes (COCs and expanded oocytes) from ovaries of slaughtered buffaloes was better with slicing (93.1%) than with aspiration (81.6%) and aspiration plus slicing techniques (78.7%). Although, the present results

Table 2: Effect of collection method on categories of buffalo oocytes

Oocyte category	Aspiration plus slicing	Slicing	Aspiration	Dissection
COCs.	51.24±0.83 <sup>b</sup>	63.17±1.19 <sup>a</sup>	51.34±0.47 <sup>b</sup>	42.03±0.90 <sup>c</sup>
Expanded	27.55±0.86 <sup>c</sup>	29.93±0.44 <sup>b</sup>	30.31±0.83 <sup>b</sup>	32.68±0.83 <sup>a</sup>
Denuded	4.93±0.98 <sup>b</sup>	1.89±0.37 <sup>c</sup>	3.34±0.54 <sup>bc</sup>	10.59±0.56 <sup>a</sup>
Partial denuded	10.89±0.77 <sup>a</sup>	3.05±0.92 <sup>b</sup>	11.01±0.84 <sup>a</sup>	3.25±0.73 <sup>b</sup>
Deg	5.39±0.39 <sup>b</sup>	1.96±0.37 <sup>c</sup>	4.00±0.39 <sup>b</sup>	11.45±0.32 <sup>a</sup>

<sup>a,b,c</sup>Means denoted within the same row with different superscripts are significantly different at  $p < 0.05$

Table 3: Effect of medium type on different maturation stages of buffalo oocytes

Medium type	Maturation stage (%)				
	Degeneration	MII	MI	GVBD	GV
TCM-199	9.10±0.95	67.1±0.64	8.4±0.83	7.3±0.81	8.10±1.02
Ham's F12	13.70±0.95	58.6±0.64	6.8±0.83	6.6±0.81	14.3±1.02
Significance	**	***	NS	NS	***

NS: Not significant, \*\*,\*\*\*Significant at  $p < 0.01$  and  $p < 0.001$ , respectively

were much higher than that obtained by Rao and Mahesh (2012), they also reported that the slicing method appeared to be superior in term of number of culture grade oocytes. The slicing method was reflected in a greater number of mean good, fair, poor quality and culture grade oocytes, being 38.46, 27.75, 33.79 and 66.21%, respectively. Also, the present results were much higher than earlier findings of some authors (Madan *et al.*, 1994; Das *et al.*, 1996; Jamil *et al.*, 2008) in buffaloes and cattle (Carolan *et al.*, 1994).

In accordance with the present results, Mehmood *et al.* (2011) found that the COC recovery rate of buffaloes was better with slicing than with the aspiration method ( $p < 0.05$ ). Also, Mistry and Dhama (2009) demonstrated that slicing was a simple effective method for collecting a high quality oocyte yield for *in vitro* culture. Also, Sianturi *et al.* (2002) found that the percentage of acceptable oocytes (cumulus oocyte complexes and expanded oocytes) of total oocytes was lower using aspiration (27.71 and 40.66%) than slicing (33.8 and 34.02%) but the difference was not significant. On the other hand, Gasparini (2002) found a higher proportion of poor oocytes using slicing technique in buffaloes due to heterogeneous population of oocytes retrieved from all follicles which distributed through the stromal of ovaries.

The recorded variation due to the different methods used for COC recovery may be associated with seasonal effects and variation in the reproductive status of the slaughtered buffaloes (Greve and Madison, 1991). Another explanation is due to the age of animal, state of ovary and the ovarian environment at time of collection (Hammam *et al.*, 1997) or age, season, nutritional status (body condition) and cyclicity of animals at the time of slaughter, size and functional status of follicles, method of oocyte retrieval (Das *et al.*, 1996; Nandi *et al.*, 2002b; Zoheir *et al.*, 2007; Amer *et al.*, 2008).

## 2nd experiment

**In vitro maturation of buffalo oocytes as affected by Medium type (TCM-199 vs. Ham's F12):** Results in Table 3 showed that overall percentages of oocytes arrested at MII (maturation rate) was higher (67.1%,  $p < 0.001$ ), while those at GV (8.1%,  $p < 0.001$ ) and Deg. (9.1%,  $p < 0.01$ ) were lower in TCM-199 than in Ham's F12, regardless hormonal addition. It is worthy noting that the highest percentage of oocytes arrested at MII stage due to TCM-199 was associated with reduction in percentage of Deg. oocytes as compared to that attained with Ham's F12 (9.2 vs. 13.7%,  $p < 0.05$ ). However, both types of media showed nearly similar percentages of oocytes at GV, GVBD or at prophase and MI stages (8.1, 7.3 and 8.4% for TCM-199 vs. 14.3, 6.6 and 6.8% for Ham's F12, respectively, Table 3).

The culture medium is one of the most important factors in maturation of follicular oocytes *in vitro* (Van de Sandt *et al.*, 1990) and expansion of cumulus cells depends largely on the culture media used for maturation of the oocytes (Nandi *et al.*, 2002b). It was reported that TCM-199 can support the maturation of buffalo oocytes (Hammam *et al.*, 1997) and most IVF laboratories routinely used TCM-199 as the basic medium for IVM of buffalo oocytes (Bavister *et al.*, 1992;

Hawk and Wall, 1993; Palta and Chauhan, 1998). A variety of media have been used for IVM and culture but TCM-199 has been used for both maturation and culture more frequently than any other medium (Hawk and Wall, 1993; Palta and Chauhan, 1998; Gandhi *et al.*, 2000).

The present study showed that nuclear maturation rates of buffalo oocytes was better with TCM-199 than with Hams-F12. Similar results were reported by Smetanina *et al.* (2000) and Leibfried and First (1979). In accordance with the present results, Jamil *et al.* (2007) studied the effect of TCM-199 and Ham's F12 as maturation media with and without the addition of Oestrus Buffalo Serum (OBS) and hormones (FSH, LH, E<sub>2</sub>) on the maturation rate of buffalo follicular oocytes. They found significant ( $p < 0.05$ ) increase in the maturation rate in TCM-199 than in Ham's F12, regardless OSB or hormones addition. The percentage of maturation rates in TCM-199 ranged from 55.4% versus 26.3% in Ham's F12. Also, Raza *et al.* (2001) revealed that TCM-199 resulted in significantly better maturation rate (73.3%) than Ham's F10 (61.6%).

In addition, the maturation rate of buffalo oocytes was higher in TCM-199 with or without LH (95.68 and 77.61%) than in Modified synthetic oviductal fluid (mSOF) medium with or without LH (57.17 and 40.92%) as reported by Barakat *et al.* (2012). In cattle, Smetanina *et al.* (2000) found that maturation rate of cattle oocyte MII was higher in TCM-199 than in Ham's F10 but maturation rates were lower than that obtained in the present study on buffalo oocytes, being 29.4 and 8.6%, respectively. However, Lonergan *et al.* (1994) concluded that TCM-199 was capable of supporting the *in vitro* maturation of bovine oocytes at high rates.

On the other hand, Gliedt *et al.* (1996) found that cumulus cells expansion was greater ( $p < 0.0001$ ) for bovine COCs that matured in (RPMI-1640) than for those that matured in TCM-199. Also, Abdoon *et al.* (2001) found that buffalo oocytes matured and cultured in CR1aa medium expressed higher cleavage (56%) than oocytes matured in TCM-199 (32%). This is in agreement with the result of Kandil *et al.* (2013), who found that the cleavage rate of buffalo oocytes matured *in vitro* in CR1aa was 52.3% versus 40.7% in TCM-199. Bovine oocytes matured in TCM-199, SFRE (serum-free medium based on TCM-199) and Minimum Essential Medium (MEM) have superior blastocyst development rates (12±19%) compared with oocytes matured in Waymouth MB 752/1, Ham's F-12 (3 and 1%, respectively (Rose and Bavister, 1992) or MeÅneÅzo's B2 (Hasler, 2000).

*In vitro* maturation media can be broadly divided into simple and complex. Simple media are usually bicarbonate-buffered systems containing physiological saline with pyruvate, lactate and glucose and they differ in their ion concentration and in the concentration of the energy sources. Complex media contains in addition to the basic components of simple media, amino acids, vitamins and purines (Bavister *et al.*, 1992; Hawk and Wall, 1993; Palta and Chauhan, 1998). The difference in maturation percentage among different types of medium may be attributed to the composition of the media (Nandi *et al.*, 2002b). TCM-199 contains both glutamine and glucose (Calder *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). The absence of glucose or pyruvate fails to support the spontaneous meiotic maturation of mouse oocytes and to maintain oocyte viability (Downs and Hudson, 2000).

**Hormonal addition to maturation media:** Results in Table 4 showed that addition of hormones increased overall percentages of oocytes arrested at MI and MII ( $p < 0.05$  and  $p < 0.001$ , respectively) and decreased those at GV and Deg. ( $p < 0.001$ ) as compared to maturation medium without hormones, regardless type of medium.

Results also showed that the highest percentage of oocytes arrested at MII stage due to hormonal addition was associated with reduction ( $p < 0.01$ ) in percentage of GV and Deg. as



Table 4: Effect of hormonal addition to maturation media on different maturation stages of buffalo oocytes

Hormones	Maturation stage (%)				
	Degeneration	MII	MI	GVBD	GV
Without	17.3±0.95	56.0±0.64	6.0±0.83	6.6±0.81	14.2±1.02
With	5.60±0.95	69.7±0.64	9.1±0.83	7.3±0.81	8.30±1.02
Significance	***	***	*	NS	***

NS: Not significant, \*,\*\*\*Significant at  $p < 0.05$  and  $p < 0.001$ , respectively

compared to maturation media without hormones (8.3 and 5.6% vs. 14.2 and 17.3%,  $p < 0.001$ ). However, hormonal addition did not affect significantly percentage of oocytes arrested at GVBD stage as compared to maturation medium without hormones (7.3 vs. 6.6%,  $\geq 0.05$ , Table 4).

Effects of hormonal addition (FSH, LH,  $E_2$ ) to maturation medium on the maturation rate of buffalo follicular oocytes was evaluated by Jamil *et al.* (2007), who found significant improvement in the percentage of maturation rates due to hormonal addition from in 33.4 to 48.2%, regardless medium type. The addition of hormone combinations (gonadotropins plus  $E_2$ ) with a source of sera such as estrous goat serum (EGS) to TCM-199 medium has also been found to be necessary for achieving high maturation rates (72.4%) for goat oocytes (Mogas *et al.*, 1992). Also, Chung *et al.* (1990) showed a positive effect of gonadotropin (LH) on nuclear maturation of *in vitro* matured buffalo oocytes. They revealed that the maturation rate using TCM-199 medium was 74.2, 62.9 and 79.5% for FSH, hCG and FSH plus Human Chorionic Gonadotropin (hCG), respectively. While with mSOF medium the maturation rate was 67.5 and 74.7% for FSH and FSH plus hCG, respectively. In general, several authors reported that adding gonadotropins (LH, FSH or their analogues, singly or in combination) in maturation media usually had beneficial effects on oocyte maturation (Ravindranatha *et al.*, 2002; Chohan and Hunter, 2003).

Germinal Vesicle Breakdown (GVBD) in many mammalian species, as meiosis-activating sterols (Tsafiriri *et al.*, 2005). As FSH and LH are the most common hormonal additives in an IVM, they are necessary for spontaneous oocyte maturation in term of improving oocyte cytoplasmic maturation by altering a range of cumulus cell activities (Sutton *et al.*, 2003). Addition of hormones such as FSH (Chauhan *et al.*, 1998) and LH and  $E_2$  (Nandi *et al.*, 2002a) to culture media has been made to cause significant increase in cumulus expansion.

FSH has a positive influence in meiosis resumption by increasing release of the intra-cellular calcium which flow down a concentration gradient, through the gap junction between follicle cells and into the oocyte to induce GVBD (Downs, 1993) and stimulating cumulus cells of oocytes-cumulus complexes to secrete a positive factor that override arrest due to hypoxanthine and that could trigger meiotic resumption (Byskov *et al.*, 1997) and also stimulating an increase of cAMP concentration and cumulus expansion (Saeki *et al.*, 1991). Moreover, Pandey *et al.* (2010) showed that FSH is essential for cumulus cell expansion and maturation of buffalo oocytes *in vitro* as FSH enhances the expansion of cumulus cells surrounding the oocytes which in turn enhances sperm capacitation and the fertilization process. Effect of LH on oocyte maturation was probably caused by FSH contamination of the LH (Choi *et al.*, 2001). Also, Abdoon *et al.* (2001) reported that IVM of buffalo oocytes in medium supplemented with FSH increased the cleavage rate and development of IVF in buffalo embryos compared with non-supplemented (control medium).

**Effect of interaction:** Effect of interaction between medium type and hormonal addition on *in vitro* nuclear maturation stages of buffalo oocytes including GV, GVBD, MI, MII and Deg. is illustrated in Fig. 1(a-e), respectively. Analysis of variance revealed that the type of

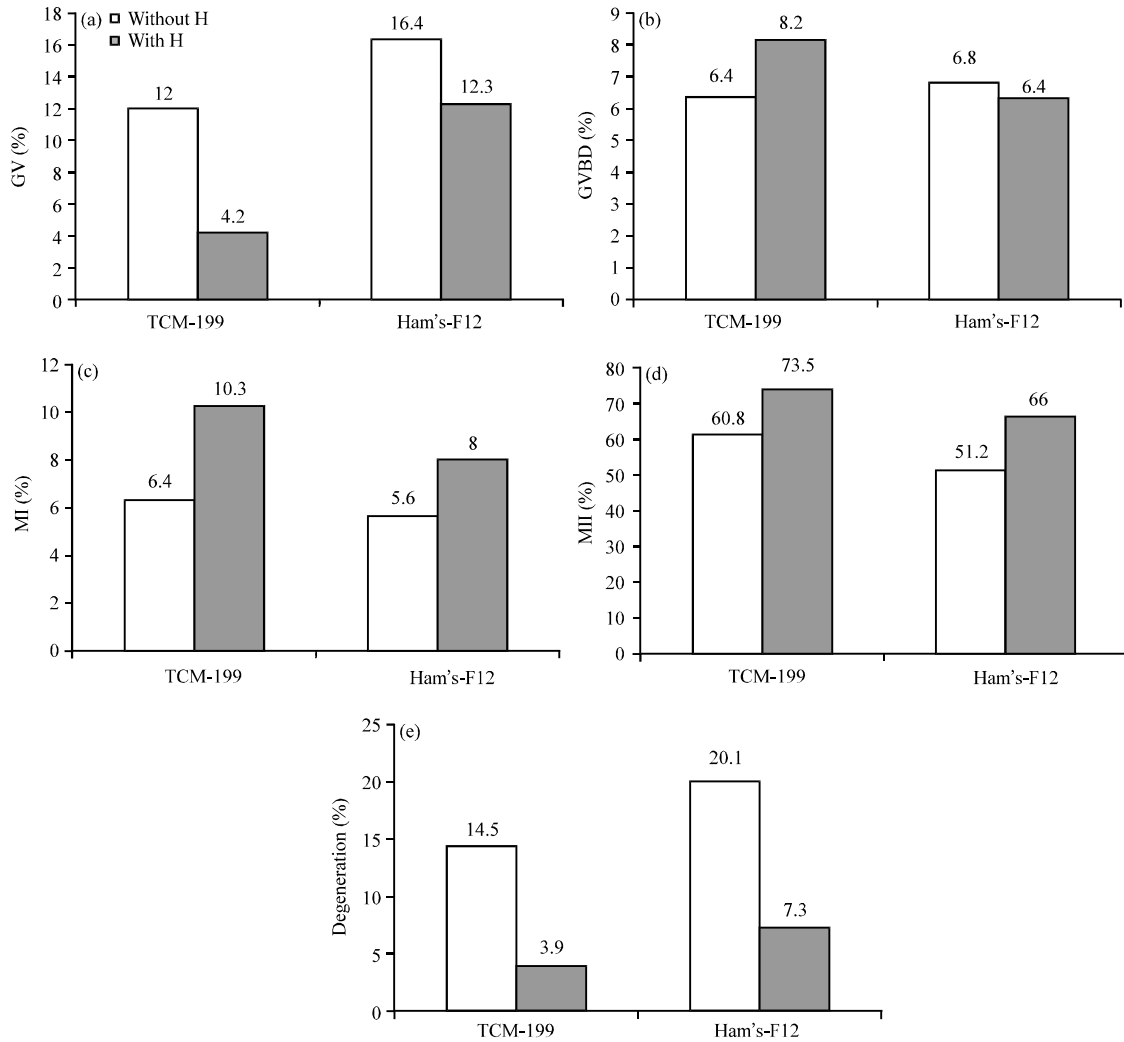


Fig. 1(a-e): Oocytes at different stages of maturation as affected by the interaction between medium type and hormonal addition, Percentage of (a) GV oocytes, (b) GVBD oocytes (c) Oocytes at MI, (d) Oocytes at MII and (e) Degeneration oocytes

medium did not interact significantly with hormonal addition on percentage of oocytes arrested at all stages of maturation. These results indicated almost beneficial effect on *in vitro* maturation of buffalo oocytes, especially on percentage of oocytes arrested at MII, using TCM-199 with hormonal addition more than using TCM-199 without hormones, Ham's F12 with or without hormones (73.5 vs. 60.8, 66.0 and 51.2%, respectively).

## CONCLUSION

In conclusion, the recovery of oocytes using slicing technique increased oocyte recovery rate and produced higher percentage of good quality oocytes (COCs and expanded oocytes) as compared to the dissection, aspiration and aspiration plus slicing techniques. Such results may indicate efficacy of slicing technique as a collection method on quantity and quality of buffalo oocytes. Also, *in vitro* maturation of buffalo oocytes was improved in TCM-199 medium supplemented with

hormones (FSH and E<sub>2</sub>). Hormonal addition, regardless medium type, had beneficial effect on *in vitro* maturation of buffalo oocytes.

## REFERENCES

- Abdoon, A.S.S., O.M. Kandil, T. Otoi and T. Suzuki, 2001. Influence of oocyte quality, culture media and gonadotropins on cleavage rate and development of *in vitro* fertilized buffalo embryos. *Anim. Reprod. Sci.*, 65: 215-223.
- Amer, H.A., A.O. Hegab and S.M. Zaabal, 2008. Effects of ovarian morphology on oocyte quantity and quality, granulosa cells, *in vitro* maturation and steroid hormone production in buffaloes. *Anim. Reprod. Sci.*, 5: 55-62.
- Barakat, I.A.H., S.A. Kandeal, H.M. El-Ashmaoui, A. Barkawi and E. El-Nahass, 2012. Effect of medium type and Luteinizing Hormone (LH) on *in vitro* maturation of Egyptian buffalo oocytes. *Afr. J. Biotechnol.*, 11: 4620-4630.
- Bavister, B.D., T.A. Rose-Hellekant and T. Pinyopumminter, 1992. Development of *in vitro* matured/*in vitro* fertilized bovine embryos into morulae and blastocysts in defined culture media. *Theriogenology*, 37: 127-146.
- Boediono, A., R. Rajamahendran, S. Saha, C. Sumantri and T. Suzuki, 1995. Effect of the presence of a CL in the ovary on oocyte number, cleavage rate and blastocyst production *in vitro* in cattle. *Theriogenology*, 43: 169-169.
- Byskov, A.G., C.Y. Andersen, A. Hossaini and X. Guoliang, 1997. Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. *Mol. Reprod. Dev.*, 46: 296-305.
- Calder, M.D., A.N. Caveney, L.C. Smith and A.J. Watson, 2003. Responsiveness of bovine Cumulus-Oocyte-Complexes (COC) to porcine and recombinant human FSH and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation *in vitro*. *Reprod. Biol. Endocrinol.*, Vol. 1. 10.1186/1477-7827-1-14
- Carolan, C., P. Monaghan, M. Gallagher and I. Gordon, 1994. Effect of recovery method on yield of bovine oocytes per ovary and their developmental competence after maturation, fertilization and culture *in vitro*. *Theriogenology*, 41: 1061-1068.
- Chauhan, M.S., S.K. Singla, P. Palta, R.S. Manik and M.L. Madan, 1998. *In vitro* maturation and fertilization and subsequent development of buffalo (*Bubalus bubalis*) embryos: Effect of oocyte quality and type of serum. *Reprod. Fert. Dev.*, 10: 173-177.
- Chohan, K.R. and A.G. Hunter, 2003. *In vitro* maturation and fertilization of water buffalo oocytes. *Buffalo J.*, 1: 91-101.
- Choi, Y.H., E.M. Carnevale, G.E. Seidel Jr. and E.L. Squire, 2001. Effects of gonadotropins on bovine oocytes matured in TCM-199. *Theriogenology*, 56: 661-670.
- Chung, Y.C., C.K. Kim, B.Y. Ryu, J.T. Yoon, H.T. Kim and K.S. Lee, 1990. Studies on the improvement of performance and reproductive efficiency in farm animal VI. Studies on improvement of development potential of *in vitro*-fertilized bovine follicular oocytes. *Korean J. Anim. Reprod.*, 14: 73-83.
- Das, G.K., G.C. Jain, V.S. Solanki and V.N. Tripathi, 1996. Efficacy of various collection methods for oocyte retrieval in buffalo. *Theriogenology*, 46: 1403-1411.
- De Wit, A.A., Y.A. Wurth and T.A. Kruij, 2000. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *J. Anim. Sci.*, 78: 1277-1283.

- Downs, S.M., 1993. Factors affecting the resumption of meiotic maturation in mammalian oocytes. *Theriogenology*, 39: 65-79.
- Downs, S.M. and E.D. Hudson, 2000. Energy substrates and the completion of spontaneous meiotic maturation. *Zygote*, 8: 339-351.
- Downs, S.M. and A. Verhoeven, 2003. Glutamine and the maintenance of meiotic arrest in mouse oocytes: Influence of culture medium, glucose and cumulus cells. *Mol. Reprod. Dev.*, 66: 90-97.
- Duncan, D.B., 1955. Multiple range and multiple *F* tests. *Biometrics*, 11: 1-42.
- Gandhi, A.P., M. Lane, D.K. Gardner and R.L. Krisher, 2000. A single medium supports development of bovine embryos throughout maturation, fertilization and culture. *Hum. Reprod.*, 15: 395-401.
- Gasparrini, B., 2002. *In vitro* embryo production in buffalo species: State of the art. *Theriogenology*, 57: 237-256.
- Gliedt, D.W., C.F. Rosenkrans Jr., R.W. Rorie, A.L. Munyon, J.N. Pierson, G.F. Miller and J.M. Rakes, 1996. Effects of media, serum, oviductal cells, and hormones during maturation on bovine embryo development *in vitro*. *J. Dairy Sci.*, 79: 536-542.
- Gordon, I., 1994. Oocyte Recovery and Maturation. In: *Laboratory Production of Cattle Embryos*, Gordon, I. (Ed.). CAB International, UK., pp: 20-142.
- Greve, T. and V. Madison, 1991. *In vitro* fertilization in cattle: A review. *Reprod. Nutr. Dev.*, 31: 147-157.
- Gupta, P.S.P. and P.V. Sharma, 2001. Oocyte recovery rates in buffaloes in relation to method of retrieval and presence of corpus luteum. *Buffalo J.*, 1: 137-143.
- Hammam, A.M., M.M. Zabal and H.A. Sabra, 1997. Effect of type of media on *in vitro* maturation, culture and fertilization of buffalo and cattle oocytes. *Beni-Swef Vet. Med. Res.*, 2: 242-259.
- Hasler, J.F., 2000. *In vitro* culture of bovine embryos in Menezo's B2 medium with or without coculture and serum: The normalcy of pregnancies and calves resulting from transferred embryos. *Anim. Reprod. Sci.*, 60-61: 81-91.
- Hawk, H.W. and R.J. Wall, 1993. Experiments to improve the yield of blastocysts from *in vitro* produced oocytes. *J. Anim. Sci.*, 72: 220-220.
- Hegab, A.O., A.E. Montasser, A.M. Hammam, E.M.A. Abu El-Naga and S.M. Zaabel, 2009. Improving *in vitro* maturation and cleavage rates of buffalo oocytes. *Anim. Reprod.*, 6: 416-421.
- Jamil, H., H.A. Samad, N.U. Rehman, Z.I. Qureshi and L.A. Lodhi, 2007. *In vitro* maturation and fertilization of riverine buffalo follicular oocytes in media supplemented with oestrus buffalo serum and hormones. *Acta Veterinaria Brno*, 76: 399-404.
- Jamil, H., H.A. Samad, Z.I. Qureshi, N.U. Rehman and L.A. Lodhi, 2008. Harvesting and evaluation of riverine buffalo follicular oocytes. *Turk. J. Vet. Anim. Sci.*, 32: 25-30.
- Kandil, O.M., A.S.S. Abdoon, C.H.D. Kacheva and B. Georgiev, 2013. Affect of *in vitro* culture media on embryo developmental competence and vitrification methods in blastocyst viability in buffalo. *Global Veterinaria*, 11: 622-629.
- Khan, I.Q., H.A. Samad and N.U. Rehman, 1997. Quantity and quality of buffalo follicular oocytes recovered by aspiration and scoring methods for *in vitro* studies. *Pak. Vet. J.*, 17: 187-189.
- Kharche, S.D., A.K. Goel, S.K. Jindal and N.K. Sinha, 2006. *In vitro* maturation of caprine oocytes in different concentrations of estrous goat serum. *Small Rumin. Res.*, 64: 186-189.
- Kim, M., S.J. Hong, J.H. Lee, C.K. Min, K.J. Hwang and R.W. Park, 2011. Comparison of *in vitro* maturation media of immature oocytes: The effectiveness of blastocyst culture media. *Fertil. Steril.*, 95: 554-557.

- Kumar, S. and S.N. Maurya, 2000. Evaluation of oocyte retrieval methods from bubaline ovaries. *Indian J. Anim. Res.*, 34: 130-132.
- Leibfried, L. and N.L. First, 1979. Characterization of bovine follicular oocytes and their ability to mature *in vitro*. *J. Anim. Sci.*, 48: 76-86.
- Lonergan, P., P. Monaghan, D. Rizos, M.P. Boland and I. Gordon, 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture *in vitro*. *Mol. Reprod. Dev.*, 37: 48-53.
- Madan, M.L., M.S. Chauhan, S.K. Singla and R.S. Manik, 1994. Pregnancies established from water buffalo (*Bubalus bubalis*) blastocysts derived from *in vitro* matured, *in vitro* fertilized oocytes and co-cultured with cumulus and oviductal cells. *Theriogenology*, 42: 591-600.
- Mehmood, A., M. Anwar, S.M.H. Andrabi, M. Afzal and S.M.S. Naqvi, 2011. *In vitro* maturation and fertilization of buffalo oocytes: The effect of recovery and maturation methods. *Turk. J. Vet. Anim. Sci.*, 35: 381-386.
- Mistry, C.N. and A.J. Dhami, 2009. Studies on follicular size and oocytes recovery rate from buffalo ovaries by slicing method. *Indian J. Field Vet.*, 5: 23-26.
- Mogas, T., A. Martino, M.J. Palomo and M.T. Paramio, 1992. Effect of method of recovery on the number and type of oocytes obtained for IVM. *J. Reprod. Fertil.*, 9: 53-53.
- Nandi, S., B.M. Ravindranatha, P.S. Gupta and P.V. Sarma, 2002a. Timing of sequential changes in cumulus cells and first polar body extrusion during *in vitro* maturation of buffalo oocytes. *Theriogenology*, 57: 1151-1159.
- Nandi, S., H.M. Raghu, B.M. Ravindranatha and M.S. Chauhan, 2002b. Production of buffalo (*Bubalus bubalis*) embryos *in vitro*: Premises and promises. *Reprod. Domest. Anim.*, 37: 65-74.
- Palta, P. and M.S. Chauhan, 1998. Laboratory production of buffalo (*Bubalus bubalis*) embryos. *Reprod. Fertil. Dev.*, 10: 379-392.
- Pandey, A., S.C. Gupta and N. Gupta, 2010. Effect of FSH and LH hormones on oocyte maturation of buffalo and gene expression analysis of their receptors and Cx43 in maturing oocytes. *Zygote*, 18: 231-234.
- Rao, M.M. and Y.U. Mahesh, 2012. Efficacy of different harvesting techniques on oocyte retrieval from buffalo ovaries. *Buffalo Bull.*, 31: 209-213.
- Ravindranatha, B.M., S. Nandi, P.S.P. Gupta and P.V. Sarma, 2001. Comparison of three different media on maturation of buffalo oocytes *in vitro*. *Indian J. Anim. Sci.*, 71: 841-843.
- Ravindranatha, B.M., S. Nandi, P.S.P. Gupta and P.V. Sarma, 2002. *In vitro* effects of different levels of commercially available PMSG on oocyte maturation. *Buffalo J.*, 1: 101-107.
- Raza, A., H.A. Samad, N.U. Rehman and E.U.H. Zia, 2001. Studies on *in vitro* maturation and fertilization of Nili-Ravi buffalo follicular oocytes. *Int. J. Agric. Biol.*, 3: 503-506.
- Rose, T.A. and B.D. Bavister, 1992. Effect of oocyte maturation medium on *in vitro* development of *in vitro* fertilized bovine embryos. *Mol. Reprod. Dev.*, 31: 72-77.
- Roushandeh, A.M., P. Pasbakhsh, Z. Alizadeh and M.H. Roudkenar, 2007. *In vitro* maturation media, cysteamine concentration and glutathione level affect blastocysts development in mouse. *Iran. J. Reprod. Med.*, 5: 159-163.
- SAS., 2001. SAS User's Guide Statistic's, Version 6.06. 4th Edn., SAS Institute Inc., Cary, NC.
- Saeki, K., M. Hoshi, M.L. Leibfried-Rutledge and N.L. First, 1991. *In vitro* fertilization and development of bovine oocytes matured in serum-free medium. *Biol. Reprod.*, 44: 256-260.
- Shirazi, A., N. Shams-Esfandabadi and S.M. Hosseini, 2005. A comparison of two recovery methods of ovine oocytes for *in vitro* maturation. *Small Rumin. Res.*, 58: 283-286.

- Sianturi, R.G., M. Thein, H. Wahid and Y.C. Rosnina, 2002. Effect of collection technique on yield of bovine oocytes and the development potential of oocytes from different grades of oocytes. *Indonesian J. Anim. Vet. Sci.*, 7: 188-193.
- Singh, L.R., K.S. Rao and K.M. Mohan, 2013. Oocyte retrieval methods, grade and percentage of oocytes in goats. *Int. J. Mol. Vet. Res.*, 3: 4-6.
- Smetanina, I.G., L.V. Tatarinova and A.S. Krivokharchenko, 2000. Influence of the culture medium composition on cattle oocyte maturation and embryogenesis *in vitro*. *Russian J. Dev. Biol.*, 31: 113-116.
- Suresh, K.P., S. Nandi and S. Mondal, 2009. Factors affecting laboratory production of buffalo embryos: A meta-analysis. *Theriogenology*, 72: 978-985.
- Sutton, M.L., R.B. Gilchrist and J.G. Thompson, 2003. Effects of *in vivo* and *in vitro* environments on the metabolism of the cumulus-oocyte complex and its influence on the oocyte developmental competence. *Hum. Reprod. Update*, 9: 35-48.
- Tamilmani, G., B.S. Rao, R. Vegdevi, D. Amarnath, B.R. Naik, M. Mutharao and V.H. Rao, 2005. Nuclear maturation of bovine oocytes in cultured preantral follicles. *Small Rumin. Res.*, 60: 295-305.
- Tsafiriri, A., X. Cao, H. Ashkenazi, S. Motola, M. Popliker and S.H. Pomerantz, 2005. Resumption of oocyte meiosis in mammals: On models, meiosis activating sterols, steroids and EGF-like factors. *Mol. Cell. Endocrinol.*, 234: 37-45.
- Van de Sandt, J.J.M., A.C. Schroeder and J.J. Eppig, 1990. Culture media for mouse oocyte maturation affect subsequent embryonic development. *Mol. Reprod. Dev.*, 25: 164-171.
- Wang, Z.G., Z.R. Xu and S.D. Yu, 2007a. Effects of oocyte collection techniques and maturation media on *in vitro* maturation and subsequent embryo development in Boer goat. *Czech J. Anim. Sci.*, 52: 21-25.
- Wang, Z.G., S.D. Yu and Z.R. Xu, 2007b. Effects of collection methods on recovery efficiency, maturation rate and subsequent embryonic developmental competence of oocytes in holstein cow. *Asian-Australasian J. Anim. Sci.*, 20: 496-500.
- Zoheir, K.M.A., A.S. Abdoon, K.F. Mahrous, M.A. Amer, M.M. Zaher, L.G. Yang and E.M. El-Nahass, 2007. Effects of season on the quality and *in vitro* maturation rate of Egyptian buffalo (*Bubalus bubalis*) oocytes. *J. Cell Anim. Biol.*, 1: 29-33.