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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (10): 1777-1781, 2004
ISSN 1028-8880

Collection and Grading of Bovine Cumulus-oocyte-complexes (COCs) from Slaughter House Ovaries in View of *in vitro* Maturation, Fertilization and Culture

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Abstracts: The research was carried out at the Animal Breeding and Genetics Laboratory under the Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh. It was conducted with a view to establish some relationship between ovarian condition in terms of the presence or absence of corpus luteum and morphological quality of the COCs as well as the relationship between follicular diameter and quality of COCs. Significantly highest ($p<0.01$) number of follicles was found in type III ovaries. The number of follicles measuring 2-6 mm diameter was observed to be significantly higher ($p<0.01$) in type III than type II and type I ovaries. Moreover, grade A and grade B COCs were significantly highest ($p<0.01$) in number in 2-6 mm diameter follicles. It was established that cumulus cells surrounding the oocytes favour to a less or greater extent the IVM (*In vitro* maturation), IVF (*In vitro* fertilization) of oocytes and subsequent IVC (*In vitro* culture) of zygotes. On the basis of the study, it is concluded that type III ovaries having no corpus luteum may be suggested for obtaining good quality Cumulus-oocyte-complexes (COCs) in experiment for IVM, IVF and subsequent IVC.

Key words: *In vitro* maturation, *in vitro* fertilization, *in vitro* culture, ovum pick up

INTRODUCTION

Genetic improvement of cattle in Bangladesh has made little progress in the last 30 years by Artificial Insemination (AI) technique. By Embryo Transfer (ET) technique outstanding cows can be used to produce remarkable number of progeny. This results in rapid genetic gain, which complements the AI program. Embryo transfer technology involves the use of superior females as donors of embryos and there are three ways of embryo transfer technology, I) Multiple Ovulation Embryo Transfer (MOET), ii) *in vivo* Ovum Pick Up (OPU) and iii) oocyte recovery at slaughter. Though the former two techniques are useful, they require huge involvement. From that standpoint, embryo production from slaughterhouse ovaries might be considered as a low cost and sustainable technology in the arena of livestock development in Bangladesh. Embryo production from slaughterhouse ovaries involves collection and grading of Cumulus-oocyte-complexes (COCs), *In vitro* Maturation (IVM), *In vitro* Fertilization (IVF) and subsequent *In vitro* Culture (IVC). *In vitro* maturation of immature oocytes from ovaries at slaughter, followed by IVF and IVC of the resulting zygotes has allowed extensive research on modern reproduction techniques in farm animals. Since Mukherjee[1] reported that mouse oocytes could be matured and fertilized *in vitro* and developed to final term, there have been intensive attempts in cattle and pigs with the technique. Hanada *et al.*[2] finally succeeded in getting calves from IVM oocytes that were fertilized *in vitro*. In pigs, Mattioli *et al.*[3] succeeded in getting piglets from IVM-IVF oocytes in 1989. Fulka and Okolski[4] first performed the IVM of mare oocytes in 1981. For *in vitro* embryo production, the efficient collection and grading of Cumulus-oocyte-complexes (COCs) are the primary steps to be done. Several methods such as aspiration of total follicular material, dissection/rupturing of individual isolated follicles with subsequent isolation of the Cumulus-oocyte-complexes (COCs) and slicing of the ovaries have been described to obtain immature oocytes from slaughterhouse ovaries. However, techniques such as slicing of the ovaries, flushing the follicles with Phosphate Buffered Saline (PBS) or rupturing the isolated

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follicles may increase the number of recovered oocytes as compared with that of aspiration of follicular materials[5]. Fulka and Okolski[4] reported that compact-cumulus equine oocytes needed more than 24 h to be matured *in vitro* and subsequent studies abroad confirmed this observation[6]. In addition, it has been shown that a comparable higher maturation rate could be reached within 24 h of culture if the oocytes had a compact-cumulus investment[7].

In addition to the quality of COCs, other factors are also responsible for the success of *In vitro* Embryo Production (IVEP). The maturation medium and the selection of protein supplements and hormones for IVM play an important role in the subsequent fertilization and development of mammalian oocytes during *in vitro* culture[8]. Several factors such as the addition of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), estradiol, granulosa cells and either of fetal calf or estrous serum to the culture medium have to be considered for maximizing the success[9]. The role of gonadotropins in the *in vitro* maturation of oocytes has also been demonstrated, since the resumption of meiosis was improved when gonadotropins were added to the culture medium[10,11]. Fukushima and Fukui [12] reported that the addition of FSH, LH and estradiol to a medium improved the fertilizability of bovine oocytes cultured *in vitro*. Addition of steroids especially estradiol, improves the completion of maturation[13]. The conditions stated above obviously have great importance for quality embryo production through IVM, IVF and IVC but selection of quality COCs for initiation of such experiment is the primary task to be done. The use of ovaries from slaughterhouse animals implies the use of COCs from females whose exact physiological state is variable and sometimes unknown and the most viable oocytes can only be selected according to their morphological characteristics. Denuded oocytes or oocytes with few cumulus cells are usually rejected because of their low capacity of fertilization and/or *in vitro* development[14]. However, a great deal of work has been done regarding collection of Cumulus-oocyte-complexes (COCs) from slaughterhouse ovaries, grading of collected oocytes, IVM, IVF of the oocytes and IVC of the zygotes throughout the world. But in Bangladesh, no such work has so far been undertaken. Slaughterhouse ovaries can be an economic source of oocytes for IVM, IVF and IVC experiment. Embryos can be produced from ovaries of the cows that are usually being slaughtered in slaughterhouses for meat purpose and the embryos thus produced can be transferred to the recipient cows. Moreover, recent advances in biotechnology have enabled the researchers to produce cloned and genetically modified animals by manipulating *in vitro* produced embryos. The present research work has been undertaken for the first time in Bangladesh as a very preliminary approach to embryo production from oocytes collected from slaughterhouse ovaries. The further approach to conduct a trial for *in vitro* maturation experiment could not be undertaken because of unavailability of some necessary instrument and apparatus at our disposal. But then it is hoped that the work would be a base line for the future researchers who will attempt to make further contribution in this field of animal biotechnology. Under the circumstances the main objectives of the present study were to know collection and grading procedures of bovine cumulus-oocyte-complexes (COCs) obtained from slaughterhouse ovaries, to establish the relationship between ovarian condition and morphological quality of the collected COCs and to establish the relationship between follicular diameter and quality of COCs.

MATERIALS AND METHODS

The experiment was conducted from January 2002 to March 2002 at the Animal Breeding and Genetics Laboratory, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Collection and processing of ovaries: Thirty-four ovaries from cows of unknown reproductive history were collected from local slaughterhouses in Mymensingh. The representative photograph of the ovaries is shown in (Fig. 1). The ovaries were kept in 0.9% normal saline in a thermoflask at 25 to 30°C and transported to the laboratory within 3 to 4 h of slaughter. The ovaries in the laboratory were kept in the same saline in sterilized petridishes. The ovaries were then grouped on the basis of the presence or absence of Corpus Luteum (CL) into 3 types: type I with functional corpus luteum, type II with regressed corpus luteum and type III without corpus luteum. The number of ovaries in each group was recorded. All visible graafian follicles were collected by incising each ovary with scissors and blades followed by scraping the ovarian tissue with forceps and needles. After collection of all visible follicles on the surface of each ovary, the ovary was dissected to expose additional follicles within it. The follicles after collection from each ovary were stored in saline at room temperatures. For each ovary, the number of follicles collected was recorded and the diameter of each follicle was measured and recorded with the help of slide calipers.

Grading of cumulus-oocyte-complexes (COCs): The follicular materials from each follicle were harvested by blunt dissection on a sterilized glass slide. The follicular materials of each follicle were then observed under microscope and COCs were classified into 4 grades as

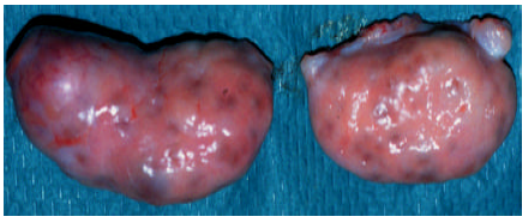

Fig. 1: Representative photograph of the ovaries

described elsewhere, briefly as grade A-homogeneous COCs; grade B-COCs not homogeneous; grade C-COCs were not found at all and grade D-expanded COCs. The number of different grades of COCs in each ovary of three types was recorded.

Statistical model and methods of data analysis: The experiment was designed as Completely Randomized Design (CRD). Data were statistically analyzed using MSTAT computer package programme in accordance with the principles of CRD[15] and least significant difference (LSD) test was done to identify the significant differences between the mean values when analyses of variance (ANOVA) showed significant differences[16]. The statistical models used for the analysis of the number of ovaries/follicles were as follows:

Statistical model: One way statistical model used in the analysis of data was as follows:

$Y_{ij} = \mu + t_i + e_{ij}$

Where:

Y_{ij} = Individual observation
μ = General mean
t_i = Treatment effect
e_{ij} = Random error term, normally and independently distributed with mean '0' and variance 'σ^2'

The statistical model for the a two factor completely randomized design was:

$Y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + e_{ijk}$

Where:

Y_{ijk} = Record of the ith ovarian type/follicular diameter and jth grade of COCs/follicular diameter in the kth observation
μ = General mean
a_i = Effect of ith ovarian type/follicular diameter
b_j = Effect of jth grade of COCs/follicular diameter
$(ab)_{ij}$ = Interaction effect of ith ovarian type/follicular diameter and jth grade of COCs/follicular diameter
e_{ijk} = Random error term, normally and independently distributed with mean '0' and variance 'σ^2'

RESULTS AND DISCUSSION

Ovaries of cows were collected from local slaughterhouses. The ovaries were classified into type I, type II and type III as described in materials and methods. In total 34 ovaries were collected. Among them 8 were categorized as type I, 7 as type II and 19 as type III. Data were collected on different parameters such as number of follicles per ovary, diameter of each follicle and microscopic state of the COCs. A total of 202 follicles was obtained from 3 types of ovary. Among them 35, 37 and 130 follicles were collected from type I, type II and type III ovaries, respectively. The COCs were classified into 4 grades based on their microscopic morphology.

Ovarian types and number of follicles per ovary: Significantly higher ($p<0.01$) number of follicles was observed in type III (6.48 ± 0.38) followed by type II (5.28±0.62) and type I (4.37 ±0.58) (Table 1). It is well established that all female mammals are born with a large store of follicles which rapidly declines as puberty approaches but whether this early losses represent a mechanism of physiological wastage is not definitely known. Follicle growth initiation is one of the most important and least understood aspects of ovarian biology and represents a major challenge for experimental study. Changes in the local microenvironment such as the pH and hormonal concentration probably occur as the follicles evolve into the primary stage but these are probably effects rather than causes[17]. In cattle, there are three wave patterns of follicular selection, although two waves or sometimes four waves can occur during the estrous cycle[18]. Each wave of follicular development is characterized by simultaneous emergence of medium sized (>4 mm in diameter) growing follicles from a pool of smaller follicles. One of these groups of follicles rapidly emerges as the dominant follicles (7-9 mm in diameter) and continues to develop; while the others undergo atresia and regress. In cattle, it usually takes 5 to 7 days for the dominant follicles to develop the ovulatory size[19,20]. Despite the overwhelming occurrence of follicular atresia the cellular and molecular mechanisms underlining this phenomenon still remain poorly understood. Growth initiation of follicles has variously been attributed to i) hormonal triggers (Gonadotrotopins), ii) stochastic processes (fluctuation in internal signal molecule) and iii) external inhibitory control from growing follicles[17]. The balance between the gonadotropins (FSH and LH) and steroids (estrogen and progesterone) might be the important criteria in this process. The highest number of follicles that were found in type III ovaries in the present study, might reflect the optimum level of gonadotropins and steroids. Type III ovaries did not contain the CL and

Table 1: Ovarian types and number of follicles per ovary

Type of ovary	Total number of ovaries	Total number of follicles	Number of follicles per ovary (Mean±SE)**
Type I	8	35	4.37 ±0.58b
Type II	7	37	5.28±0.62ab
Type III	19	130	6.48±0.38a

**Means with different superscripts differ significantly from each other within the column (p<0.01)

Table 2: Ovarian types and number of COCs

Types of ovary	Number of COCs**			
	Grade A	Grade B	Grade C	Grade D
Type-I	1.71cd	0.71d-f	0.42ef	1.28c-f
Type-II	2.85ab	1.42c-e	0.57ef	0.42ef
Type-III	3.57a	1.85bc	0.28f	1.71cd

**Means with different superscripts differ significantly from each other both in columns and rows (p<0.01)

Table 3: Number of follicles of different diameter

Types of ovary	Number of follicles**		
	<2 mm diameter	2-6 mm diameter	>6 mm diameter
Type–I	0.57c	3.14b	0.57c
Type–II	0.28c	4.71a	0.28c
Type–III	1.28c	5.85a	0.14c

**Means with different superscripts differ significantly from each other both in columns and rows (p<0.01)

Table 4: Number of COCs of different grades for different follicular diameter

Follicular diameter	Number of COCs**			
	Grade A	Grade B	Grade C	Grade D
<2 mm	0.58cd	0.08ef	0.14ef	0.12ef
2-6 mm	2.03a	1.32b	0.41de	0.88c
>6 mm	0.03f	0.06ef	0.12ef	0.15ef

**Means with different superscripts differ significantly from each other both in columns and rows (p<0.01)

the negative effect of progesterone on anterior pituitary was not functional in this type of ovaries. Similarly, second highest number of follicles in type II and least number in type I ovaries further confirmed the above statement as regressed CL was found in type II and functional CL was found in type I ovaries. As the ovaries were collected from slaughterhouses it was impossible to confirm the cyclic state of the ovaries, so there might have some discrepancies in the present result.

Ovarian types and number of COCs: Significantly highest (p<0.01) number of grade A and grade B COCs was observed in type III followed by type II and type I ovaries (Table 2). Hafez[21] reported that progesterone secreted by the luteal cells of the CL inhibited estrus and gave the negative feedback on the anterior pituitary to secrete FSH. As a result, the growing follicles regressed and became atretic. Though in the present study the effect of progesterone on follicular growth could not be investigated, it can be assumed that the higher number of grade A and B COCs in type III and medium to low number of COCs in type II and type I ovaries might arise from the activity of CL. These results further confirmed the previous findings described in Table 1. Slightly higher number of grade C COCs was found in type II and medium to fewer number in type I and type III, respectively. But the differences among the types did not reach in significant level. As progesterone was reported to be responsible for follicular atresia[21], the findings are very much in conformation. The less number of grade C COCs found in type I ovary might arise from other factors causing atresia. In respect of the number of grade D COCs, the unexpected highest value was found in type III and second highest was in type I though the differences did not reach significant level. The lowest number of this grade of COCs was found in type II ovaries. This discrepancy might come from lack of recording of the ovarian state as slaughterhouse ovaries were used in the present study.

Number of follicles on the basis of diameter: Follicles were classified into 3 types based on their diameter: <2 mm, 2-6 mm and >6 mm diameter (Table 3). Significantly highest number of follicles of 2-6 mm diameter was observed in type III, but the difference between type III and type II did not reach in significant level (p<0.01). No significant difference was observed in the number of follicle of <2 mm and >6 mm diameter in three types of ovaries. Oocytes collected from the follicles of 2-6 mm diameter were usually used for IVM and IVF and subsequent IVC study. Totey *et al.*[22] in buffalo and Rath *et al.*[23] in cow collected 2-6 mm diameter follicles to determine the efficiency of *in vitro* maturation and fertilization experiment and found the desirable results. The highest number of 2-6 mm and <2 mm diameter follicles obtained in type III ovaries indicated that the recruitment and selection of ovarian follicles were functional in usual manner due to lack of progesterone activity. Comparatively less number of <2 mm and 2-6 mm diameter follicles obtained in type I and type II explains the reverse effect of progesterone activity. The higher and medium number of >6 mm diameter follicles in type I and type II might indicate the growing follicles of the luteal phase but a further study to confirm it would be helpful.

Number of COCs of different grades: Significantly highest number of grade A COCs followed by grade B COCs were observed in 2-6 diameter follicles (Table 4). The most notable observation was that the less number of grade A and grade B COCs was obtained from >6 mm diameter follicles. Cumulus cells during maturation period supported the IVM of oocytes to the metaphase II stage and development to the blastocyst stage in cattle[24]. Porcine cumulus free oocytes could not be developed beyond 4-cell stage[25]. In the current study grade A and grade B COCs were found in significantly highest number

in type III ovaries as well as in follicles with 2-6 mm diameter. So follicles of 2-6 mm diameter from type III ovaries can be an important source of oocytes for IVM, IVF and subsequent IVC study.

ACKNOWLEDGMENTS

This study was carried out at the Animal Breeding and Genetics laboratory under the Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh. The authors are grateful for the financial support of the University Authority and the Department of Animal Breeding and Genetics for Research Cooperation. The authors would also like to thank Bangladesh Agricultural University for allowing us to carry out the study and to research assistants for their cooperation.

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