INFLUENCE OF DIFFERENT QUALITY OF BUFFALO OOCYTES ON *IN-VITRO* MATURATION AND FERTILIZATION

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ABSTRACT

The study was conducted on 499 freshly collected abattoir ovaries of Surti buffaloes. The total number of different grades of oocytes recovered by slicing method was 1046. The mean numbers of oocytes per ovary of grade A (2.92 ± 0.14) and B (2.49 ± 0.13) were significantly higher (P<0.05) followed by grade C (1.66 ± 0.09) than that of grade D (1.32 ± 0.10) oocytes. The overall maturation rate achieved for different grades of oocytes was 80.97 per cent in TCM-199 supplemented with 0.6 per cent BSA. The percentage of cytoplasmic maturation observed in oocytes of grade A, B, C, and D was highest for good (50.30 %), fair (37.70 %) and poor (33.30 %) and 28.60 %) quality of oocytes, respectively. A significantly higher number of grade A (>3 layer of cumulus cells) and grade B (1-3 layer of cumulus cells) quality of oocytes attained good cytoplasmic maturation than grade C (less compact cumulus cells) and grade D (nude oocytes). The total maturation rate from grade A oocytes was highest (87.94 %) followed by oocytes of grade B (82.91 %) and grade C (72.22 %). The nuclear maturation was evaluated in 528 oocytes by Hoechst 33342 stain. The highest number of grade A oocytes (26.00 %) reached to M-II stage, followed by grade B (24.21 %) and grade C (14.28 %) and none of the oocytes from grade D reached to M-II stage; they were mainly arrested at GV and GVBD stage. Oocytes of grade A (n=181) and B (n=305), which had cytoplasmic maturation, were utilized for *in-vitro* fertilization. The overall fertilization rate observed was 20.16 per cent for grade A and B oocytes. The higher fertilization rate was observed for grade A (21.00 %) oocytes than that in grade B (19.67 %). The study revealed that the presence of cumulus layer around the oocytes affected the maturation and fertilization rate of buffalo oocytes. Significantly (P<0.05) greater number of oocytes per ovary was recovered (3.31 ± 0.36) when the CL was absent compared with ovaries on which CL was present (1.01 ± 0.05). Thus, the effect of presence Vs absence of CL on the ovaries had significant effect on recovery rate of buffalo oocytes.

Key words: Buffalo oocytes, *In-vitro* maturation, *In-vitro* fertilization, Cumulus Cells, TCM-199 media

INTRODUCTION

The success of *in-vitro* production of buffalo embryo has been hampered by many factors including low number of follicles on the ovaries (Ty *et al.*, 1989; Jainuddin *et al.*, 1993), poor recovery rate of oocytes (Kumar *et al.*, 1997) and poor *in-vitro* fertilization efficiency of buffalo oocytes (Holm *et al.*, 1999; Farin *et al.*, 2001). Whether these problems arise due to intrinsic quality of buffalo follicular oocytes themselves or due to inadequacies of culture system has yet to be understood. It has been noted that a decrease of storage temperature for bovine ovaries resulted in slower *in-vitro* embryonic development (Yang *et al.*, 1990; Aman and Parks, 1994). Research has also shown that the increase in storage time of the ovary decreases the embryonic development of oocytes aspirated from water buffalo (Ravindranatha *et al.*, 2003). *In-vitro* maturation (IVM) and *in-vitro* fertilization (IVF) procedures performed on oocytes obtained from slaughter house derived ovaries have recently provided a practical means for producing large number of bovine zygotes at low cost for research and commercial settings (Hansen, 2006).

MATERIALS AND METHODS

Ovaries from the adult Surti buffaloes were collected within half an hour of their slaughter from the local abattoir and were transported within an hour to the laboratory in a vacuum flask containing normal saline (0.9 % NaCl) at pH 7.0 supplemented with gentamicin 50 µg/ml (Sigma, G 3632) and the temperature of the solution was maintained at 25-30°C (Totey *et al.,* 1992). Upon arrival at the laboratory, extraneous tissue and fat were removed and ovaries were washed with 70 per cent ethanol to check contamination, followed by three washes of the normal saline (39°C). Ovaries were finally dried with sterilized paper towel.

After measurement of follicle size and evaluation of CL, ovaries were sliced with a fine BP blade and transferred in 100 mm disposable searching petri dish (Tarson® INDIA) with warm normal saline (Das *et al.*, 1996). The contents of all sliced ovarian follicles recovered were searched for cumulus oophorus complexes (COCs) in 100 mm petri dish. A stereozoom microscope (Olympus SZX9, Tokyo, Japan) was used to identify oocytes. The oocytes were transferred to pre-warmed drops of 100 µl of gonadotrophin free hepes-buffered TCM-199 medium, which was covered with 3 ml of silicon oil (Sigma) in 35 x 10 mm (Sonar® Axiva) plastic petri dish.

Classification of oocytes was done on the basis of cumulus investment and ooplasm homogeneity (Gordon, 1995). They were classified on the basis of cumulus investment and ooplasm homogeneity, viz. grade A (>3 layers of cumulus cells), grade B (1-3 layers of cumulus cells), grade C (less compact cumulus) and grade D (nude) oocytes.

The oocytes of different grades selected for maturation were kept in tissue culture medium (TCM-199). Oocytes were washed three times with the *in-vitro* maturation (IVM) medium that consisted of TCM-199 supplemented with bovine serum albumin (BSA) without gonadotrophin. The maturation was achieved by keeping 2-4 oocytes per 5 µl drop of IVM medium, overlaid with 3 ml of silicon oil, in 35 mm petri dish for 24 h in CO_2 gas incubator (5 % each of CO_2 and O_2) at 39°C.

After culturing for 24 h, COCs were denuded for 5 min with gentle pipetting or votexing to remove cumulus cells. Denuded oocytes that reached the M-II stage with consecutive stages of germinal vesicle breakdown (GVBD), metaphase I (M-I) and metaphase II (M-II) were considered as matured. This was confirmed by staining with 0.1 per cent bis-benzimide (Hoechst 33342). The maturation of oocytes was assessed under the inverted microscope with florescence unit (Leica DMIL, Germany).

In swim up method (Parrish *et al.*, 1988), 0.25 ml of fresh semen was deposited at the bottom of 1.5 ml of incubated wash medium (TALP: modified calcium-free Tyrode's Albumin Lactate Pyruvate made with 6 mg/ml BSA fraction-V, 50 IU/ml penicillin and 50 µg/ml streptomycin, pH 7.4). After washing of sperms, motile spermatozoa were added with capacitation agent (heparin, hypotaurine and epinephrine) and kept for capacitation in the incubator for 20 minutes.

Following capacitization and counting, the sperms were added to the fertilization drop in the concentration of $1x10^6$ sperm/ml. The fertilization drops contained modified TALP with 0.2 µmol/l penicillamine, 0.1 µmol/l hypotaurine, 0.02 µmol/l epinephrine, 6 mg/ml fatty-acid free BSA, 30 µg/ml heparin and 50 µg/ml Gentamicin. The oocytes transferred from maturation dishes to a petri dish containing 2 ml wash medium (Hepes-buffered TALP supplemented with 3 mg/ml BSA, fraction-V). The oocytes were washed in wash medium and then were added to fertilization droplets (100 µl). Oocytes and spermatozoa were co-incubated (38.5°C, 5 % CO_2 in air with maximum humidity) under silicon oil for 18-20 h. The fertilization was confirmed by staining with 0.1 per cent bis-benzimide (Hoechst 33342). The fertilization of oocytes was assessed under the inverted microscope with florescence unit (Leica DMIL, Germany).

RESULTS AND DISCUSSION

The mean numbers of oocytes per ovary of grade A (2.92 ± 0.14) and B (2.49 ± 0.13) were significantly higher (P<0.05) followed by grade C (1.66 ± 0.09) than that of grade D (1.32 ± 0.10) oocytes. The overall mean number of oocytes recovered per ovary was 2.28 ± 0.07. The oocytes recovery in the present study was higher than that reported by Makwana and Shah (2009). They reported significantly higher (P<0.05) mean number of oocytes per ovary of grade A (0.82 ± 0.04) and B (0.79 ± 0.04) followed by grade D (0.76 ± 0.05) than grade C (0.64 ± 0.04) oocytes. The findings of the present study are in agreement with the report of Sarvaiya (1997, unpublished data), who recovered 3.55 oocytes per ovary by aspiration and slicing method. The high proportion of *in-vitro* recovery of the oocytes in the present study might be due to the good body condition and reproductive status of the slaughtered buffaloes.

Cytoplasmic maturation and its quality were evaluated on the basis of expansion of COCs. The percentage of cytoplasmic maturation of grade A, B, C and D oocytes was highest for good (50.30 %); fair (37.70 %) poor (33.30 %), and poor (28.60 %) quality, respectively. The overall cytoplasmic maturation rate was the highest (87.94 %) from grade A oocytes followed by grade B oocytes (82.91 %) and grade C oocytes (72.22 %). The overall *in-vitro* maturation rate of all the four grades of oocytes in Surti buffalo was 80.97 per cent, whereas 19.00 per cent oocytes failed to mature. The result indicated that quality of matured oocytes was dependent on grades of oocytes selected for maturation. These findings compared with those of Shah and Makwana (2009). They found 78.05 per cent maturation rate in TCM-199 supplemented with 0.6 per cent BSA and reported the highest percentage of cytoplasmic maturation for oocytes of grade A, B, C and D for good (54.93 %), fair (43.34 %), poor (40.68 %) and poor (44.66 %) quality of oocytes, respectively. Our finding of maturation rate is in agreement with the finding of Ocana-Quero *et al.* (1999), who found 85 per cent maturation rate with TCM-199 medium supplemented with BSA. TCM-199 is the most widely used complex medium for performing IVM of buffalo oocytes, except for a few studies in which Ham's F-10 has been used (Totey *et al.*, 1992).

The nuclear maturation was evaluated in 528 oocytes by staining the matured oocytes with Hoechst 33342 stain. The highest number of grade A oocytes (26.00 %) reached to M-II stage, followed by grade B (24.21 %), and grade C (14.28 %) and none of the oocytes from grade D reached to M-II stage. They were mainly arrested at GV and GVBD stage. The grade D oocytes did not mature and maximum percentage of degenerated oocytes (72.50 %) was found in this category. Shah and Makawana (2009) reported the highest number of grade B oocytes (44.91 %) which reached to M-II stage, followed by grade A (42.12 %) and grade C (8.28 %). The grade D oocytes did not mature and maximum percentage of oocytes got degenerated (42.21 %). Amer *et al.* (2008) observed 58 per cent of oocytes with >3 layers of cumulus reaching to M-II stage and 5 per cent of oocytes with no cumulus reaching to M-II stage. The nuclear maturation rate was higher than the present study. This might be attributed to the culture media used, handling of the oocytes, culture condition and laboratory facilities.

Oocytes of grade A (n=181) and B (n=305), which had cytoplasmic maturation were utilized for *in-vitro* fertilization. The fertilization droplet contained modified TALP medium with good quality of matured oocytes in which motile swim up separated and capacitated sperms ($1x10^6$ /ml) were added. An assessment of fertilization of oocytes was done on the basis of the penetration of sperms into matured oocytes and was confirmed with Hoechst 33342 stain. In the present study, overall 20.16 per cent of fertilization for grade A and B was observed. A little higher fertilization rate was observed for grade A (21.00 %) oocytes than that of grade B (19.67 %) oocytes, but the difference in fertilization rate was found to be non-significant. The *in-vitro* fertilization rates of the present study are lower than those of Goto *et al.* (1989) who found 53 to 55 per cent fertilization rate in bovine with BO medium (Brackett and Oliphant, 1975) supplemented with 5 mM caffeine without bovine serum albumin, whereas

Ijaz and Hunter (1989) found higher fertilization rate (82.5 %) of the oocytes with the Ca^{2+} free Tyrode's medium. Ball *et al.* (1983) reported that the presence of cumulus cells was not necessary for penetration of ova, but they have also observed that when cumuli were present, the frequencies of ova with both male and female pronuclei were increased ($P<0.05$). Comparatively lower fertilization rate of *in vitro* matured oocytes observed in the present study could be attributed to difference in the experience of operator and the laboratory/incubation conditions.

Three hundred and sixty seven ovaries were examined to know the effect of presence or absence of corpus luteum (CL) over the ovary on oocyte recovery rate. A careful examination of the ovaries revealed that CL was present on 73 and absent on 294 ovaries, which yielded 74 and 972 oocytes, respectively. Significantly ($P<0.05$) greater number of oocytes per ovary was recovered (3.31 ± 0.36) when the CL was absent compared with ovaries on which CL was present (1.01 ± 0.05). Thus, the effect of presence Vs absence of CL on the ovaries had significant effect on recovery rate of buffalo oocytes. Nandi *et al.* (2000) reported decreased recovery rate of oocytes when ovary had a CL. This is because the follicular development is restricted as the lutein cells occupy most of the portion of the ovary (Kumar *et al.*, 1997). The dominant follicle is usually observed in the CL bearing ovaries and the other follicles are very small and remain mostly inaccessible (Gasparrini *et al.,* 2000). The present study revealed significantly higher recovery rate from ovaries with absence of CL.

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