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Quality assessment of yak oocytes for *in vitro* production of yak embryos

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*T*he efficiency of yak *in vitro* production (IVP) is low (Zi *et al*. 2004). Therefore, the present study was undertaken to investigate the influence of cumulus cells support for *in vitro* maturation, fertilization and embryonic development of yak oocytes.

Yak ovaries were transported to the laboratory in a thermosflask containing sterile normal saline solution with penicillin (100 IU/mL) and maintaining temperatures of 26–37ºC within 2–3 h of collection. After the removal of extraneous tissues and ovaries were washed 3–4 times with normal saline containing antibiotic for further processing. For collection of oocytes, TCM-199 medium was used separately in conjugation with BSA (3700µg/ml), L-glutamine (100µg/ml) and gentamicin (50µg/ml). Media were incubated in CO_2 incubator for 2 h at 38.5ºC at 5% level of CO_2 with 90–95 % humidity before use and were filtered through 0.2µm filter. The cumulus oocyte complexes (COCs) were recovered from the ovarian follicles by using both aspiration and post-aspiration slicing techniques. The collected oocytes were washed 3–4 times in 35 mm petri-dishes containing TCM-199 as a basic medium in conjugation with FBS 1ml/10ml, sodium pyruvate 0.9mg/10ml and cysteamine 50µM. After washing, the oocytes were categorized as good, fair and poor quality based on their gross morphology and integrity of cumulus cells (Pawshe 2010): (i) oocytes with more than 4 layers of compact cumulus cells with evenly distributed cytoplasm were rated as good quality; (ii) oocytes with one or two layers or with partial cumulus cells with uniform cytoplasm were classified as fair; (iii) oocytes without cumulus cells with uniform cytoplasm were classified as poor quality oocytes. The selected oocytes were washed 3–4 times in washing media followed by 1–2 washed in *in vitro* maturation media, i.e., tissue culture medium (TCM-199) supplemented with 10% follicular fluid, 0.2 mM sodium pyruvate, L- glutamine, 10% heat inactivated oestrus cow serum (HIOCS), 10 µg/mL follicle-stimulating hormone (pFSH) and 1 µg/mL 17ß estradiol. The oocytes were transferred into 50µl droplets (10 oocytes/droplet) of IVM media in a 35 mm Petri-dish and incubated in a CO_2 incubator maintaining 5% CO_2, 38.5°C with 90–95% relative humidity, for 24 h. For the study of the maturation rate of the oocytes, after 24 h of maturation some of the oocytes were fixed in acetic acid: ethanol (1: 3), stained with aceto-orcein and classified as germinal vesicle (GV), metaphase I (M-I) and metaphase II (M-II). Oocytes with a cumulus cell expansion were selected for *in vitro* fertilization from the media and carefully transferred to IVF droplets (BO media + BSA) after washing 2–3 times in B.O media.

Frozen yak semen straws (2) were thawed and poured into a round-bottom centrifuge tube (swim-up tube) containing 2– 3ml of filtered working BO medium (Brackett and Oliphant 1975) and BSA (0.01g/ml). The tubes were incubated for 1 h (swim-up) at 38.5ºC and 5% CO_2 in a 90–95 % humidified atmosphere. After 1 h, the top layer containing live/motile spermatozoa were transferred into a clean centrifuge tube containing fresh 3– 4 ml of working BO medium and centrifuged at 2,700–2,800 rpm for 10 min. The supernatant was removed and the pellet of spermatozoa was resuspended in 3–4 ml of BO medium and centrifuged again at 2,700–2,800 rpm for 5 min. Finally, the sperm suspension was added into each drop containing oocytes. Oocytes were co-incubated with sperm for 18–22h at 38.5°C and 5% CO_2 in 90–95 % humidified incubator. The processed semen was added in IVF droplets. Oocytes and spermatozoa (40% or more motility) were co-incubated for 18–22 h at 38.5°C under 5% CO_2 in 90–95 % humidified incubator. For the study of the fertilization rates, some of the oocytes were fixed in acetic acid: ethanol (1: 3) after 18–22 h of fertilization, stained with lacto-acetic lacmoid and evaluated for the presence of pronuclei. After 18–22 h of sperm-oocyte co-incubation, oocytes were placed in 35 mm petri-dishes containing 2–3 ml IVC media (mCR2aa stock containing BSA fraction V @0.08g, FBS 1ml/10ml and gentamicine 50µg/ml) and washed for 3–5 times by repeated pipetting. If necessary, the presume zygotes were also vortex for 2–3 min in an eppendorf containing IVC media for removal of dead sperms and cumulus cells adhered on the surface of the zygotes; 10–12 numbers of zygote were randomly distributed to droplet into 50 µl of IVC droplets for 5 to 7 days. These zygotes were incubated

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in CO_2 incubator and IVC media were replaced with fresh IVC media in every 48 h. The confirmation of IVF and cell division of the zygotes were checked in every 24 h of interval. The effect of IVM, fertilization, and embryonic development of morphologically classified yak oocytes were analyzed by using one-way ANOVA test.

The recovery rates of oocytes per ovary were 4.14 and 2.14 in aspiration and post-aspiration slicing technique respectively. Guo *et al*. (2006) reported the rate of recovery of yak oocytes to be 4.70 which was almost similar with the present study but Kumar and Maurya (2000) in bubaline ovaries and reported much lower recovery rate (0.46/ovary) by post aspiration slicing method than the present study. The recovery rate of good, fair and poor quality oocytes were 58.17, 28.76 and 13.07%, respectively, in aspiration and 24.05%, 34.18 and 41.77%, respectively, using the post-aspiration slicing technique. Overall recovery rates of good, fair and poor quality oocytes were 46.55, 30.6 and 22.84%, respectively, and total oocytes recovery per ovary was 6.27.

The per cent maturation (M-II) rate of good quality oocytes (66.99%) was significantly higher (P<0.01) as compared to fair and poor quality oocytes but in metaphase-I the maturation rate was higher (P<0.05) in fair (25.81%) than in good and poor quality oocytes (Table 1). The per cent degeneration was higher (P<0.01) in poor as compared to good and fair quality oocytes (Table 2). There are several essential components required for completion of both nuclear and cytoplasmic maturation, the most important of them being the presence of cumulus cells (Trounson 1992). Our findings on the maturation rate are in agreement with those reported in buffaloes (Chauhan *et al*. 1999) and also in agreement with other studies that showed a reduced maturation percentage in unselected, fair quality and denude bovine oocytes *in vitro* as compared to selected oocytes, which had a compact cumulus cells investment (Im *et al*. 1995).

After 18–22 h of co-incubation, oocytes were evaluated for evidence of total fertilization, normal fertilization and abnormal fertilization. The total fertilization rate was 59.09, 45.24 and 20.0% in good, fair and poor quality oocytes, respectively. The normal fertilization rate, with oocytes containing 2 pronuclei, was higher (P<0.01) in good quality oocytes than in fair and poor quality oocytes. There was a significant (P<0.01) higher total fertilization rate in good (59.09%) than in fair (45.24%) and poor (20%) quality oocytes. Our observation is in overall consonance with the normal fertilization rate (67.4–84.3%) in bovine oocytes (Im *et al*. 1995). The difference in fertilization and normal fertilization rates in yak oocytes containing more than 4 layers of cumulus cells towards the lower range compared to bovine oocytes might be attributed to species differences. Conversely, the percentage of abnormal fertilization was higher in poor (13.33%) than in good and fair quality oocytes (Table 1). The present study observed that the abnormal fertilization rate is higher in cumulus free or denuded oocytes in mice (Downs *et al*. 1986). Fertilization abnormalities and low development of *in vitro* matured and fertilized bovine oocytes (Pavlok *et al*. 1992) are likely due to deficiencies in the cytoplasmic maturation and may be related to the type of oocytes and the size of follicles from which the oocytes are collected. The difference in fertilization rates of different types of oocytes, as observed by different workers, may be attributed to a different composition of media for IVM and IVF, as well as species differences.

The cleavage rate of fertilized oocytes was significantly

Table 1. Percentage rates of *In vitro* maturation of morphologically classified yak oocytes.

Morphological classification of yak oocytes	Total number of oocytes	Maturation (%)			
		Germinal vesicle	Metaphase I	Metaphase II	Degeneration
Good	103	8(7.77)	20(19.41)	69 (66.99)a	6(5.83)a
Fair	62	5(8.06)	16(25.81)	28 (45.16)b	13(20.97)b
Poor	41	7(17.07)	6(14.63)	7(17.07)c	21(51.22)c
F-value	0.22^{NS}	4.58*	18.73**	10.15**	

**P<0.01, *P<0.05, ^{NS}Nonsignificant. a, b, c mean value in the same column with different superscripts differ significantly (P<0.01).

Table 2. Percentages of *In vitro* cleavage and embryonic development of morphologically classified yak oocytes

Morphological classification of yak oocytes	Total number of oocytes	Development (%)		
		Cleavage	Morula	Blastocyst
Good	66	45 (68.18)a	11 (16.67)	27 (40.9)a
Fair	42	19 (45.23)b	8 (19.05)	7 (16.67)b
Poor	30	6 (20.0)c	2 (6.67)	0(0.0)
F-value	13.37**	2.65^{NS}	18.02**	

**P<0.01, ^{NS}Nonsignificant. a, b, c mean value in the same column with different superscripts differ significantly (P<0.01).

($P<0.01$) higher in good quality oocytes as compared to that in fair and poor quality oocytes in the present experiment (Table 2). The rates of development to morula stage of embryos from good, fair and poor quality oocytes were 16.67%, 19.05% and 6.67%, respectively (Table 2). Interestingly, there was no significance difference in morula development between different categories of oocytes (Table 2). However, blastocyst development from morula stage of embryos was significantly ($P<0.01$) higher in good than fair and poor quality oocytes (Table 2). The present finding of a 68.18% cleavage rate obtained from good quality oocytes is very similar to that previously reported in yaks (62.7–65%) (Zi *et al*. 2009); however, the authors reported a higher blastocyst formation. Similarly, a higher percentage of cleavage, morula and blastocyst development are observed in good quality oocytes as compared to fair and poor quality oocytes in cattle (Fukui 1990). The lower cleavage rate and embryonic development observed in the present study may be attributed to the hypothesis investigated by other working on development competence and cleavage formation in bovine oocytes (Pavlok *et al*. 1992). Therefore, oocytes containing compact cumulus cells should be selected to achieve maximum maturation and fertilization rates, leading to the development of transferable embryos (morula and blastocyst) in yaks.

In vitro embryo production in yaks yet remains in a promising early stage, and protocols related to this technique need to be standardized. This is the first of such efforts, which might lead to better outcomes in the near future for the *in vitro* production of yak embryos.

SUMMARY

Yak oocytes were isolated and morphologically classified into 3 categories, i.e. good, fair and poor quality oocytes according to the compactness of the cumulus cells, and were assigned for *in vitro* maturation, fertilization and embryonic development. Oocytes were retrieved by both aspiration and post-aspiration slicing technique, and the overall recovery rates of good, fair and poor quality oocytes were estimated at 46.55, 30.6 and 22.84% respectively. Maturation rates (M II) in good quality oocytes were significantly higher as compared to that in fair and poor quality oocytes. Total and normal fertilization rates were also higher in good quality oocytes than in fair and poor oocytes. Good quality oocytes led to embryonic development with significant numbers of transferrable embryos (*i.e*. morula and blastocyst); 60% of morula was further developed to blastocyst. Thus, it is concluded that the selection of good oocytes according to cumulus cells compactness is an important criterion for *in vitro* maturation, fertilization, and embryonic development in yaks.

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