Developmental Competence of Oocytes Recovered from Postmortem Ovaries of the Endangered Indian Blackbuck (*Antilope cervicapra*)

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Abstract. The ability to rescue gametes from endangered or wildlife species and to subsequently produce viable embryos holds tremendous potential as a means to increase the population size of endangered or wildlife species. The objective of this study was to assess the meiotic and developmental competence of oocytes recovered from postmortem ovaries of the Indian blackbuck. Oocytes collected from the ovaries of dead blackbucks were allowed to mature *in vitro* and then tested for developmental potential by activation with ionomycin followed by treatment with 6-dimethylaminopurine. The average number of oocytes recovered per ovary was 10.9, and recovery of the oocytes did not depend on the presence or absence of the corpus luteum, on the side, size and weight of the ovaries or on the type of oocytes recovered. The proportion of good quality oocytes showing cumulus expansion and extrusion of the first polar body were 79.3% and 46.1% when cultured with gonadotropins. *In vitro* maturation studies indicated that the proportion of oocytes that reached MII stage was significantly higher when good quality oocytes (68%) were used compared with fair quality oocytes (48%) when cultured in the presence of gonadotropins. Furthermore, fifty eight percent of the *in vitro* matured oocytes cleaved, and thirteen percent of the cleaved oocytes developed into blastocysts. These findings suggest that the oocytes recovered from postmortem ovaries of the blackbuck can be utilized for production of embryos.

Key words: Blackbuck, *In vitro* maturation, Parthenogenetic activation

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The blackbuck (*Antilope cervicapra*) is an endangered antelope native to the Indian subcontinent and has been listed as a Schedule I (endangered) animal under the Indian Wildlife Protection Act, 1972. The International Union for Conservation of Nature (IUCN) also lists the blackbuck as a near threatened animal [1].

Assisted reproductive technologies such as artificial insemination, *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF), cryopreservation of oocytes and embryos, somatic cell nuclear transfer and embryo transfer are becoming increasingly important tools in the conservation and management of wild/endangered species [2–7]. However, the application of reproductive biotechnologies for the preservation of endangered mammalian species is limited by several factors [8, 9] such as the lack of availability of species-specific biological material required for a better understanding of the fundamental biology of the male and female gametes [10]. It is in this context that rescue of gametes from wild/endangered animals that have died unexpectedly is a worthwhile research tool for understanding the fundamental physiology of the species concerned and also for development of species-specific

protocols for application of new emerging assisted reproductive technologies in endangered species. In addition, production of competent oocytes by *in vitro* maturation (IVM) will positively facilitate IVF and somatic cell nuclear transfer (SCNT) technology for conservation of endangered animals.

Little research has been performed so far on collection and preservation of oocytes and on *in vitro* production of antelope embryos. To date, very few attempts have been made to explore the possibility of recovery, IVM and IVF of oocytes collected from ovaries of antelopes either from supervised culls or after death as reported earlier in klipspringer [11], blesbok [12, 13], addax antelope [14], blackbuck [15], black wildebeest [13], dorcas gazelle [16] and springbok [13, 17]. To our knowledge, no information is available on oocyte recovery, morphology or IVM of oocytes in the blackbuck. It is in this context, the present study aims to present information on ovarian biometry, oocyte recovery, oocyte characteristics and IVM of oocytes collected from ovaries of the Indian blackbuck (*Antilope cervicapra*) postmortem.

Materials and Methods

Preparation of media

All the media, hormones, fetal bovine serum (FBS) and other chemicals used were purchased from Sigma Chemical, USA, and plasticware was obtained from Nunc, Denmark. HEPES buffered

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tissue culture medium 199 (TCM 199H) with 10% FBS, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) was used for collection, washing and handling of oocytes. The washed oocytes were then incubated either in the control medium or gonadotropin-treatment medium. The control medium was prepared by supplementing bicarbonate buffered TCM 199 (TCM 199B) with 10% FBS, 0.22 mM sodium pyruvate, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). The gonadotropin medium was prepared by supplementing the control medium with 10 μ g/ml of follicle stimulating hormone (FSH) and 6 IU/ml luteinizing hormone (LH). All media were sterilized by filtration through 0.22 μ m filters (Millipore, USA) and equilibrated with 5% carbon dioxide in air in a humidified atmosphere at 38.5 C for at least 2 h prior to use.

Source of ovaries and oocyte collection

Blackbucks were reared in captivity at the Nehru Zoological Park, Hyderabad, India. Female blackbucks were reared together in four adjoining indoor pens (2.5 $\times$ 2.5 $\times$ 2.0 m each) at night and were set free in a paddock (13 $\times$ 12 m) during the day. Animals were exposed to the natural photoperiod and fed once in the morning with commercial cattle feed (Poshak Feeds, Hyderabad, India) supplemented with a mineral mixture (Agrimim; GlaxoSmithKline India, Mumbai, India). Green fodder was also provided twice a day, and the animals had free access to clean drinking water throughout the day. Ovaries from six female blackbucks aged 3–9 years that had died due to shock from sudden disturbances in the surroundings were collected 6–12 h after death at postmortem. The ovaries were placed individually in labelled, screw-top plastic tubes containing prewarmed (35 C) Dulbecco's phosphate buffered saline (D-PBS; Gibco, Invitrogen Corporation, USA) supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin and transported to the laboratory in a thermos flask maintained at 38.5 C within 1–2 h of collection. After reaching the laboratory, the ovaries were trimmed of adjacent fat and ligaments, and each ovary was examined for the presence of visible follicles and corpora lutea (CLs). Ovarian dimensions (length, width and thickness) were measured with Vernier callipers, and the weight was recorded. Each ovary was rinsed (2–3 sec) once in 70% alcohol and thrice in prewarmed (37 C) D-PBS with antibiotics. Ovaries were placed into a 60-mm culture dish containing 5 ml of the oocyte handling medium supplemented with 25 IU/ml heparin (collection medium). Each ovary was processed individually. Incisions were made along the ovarian surface using a sterile surgical blade to release the cumulus-oocyte complexes (COCs) into a culture dish containing the oocyte collection medium. After slicing, each ovary was washed with the collection medium. Culture dishes were then kept undisturbed for 5 min to allow the oocytes to settle. The excess medium was then removed using a sterile syringe without disturbing the oocytes at the bottom of the culture dish. Thereafter, the culture dishes were examined for COCs under a stereozoom microscope (Nikon, Japan). Isolated COCs were then washed extensively in fresh droplets of handling medium and classified as good, fair or poor quality according to criteria used by Rao *et al.* [18]. Good COCs included oocytes with a homogeneous cytoplasm surrounded by more than three layers of compact cumulus cells. Fair COCs were similar to good COCs but were surrounded

by at least one layer of cumulus cells. In the poor COCs, the oocyte cytoplasm was fragmented and had few or no cumulus cells. The poor COCs were not used.

Measurement of oocyte diameter

The average diameter of each oocyte with and without a zona (ooplasm) was measured at three different sites of the oocyte before and after *in vitro* maturation. Zona thickness was measured as the average thickness of the zona at five different sites of each oocyte. Oocyte diameter and zona thicknesses were determined using the XYClone software (Hamilton Thorne, USA).

In vitro maturation of cumulus-oocyte complexes

Good and fair quality COCs were selected for *in vitro* maturation. For this purpose, the selected COCs were washed extensively in fresh droplets of handling medium and then with the *in vitro* maturation medium. The washed COCs (in groups of 5–10) were then transferred into a 50- μ l droplet of either pre-equilibrated control or gonadotropin maturation medium and overlaid with equilibrated mineral oil. These droplets containing COCs were cultured in a incubator with 5% CO₂ under humidified air at 38.5 C for 24 h.

Evaluation of oocytes following in vitro maturation

At the end of the *in vitro* maturation period, COCs were examined for cumulus cell expansion. Subsequently, the oocytes were denuded of cumulus cells by repeatedly passing through a fire-polished narrow-bore glass pipette and examined for extrusion of the first polar body. Oocytes were also evaluated for nuclear status according to method used by Rao *et al.* [18]. Briefly, oocytes were washed thrice in microdroplets of handling medium containing Hoechst fluorescent dye (5 μ g/ml) and incubated for 15 min at 38.5 C. Subsequently, oocytes were examined using a fluorescence microscope (Olympus IX 70, Olympus Optical, Japan). The nuclear status of each oocyte was classified into one of five stages: (1) germinal vesicle (GV); (2) germinal vesicle breakdown (GVBD); (3) metaphase I (MI); (4) telophase I/metaphase II (MII); or (5) degenerate (Fig. 1). Oocytes exhibiting cumulus cell layer expansion, extrusion of the first polar body and presence of the MII stage were considered to be mature oocytes. Nuclear maturation was defined by the number of oocytes that had extruded the first polar body and the number of oocytes that had matured to the MII stage relative to the total number of oocytes that were cultured *in vitro*. Oocytes that were arrested at the GVBD stage or only progressed to MI were considered to be immature. Oocytes with fragmented cytoplasm or without chromatin were classified as degenerate.

Activation of oocytes

Oocytes that had extruded the first polar body were selected for activation. These oocytes were exposed to ionomycin (5 μ g/ml) in the culture medium (TCM199B containing 15% FBS) for 5 min, washed thoroughly in handling medium and then incubated in culture medium supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) for 5 h in a humidified atmosphere of 5% CO₂ in an incubator at 38.5 C. After completion of 5 h incubation in 6-DMAP, the

Fig. 1. *In vitro* maturation of blackbuck oocytes recovered from the ovaries (a) of dead Indian blackbucks. Corpora lutea (black arrow) and follicles (white arrow) were visible in the ovaries. Freshly collected culture grade oocytes (b) before IVM and various stages of oocyte maturation, such as the oocyte with cumulus cell expansion (c), oocyte with the first polar body (d), the germinal vesicle stage (e), germinal vesicle breakdown (f), metaphase I (g), telophase I (h) and metaphase II and the first polar body (i), are shown. *In vitro* development of 2–4 cell embryos (j) and a blastocyst (k) following parthenogenetic activation of IVM oocytes. The oocytes were stained with Hoechst 33342.

oocytes were washed thoroughly and cultured in culture medium for 7 days for further development.

Statistical analysis

The data were analyzed by using the SPSS version 16.0 software (SPSS, USA). Oocyte recovery and morphometry was analyzed by the Student's *t*-test. For *in vitro* maturation, the percentage data were arcsine transformed and analyzed by two-way analysis of variance followed by a least square difference (LSD) test. Differences in means were considered significant at $P < 0.05$.

Results

A total of 12 ovaries were collected from six female blackbucks postmortem. The average length, width, thickness and weight of

the ovary were 1.43 ± 0.10 cm, 1.09 ± 0.08 cm, 0.75 ± 0.06 cm and 0.75 ± 0.12 g, respectively. The mean number of visible follicles (>2 mm diameter) present on the ovaries was 5.33 ± 0.84 (range: 1 to 11).

Oocyte recovery

A total of 131 oocytes were collected from 12 ovaries, out of which 45.8% were good ($n=60$), 25.2% were fair ($n=33$) and 29.0% were considered poor ($n=38$). The good and fair quality oocytes ($n=93$) were used in all subsequent studies. The number of good and poor quality oocytes recovered per ovary did not depend on the size, weight, presence or absence of a CL or location of the ovary (left or right side; Table 1). However, the presence or absence of a CL and size of the ovary had a significant effect on the recovery of fair quality oocytes (Table 1).

Table 1. Factors affecting oocyte recovery rate in blackbuck ovaries*

Attributes	Number of oocytes recovered (Mean $\pm$ SE)				
	Total	Good	Fair	Poor	Culture grade
I) Ovarian status					
CL present (n=3)	10.0 $\pm$ 1.0 ^a	6.0 $\pm$ 1.0 ^a	1.3 $\pm$ 0.3 ^a	2.7 $\pm$ 0.3 ^a	7.3 $\pm$ 1.2 ^a
CL absent (n=9)	11.2 $\pm$ 1.7 ^a	4.7 $\pm$ 0.8 ^a	3.2 $\pm$ 0.8 ^b	3.3 $\pm$ 0.8 ^a	7.9 $\pm$ 1.0 ^a
II) Side of ovary					
Right (n=6)	10.0 $\pm$ 1.6 ^a	4.7 $\pm$ 1.0 ^a	2.3 $\pm$ 0.9 ^a	3.0 $\pm$ 0.3 ^a	7.0 $\pm$ 1.4 ^a
Left (n=6)	11.8 $\pm$ 2.1 ^a	5.3 $\pm$ 0.8 ^a	3.2 $\pm$ 0.9 ^a	3.3 $\pm$ 1.2 ^a	8.5 $\pm$ 1.2 ^a
III) Ovarian size					
< 8 $\times$ 11 $\times$ 15 mm ³ (n=6)	12.5 $\pm$ 1.0 ^a	4.7 $\pm$ 0.8 ^a	4.2 $\pm$ 0.9 ^a	3.7 $\pm$ 1.2 ^a	8.8 $\pm$ 1.2 ^a
> 8 $\times$ 11 $\times$ 15 mm ³ (n=6)	9.3 $\pm$ 1.5 ^a	5.3 $\pm$ 1.1 ^a	1.3 $\pm$ 0.3 ^b	2.7 $\pm$ 0.3 ^a	6.7 $\pm$ 1.3 ^a
IV) Ovarian weight					
Less than 750 mg (n= 6)	12.5 $\pm$ 1.0 ^a	4.7 $\pm$ 0.8 ^a	4.2 $\pm$ 0.9 ^a	3.7 $\pm$ 1.2 ^a	8.8 $\pm$ 1.2 ^a
Greater than 750 mg (n=6)	9.3 $\pm$ 1.5 ^a	5.3 $\pm$ 1.1 ^a	1.3 $\pm$ 0.3 ^b	2.7 $\pm$ 0.3 ^a	6.7 $\pm$ 1.3 ^a

* Values are expressed as means $\pm$ SEM. Means with different superscripts within a characteristic differ significantly (P<0.05).

Table 2. Micrometry of different grades of oocytes recovered from the ovaries of blackbucks*

Grade	Oocyte diameter (μ m)		Ooplasm diameter (μ m)	Zona thickness (μ m)
	With cumulus cells	Without cumulus cells		
Good	222.6 $\pm$ 1.8	154.8 $\pm$ 1.9 ^a	126.7 $\pm$ 1.7 ^a	13.3 $\pm$ 0.1 ^a
Fair	194.4 $\pm$ 2.9	147.5 $\pm$ 2.6 ^b	118.4 $\pm$ 2.5 ^b	13.3 $\pm$ 0.2 ^a
Poor	—	156.9 $\pm$ 2.5 ^a	127.1 $\pm$ 2.5 ^a	13.9 $\pm$ 0.6 ^b
Overall	212.2 $\pm$ 1.7	153.7 $\pm$ 1.3	124.8 $\pm$ 1.3	13.4 $\pm$ 0.1

* Values are expressed as means $\pm$ SEM. Means with different superscripts within a column differ significantly (P<0.05).

Table 3. Maturation of blackbuck oocytes after *in vitro* maturation in the presence or absence of gonadotropins*

Oocyte quality	Gonadotropins	Number of oocytes	Morphological assessment		Nuclear assessment				
			CCE**	PBE**	GV** (%)	GVBD** (%)	MI** (%)	TI/MII** (%)	Deg ** (%)
Good	—	15	60.4 $\pm$ 6.2 ^a	33.3 $\pm$ 5.8 ^a	6.2 $\pm$ 3.1 ^a	20.8 $\pm$ 7.2 ^a	33.3 $\pm$ 5.8 ^a	39.6 $\pm$ 6.25 ^a	0.00 ^a
Fair	—	11	45.8 $\pm$ 7.9 ^a	27.2 $\pm$ 6.3 ^a	8.3 $\pm$ 4.1 ^a	20.8 $\pm$ 12.5 ^a	25.0 $\pm$ 8.3 ^a	33.3 $\pm$ 13.6 ^a	12.5 $\pm$ 6.2 ^a
Good	+	45	79.3 $\pm$ 5.2 ^b	46.1 $\pm$ 3.3 ^a	3.4 $\pm$ 2.1 ^a	9.6 $\pm$ 1.6 ^b	15.1 $\pm$ 2.7 ^b	68.0 $\pm$ 5.2 ^b	3.6 $\pm$ 3.5 ^a
Fair	+	22	65.6 $\pm$ 7.5 ^a	38.8 $\pm$ 6.8 ^a	8.5 $\pm$ 5.1 ^a	13.9 $\pm$ 5.2 ^{ab}	18.9 $\pm$ 5.8 ^{ab}	48.0 $\pm$ 6.9 ^{ab}	10.4 $\pm$ 6.2 ^a

*Values expressed as means $\pm$ SEM. Means having different superscripts with in a column differ significantly (P<0.05). **CCE, cumulus cell expansion; PBE, polar body extrusion; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; TI/MII, telophase I/metaphase II; Deg, degenerated.

Oocyte morphometry

Morphometric data such as oocyte diameter with and without cumulus cells, ooplasm diameter and zona thickness of the good, fair and poor quality oocytes are shown in Table 2. The results indicated that the mean diameter of the oocytes with cumulus cells did not depend on the quality of the oocytes, but when the mean diameter of the oocytes without cumulus cells was compared between the different groups of oocytes, it was significantly higher in the good and poor quality compared with the fair quality oocytes. The highest mean diameter of the ooplasm in the poor and good quality oocytes was similar (about 127 μ m) but significantly higher than that of the fair quality oocytes (118.46 $\pm$ 2.56 μ m; Table 2). The zona thickness of the good and fair quality oocytes was not significantly different. However, the zona thickness of the poor quality oocytes was significantly higher than those of the good and

fair quality oocytes (Table 2).

In vitro maturation (IVM)

In vitro maturation studies using good and fair quality indicated that the proportion of oocytes at different stages of development, such as those that exhibited cumulus cell expansion, polar body extrusion, presence of germinal vesicle and degeneration (Fig. 1), in both the control and gonadotropin treated groups were similar (Table 3). However, in the gonadotropin treated oocytes, the percentage of oocytes that exhibited cumulus cell expansion, germinal vesicle breakdown, Metaphase I and Telophase I and Metaphase II was higher (P<0.05) in the good quality oocytes compared with the good and fair quality oocytes of the control group. Furthermore, the good and fair quality oocytes of the gonadotropin treatment group differed significantly only with respect to the percentage of

cumulus cell expansion (Table 3).

Development of in vitro matured blackbuck oocytes after parthenogenetic activation

A total of 38 oocytes that extruded the first polar body were subjected to activation with ionomycin followed by treatment with 6-DMAP. Fifty eight percent of the oocytes cleaved (22/38), and thirteen percent of the cleaved oocytes developed to the blastocyst stage after 7 days.

Discussion

IVM followed by IVF and embryo transfer has resulted in live offspring only in selected wild species with a low success rate [10], and this has been attributed to problems related to the total absence or incomplete knowledge of the fundamental biology of male and female gametes and embryos [9]. Thus far, only one report has been published on *in vitro* development of blackbuck embryos derived from male and female gametes collected postmortem [15]. However, basic information regarding ovarian biometry, oocyte recovery, oocyte morphometry, IVM and activation of oocytes in the blackbuck has not yet been reported. In the present study, the average number of culture grade oocytes (fair and good quality) recovered per ovary was 7.75, and this number is similar to the number of oocytes that were recovered from the dorcas gazelle [16] and white tailed deer [19] but higher than that observed in the blesbok [12] and springbok [17], in which 5 and 2.7 oocytes were recovered, respectively. Furthermore, it is also known that the number of oocytes recovered depends on the species, age, season and reproductive status of the donor animal [20, 21]. The presence of a CL is also known to adversely affect the number of oocytes recovered in cattle [22], sheep [23], goats [21] and buffalo [24], and the cause has been attributed to the fact that progesterone secreted from the CL inhibits the growth of follicles and increases their atresia [25]. However, in bovine species, oocyte yield per ovary was higher for ovaries with a CL than without a CL [26, 27]. The reasons for this observed discrepancy are not clear. In the present study, oocyte recovery was not affected by the presence of a CL. The reason for this could be that both completely regressed CLs and regressing CLs were observed in the present study, and it is known that the progesterone levels secreted by a regressing CL are lower than those secreted by a functional CL. Therefore, it is likely that the low level of progesterone produced by the regressing CL may not be sufficient to inhibit growing follicles in the ovary. Indeed, this may be so, the case, and the observation that the proportion of good quality oocytes was higher in the CL group than in the CL absent group supports this hypothesis.

The large ($> 8 \times 11 \times 15 \text{ mm}^3$) ovaries and ovaries $> 750 \text{ mg}$ in weight yielded fewer oocytes than the ovaries that were small ($< 8 \times 11 \times 15 \text{ mm}^3$) and $< 750 \text{ mg}$ in weight. This is in contrast to the ovaries of sheep, in which the mean number of oocytes recovered did not depend on the size of the ovaries [23].

The mean diameters of blackbuck oocytes with and without a zona (ooplasm) were found to be smaller than those of eland antelope [28] and Asian buffalo oocytes [29] aspirated from antral follicles. The mean thickness of the blackbuck zona pellucida was

lower than that of the eland antelope [28] and higher than that of the goat [30]. It has been observed that the mean oocyte diameter increased from 96 ± 0.3 to $136 \pm 0.6 \mu\text{m}$ as follicle size increased from 0.5 to 2–3 mm in the goat [31].

This is the first report of successful IVM and activation of oocytes collected from blackbuck ovaries after death using TCM 199 medium supplemented with pyruvate, gonadotropins and fetal calf serum. This modified TCM199 medium was used previously for IVM of oocytes of antelopes, such as the blesbok [12, 13], addax antelope [14], blackbuck [15], black wildebeest [13], dorcas gazelle [16], springbok [13, 17], eland and bongo [32]. Oocytes that were induced to mature *in vitro* (postmortem derived) developed to morulae/blastocysts following IVF in klipspringer [11], blackbuck [15] and springbok [17] antelopes. In the present study, the proportion of oocytes (68%) that reached the MII stage after 24 h of culture was similar to that of the eland antelope (63%) [32], higher than the proportions observed in other antelopes, such as the springbok (35%) [13], blesbok (39%) [13], dorcas gazelle (47%) [16] and black wildebeest (52%) [13], and lower than that the proportions observed in white tailed deer (77%) [19] and bongo antelope (81%) [32]. The low maturation rates may be due to the method of collection, since it has been recorded that slicing probably leads to the inclusion of non-competent oocytes from small diameter follicles [33], or to delay in collection of the ovaries from the animals after death. The time interval between animal death and collection of ovaries is important especially in wild animals because the onset of autolytic changes may cause degenerative changes in the follicles. In the present study, since the ovaries were collected due to the unexpected deaths of the blackbucks, the delay ranged between 6 to 12 h after death of the animals. Such delays could affect the ovaries and COCs that are recovered [34]. Other factors known to influence the success of *in vitro* maturation of oocytes include age, reproductive status, health of the oocyte donor, proper supplementation of the culture medium and quality of the oocytes [35, 36].

Concomitant with nuclear maturation during which the oocytes achieve the MII stage, the oocytes also undergo cytoplasmic maturation, which is a prerequisite for normal fertilization and development [37]. Granulosa and cumulus cells play a major role in oocyte growth, acquisition of meiotic competence and cytoplasmic maturation [38]. It has also been demonstrated that physical contact between the oocyte and cumulus cells is necessary for transfer of nutrients and factors essential for oocyte development [24, 39]. This may be the reason why the blackbuck oocytes with homogenous cytoplasm surrounded by compact layers of cumulus cells in the present study had a significantly higher maturation rate.

In the present study, ionomycin followed by immediate exposure to 6-DMAP was used to induce oocyte activation. The only other report on activation of *in vitro* matured oocytes in antelopes was carried out using oocytes of the dorcas gazelle, and in that study, ethanol was used for activation [16]. Ionomycin followed by 6-DMAP has also been shown to be effective for inducing parthenogenesis of bovine [40], goat [41] and sheep [42] oocytes.

The differences in the potential of oocytes to develop into embryos may indicate differences with respect to cytoplasmic maturation, since oocyte competence to develop tends to increase with

increasing follicle size [43, 44] and oocyte diameter [29, 45]. In the present study, the poor developmental rates after activation of *in vitro* matured oocytes may have been due to the variation in the size of the oocytes, since it has been reported that slicing probably leads to the inclusion of small diameter oocytes [33]. Although the oocytes reached the MII stage, only 13% of the cleaved oocytes developed to the blastocyst stage, and this may be due to improper cytoplasmic maturation. Miao *et al.* [46] demonstrated that the number of cells per blastocyst and blastocyst rates declined remarkably after *in vitro* maturation and activation of oocytes when the time interval between removal of ovaries and animal death increased. This may indeed be the case in the present study. Furthermore, it is also known that oocyte recovery, IVM and development to embryos depend on the species, age, season, reproductive status, nutrition and health of the donor animal [20, 21, 47–53]. The results also demonstrate that oocytes recovered from blackbucks after sudden death of the animals possess competence to develop into embryos following *in vitro* maturation and activation.

Reproduction by cloning could be one of the methods used to increase the number of endangered/wild [54] or extinct species [55] in a population, for which competent oocytes for successful reprogramming of transferred nuclei is a must. The present study could thus be considered a step in this direction to generate oocytes competent for somatic cell nuclear transfer for production of cloned offspring of endangered animals.

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