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DYSMORPHIC GOAT OOCYTES SHOWED POOR EMBRYO DEVELOPMENTAL CAPACITY FOLLOWING INTRACYTOPLASMIC SPERM INJECTION

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ABSTRACT

Embryo developmental competence of dysmorphic goat oocytes recovered from abnormal ovaries was evaluated following intracytoplasmic sperm injection (ICSI). Cumulus-oocyte complexes (COCs) were obtained by slicing of abattoir-derived goat ovaries and categorized according to morphology of the ooplasm into four groups as normal, paler, oocyte with big fat globules (BFG) and oocyte with darker zone (DZ). The COCs were subjected to *in vitro* maturation (IVM) in presence of CO_2 (5%) at 35-38°C for 27 hours (h). The ICSI was performed with immobilized sperm on the lid of a culture dish (35 mm) under an Olympus inverted microscope fitted with Narishige micromanipulators. Injected oocytes were cultured for <192 h in modified synthetic oviductal fluid (mSOF) medium in CO_2 incubator. The maturation rates (mean±SEM) were 54.6±2.7, 56.7±4.9, 69.0±6.2 and 73.7±4.2% (P<0.05) for paler oocyte, oocyte with BFG, oocytes with DZ and normal oocytes, respectively. No significant difference was found between dysmorphic and normal oocytes in fertilization and cleavage rates. However, dysmorphic oocytes were arrested or degenerated at around 5-to 8-cell stage; whereas morula (12.6%, P<0.05) were obtained from normal oocytes. In conclusion, dysmorphic oocytes were less competent than normal oocytes in terms of embryo developmental capacity after ICSI in goat.

Keywords: Goat, COCs, oocyte, ooplasmic dysmorphism, IVM, ICSI, embryo

Introduction

Oocyte quality is a key limiting factor in female fertility which is primarily reflected in morphological features. It is an important prognostic factor as the nuclear and ooplasmic maturity of the oocyte may be directly related to the success rate of intracytoplasmic sperm injection (Kahraman *et al.* 2000). A search of available literature could not generate any work that relates oocyte quality or morphology with the outcome of ICSI particularly in goat. However, there are a number of studies reported in human in this regard. Oocyte denudation prior to an ICSI procedure has revealed that a significant proportion of oocytes exhibit different morphological abnormalities, such as excessive granularity, vacuolarization, clustering of smooth endoplasmatic reticulum, refractile bodies, large perivitelline space and fragmented polar body (PB). Although morphological appearance of goat oocyte is quite different from that of human, it is likely that a similarity in oocyte dysmorphism may exist. The extent to which these abnormalities can interfere with the normal process of fertilization following ICSI is controversial. Some authors did not find any correlation between oocyte morphology, fertilization rates and embryo quality (De Sutter *et al.* 1996, Balaban *et al.* 1998), others have reported a significant influence of oocyte morphology on fertilization rates and embryo quality after ICSI (Xia 1997, Sehral *et al.* 1997). Although not with ICSI, a few reports on oocyte quality and embryo developmental competence after *in vitro* fertilization (IVF) in cattle have been published. Except one study (Nagano *et al.* 1999), others were in the conclusion that poor quality oocytes had lower developmental competence than those of good quality oocytes (Nagano *et al.* 2006, Nagano *et al.* 2007).

An optimum supply of oocytes is a prerequisite for the production of embryos *in vitro*. This supply is normally accomplished from abattoir source. Unlike other countries, abattoir-derived ovaries in Malaysia are not readily available due to low slaughtering of female goats (or does) as a result of acute shortage of breeding stocks in Malaysia (Rajikin 1995). Therefore, doe those are either very old or

culled from breeding program are generally slaughtered. Oocytes from these goats are also of poor quality and most of these are not generally used. Considering the situation in Malaysia, if the developmental capacity of these oocytes can be known then these may also be useful for *in vitro* embryo production studies like in human. Moreover, no reports have been published on ICSI outcome in relation to oocyte quality in goats, therefore, the objectives of the present study were to determine the: (i) *in vitro* maturation rates of dysmorphic goat oocytes collected from abattoir source and (ii) embryo developmental competence of mature dysmorphic oocytes following ICSI.

Materials and Methods

Chemicals, reagents and media: All chemicals and media used in this study were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. For all media, the pH was adjusted to 7.2-7.4 and osmolarity to 280 mOsm/kg.

Oocyte recovery and grading: The ovaries from slaughtered does were obtained from a local abattoir and transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS) solution supplemented with gentamicin sulfate (50 mg/mL) at 35-38°C within 1-2 h of slaughter. The ovaries were rinsed in the same solution. Most of the ovaries were of poor quality as evidence by the presence of fluid-filled cysts, corpus lutei (CL) in the ovaries; and some of the ovaries had unusual shape (Figure 1). Cumulus-oocyte complexes (COCs) and cumulus-free oocytes (CFOs) were recovered after slicing the ovaries submerged in slicing medium comprised of HEPES-buffered M199 with Earle's salt, sodium bicarbonate and L-glutamine (M4530, Sigma-Aldrich) supplemented with gentamycin sulfate (50 mg/mL) at 38.5°C. The oocytes (COCs or CFOs) were visually assessed and most of them were found ill-looking and of bad quality. The oocytes were classified into three morphological categories related to ooplasm as paler oocytes, oocytes with big fat globules (BFG) and oocytes with darker zone (DZ) as described by Rahman *et al.* (2007) and Rahman (2008) (Table 1 and

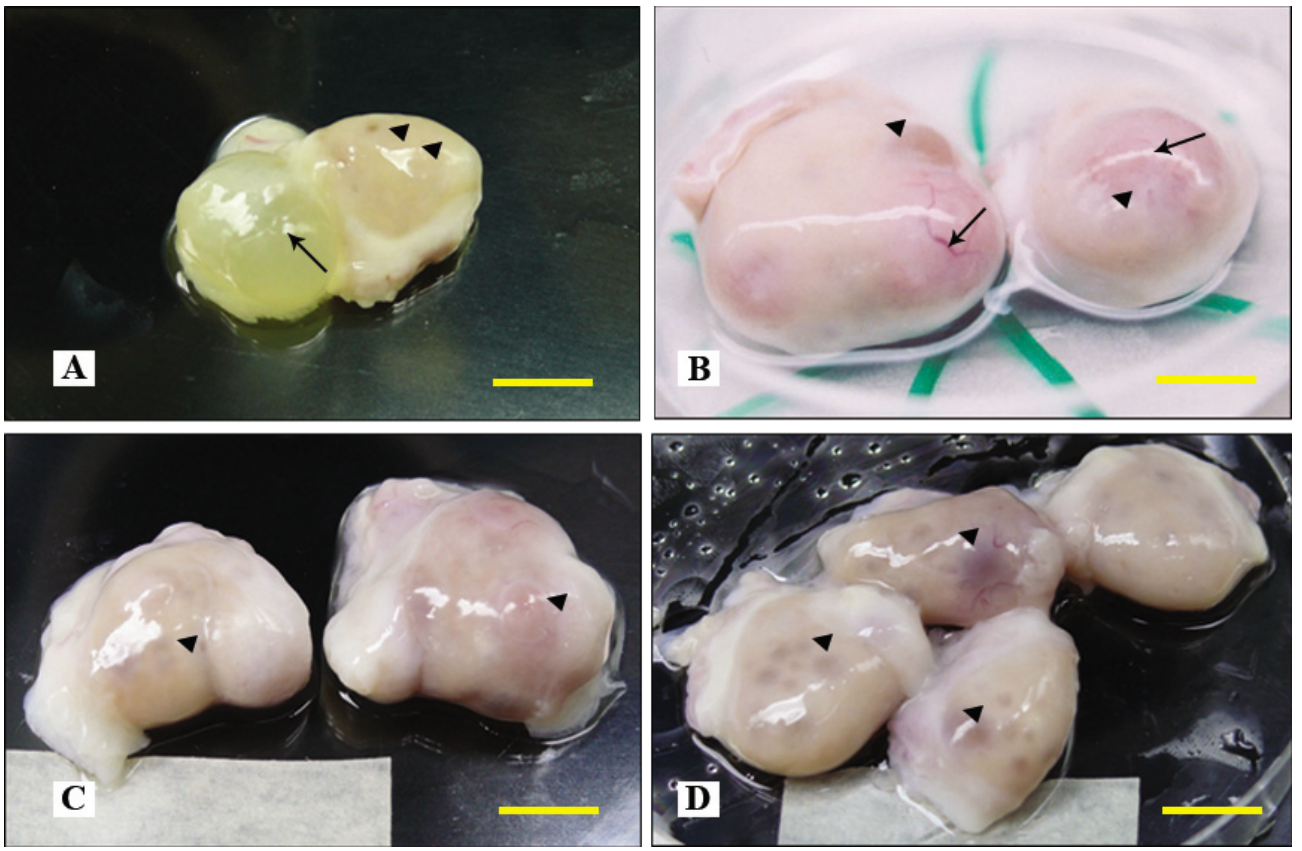


Figure 1. Goat ovaries of poor quality. A) Ovary with a big cyst (Arrow), B) ovaries with corpus lutei (small arrow), C) unusual kidney-shaped ovaries and D) normal ovaries. Arrow head = follicle. Scale bar = 10 mm.

Figure 2). Morphological deformity (*e.g.,* abnormal size, shape, PB or zona deformity) other than ooplasm were not considered in the present study. Oocytes with other types of ooplasmic dysmorphism including highly granulated ooplasm or dark markings at the periphery or entirely dark ooplasm were also retrieved. These oocytes were not included as their numbers were insufficient for the experiment. Along with dysmorphic oocytes, a very few normal-looking oocytes were also recovered from bad quality ovaries. Because the number of normal oocytes was insufficient, therefore, these were not included in the present study. Some normal ovaries were also sliced as control group and viable oocytes retrieved from these ovaries with finely granulated homogeneous ooplasm

Table 1. Grading of recovered oocytes in different categories according to ooplasmic morphology

Characteristics of COCs and CFOs	Category
COCs with 1-5 or >5 complete or incomplete layers of CCs and CFOs with finely granulated homogeneous ooplasm, golden, golden-yellow or brownish in color and evenly distributed dark fat globules	Normal oocytes (Normal)
COCs with 1-5 or >5 complete or incomplete layers of CCs and CFOs having a paler or semi-translucent ooplasm, central portion of some of those have clear appearance due to lack of ooplasmic organelles	Pale oocytes (Paler)
COCs with 1-5 or >5 complete or incomplete layers of CCs and CFOs having big fat globules of different sizes in any part of the ooplasm	Oocytes with bigger fat globules (BFG)
COCs with 1-5 or >5 complete or incomplete layers of CCs and CFOs having a darker or black zone or appearance at one side or end of the ooplasm	Oocytes with dark zone (DZ)

Source: Rahman *et al.* (2007) and Rahman (2008)

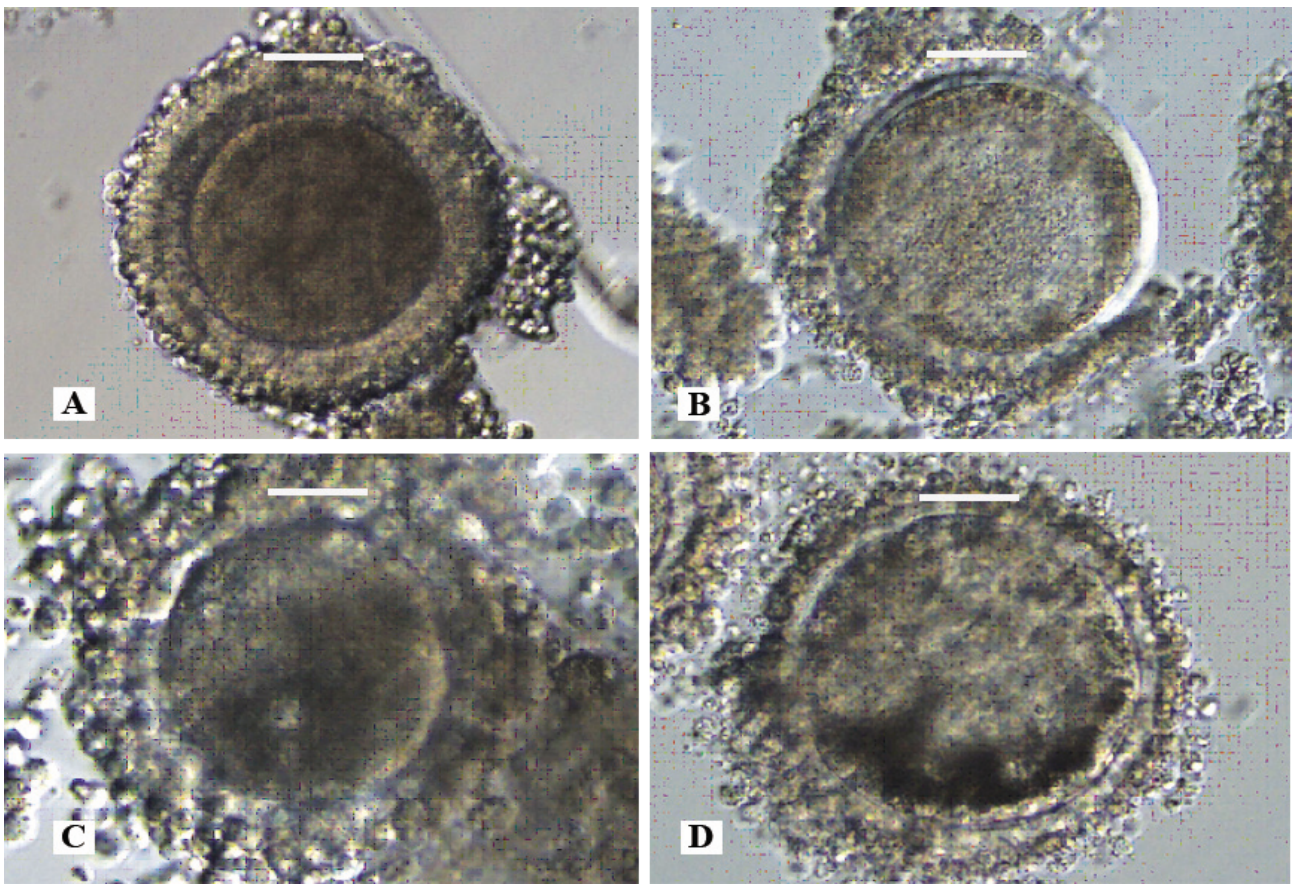


Figure 2. Goat oocyte grading according to morphology of the ooplasm. A) Normal oocyte, B) paler oocyte, C) oocyte with BFG and D) oocyte with DZ. P = paler oocyte, BFG = big fat globule, DZ = darker zone. Scale bar represents 50 mm.

were categorized as normal (Table 1 and Figure 2). Viable oocytes having heterogeneous and dysmorphic ooplasm (paler, BFG and DZ) and normal oocytes having finely granulated and homogeneous ooplasm were selected for *in vitro* maturation (IVM).

***In vitro* maturation:** The selected oocytes were washed three times in IVM medium, which consisted of M199 with Earle's salt, sodium bicarbonate, and L-glutamine (Sigma, M-4530) as a base medium, supplemented with ovine LH (10 mg/mL; Sigma L5269), ovine FSH (10 mg/mL; Ovagen), 17b-estradiol (1 mg/mL; Sigma E2257), sodium pyruvate (275 mg/mL; Sigma P3662), gentamycin sulfate (50 mg/mL; Sigma G3632), cysteamine (100 mM; Sigma M9768) and 10% (v/v) heat-inactivated estrus goat serum (EGS). Groups of 10 oocytes were matured in 100 mL of IVM medium overlaid with light mineral oil (Sigma M8410) for 27 h at 38.5°C and 5% CO_2 in air.

Assessment of oocyte maturation: After IVM, oocytes were treated with 100 mL of hyaluronidase (80 IU/mL, H4272, Sigma-Aldrich) in HEPES-buffered mSOF medium (Takahashi and First, 1992). The oocytes were visualized under a dissecting microscope and the cumulus-corona cells were carefully stripped off the zona pellucida by sequential denuding through glass pipettes of 250 mm and 200 mm inner diameters, respectively. Denuded oocytes were assessed for maturation on an inverted microscope. Oocytes with a clear first PB were considered as mature and meiotically competent. Viable mature oocytes from all 4 groups were then cultured in the incubator (5% CO_2 and 38.5°C) until the ICSI procedure.

Sperm preparation: Sperm motility was evaluated under an inverted microscope and motile sperm were selected by swim-up. Briefly, 500 mL of frozen buck semen from the Jermasia breed (a breed developed at the University of Malaya, Malaysia) of proven quality was thawed and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 for 60 minutes (min) in 2 mL of mSOF medium supplemented with 20% (v/v) goat serum (GS). After incubation, the supernatant was removed and centrifuged at 200 ´ *g* for 10 min. The sperm pellet was washed twice and re-suspended with an equal volume of mSOF medium containing heparin (50 mg/mL, Sigma, H0777) and incubated for another 15 min in a humidified atmosphere of 5% CO2 at 38.5°C.

Intracytoplasmic sperm injection: The ICSI procedure was performed on an Olympus IX71 inverted microscope fitted with Narishige hydraulic micromanipulators (Narishige Scientific Instrument, Tokyo, Japan). The holding pipettes were made from borosilicate glass tubing of 1.0 mm outside diameter (OD) using a Sutter Micropipette Puller (P-97, Sutter Instrument Co., Novato, CA, USA), in combination with a Narishige microforge (Model MF-9) and Narishige microgrinder (Model EG-4, Narishige Scientific Instrument, Tokyo, Japan). The holding pipettes were pulled to an OD of 150 mm and then fire-polished to create an inside diameter (ID) of 30–40 mm, with pipette tips bent to an angle of 35°. Injection needles (Humagen, Charlottesville, VA, USA) had an ID of 6–7 mm and OD of 8 mm, with a sharp spike and a tip bent at 35°.

An aliquot (1–2 mL) of washed sperm suspension was added to a 5 mL elongated microdroplet of ICSI medium (HEPES-buffered mSOF medium) prepared on the left side of the ICSI dish. A motile sperm was aspirated into the injection needle and brought into a microdroplet containing 10% polyvinylpyrrolidone (PVP), located in the center of the dish. The sperm was immobilized by crushing its tail against the bottom of the dish with the injection needle. The immobilized sperm was then aspirated, tail-first, into the injection needle. A mature oocyte was orientated with its first PB at the 6 or 12 O'clock position, by the holding pipette in a microdroplet located on the right side of the ICSI dish. The sperm was injected head-first, into the ooplasm of the oocyte (at the 3 O'clock position). During ICSI, ooplasm was aspirated into the injection needle to break the oolemma (and ensure that the needle was in the ooplasm). The sperm was then injected into the ooplasm with a minimal volume (<5 pL) of PVP. Sham test controls were not performed due to the availability of small number of IVM oocytes for ICSI and as in our previous studies none of the oocytes were activated by the microinjection of ICSI medium without a sperm (Rahman *et al.* 2007, Rahman 2008, Abdullah *et al.* 2008, Rahman *et al.* 2009).

***In Vitro* Culture:** Injected goat oocytes were cultured *in vitro* in mSOF medium supplemented with 10% heat-inactivated goat serum in 5% CO_2 at 38.5°C. After 48 h of *in vitro* culture (IVC), presumptive zygotes were cultured in freshly prepared mSOF medium and embryos were cultured for <192 h. Once daily, embryos were assessed and their developmental stage recorded.

Experimental design: Abattoir-derived bad quality ovaries (Cystic ovaries, ovaries with CL or unusual shape) were sliced and the recovered oocytes were categorized into paler, oocytes with big fat globules (BFG) and oocytes with darker zone (DZ) according to their ooplasmic morphology. Ovaries with normal size and shape were also sliced; oocytes retrieved from these ovaries with finely granulated homogeneous ooplasm were categorized as normal (control group). Oocyte maturation and embryo development following ICSI from all these categories were recorded. The experiment was replicated at least four times for each treatment.

Statistical analysis: The effect of ooplasmic dysmorphism on IVM and embryo developmental competence following ICSI was determined with one-way ANOVA; significant differences between the means were located with Duncan's Multiple Range Test. Analyses were carried out with SPSS (Statistical Packages for Social Sciences) for Windows, Version 11.5, SPSS Inc. (2002), Chicago, IL, USA. All data are presented as mean±SEM.

Results

There was no significant difference in oocyte survival rates among four different morphologic categories when they were matured in vitro. Compared to paler oocytes and oocytes having BFG in the ooplasm, higher proportion (P<0.05) of normal oocytes were matured; but the difference with DZ was insignificant (Table 2). There was no significant difference in maturation rate among three different categories of dysmorphic oocytes. Mature oocytes from different morphologic categories including normal oocytes have been shown in Figure 3 (A-D).

Following ICSI, no significant difference in fertilization and cleavage rates among the three categories of dysmorphic and the normal oocytes were found (Table 3). Cleaved embryos from oocytes having all forms of ooplasmic dysmorphism were developed until 5-to 8-cell stage (data not shown) and after that development of paler oocytes and oocyte having BFG were either arrested or degenerated. Although some oocytes with DZ (10.0%) were developed to 9-to 16-cell stage, but no categories of dysmorphic oocytes developed to morula which was significantly (P<0.5) different than that from normal oocytes (12.6%) (Table 3). Four-cell stage embryos developed from dysmorphic and normal zygotes have been shown in Figure 4 (A-D). A representative microphotograph of a morula from a normal oocyte following ICSI is shown in Figure 5.

Discussion

The data obtained in the present study showed that the oocyte morphology was related to the ovarian morphology as evidenced by the recovery of large number of ill-looking or dysmorphic oocytes (ooplasmic dysmoprhism) from abattoir-derived poor quality or dysmorphic ovaries. Although, no reports were found on goat oocyte morphology in relation to ovarian morphology,

Table 2. *In vitro* maturation (Mean±SE) of goat oocytes according to ooplasmic morphology

Oocyte category	No. of oocytes cultured	No. of oocytes died during IVM	Survival rate (%)	Maturation rate (%)
Paler	48	7	88.1±7.9a (*n* = 41)	54.6±2.7a (*n* = 22)
Big fat globules	59	6	91.6±3.9a (*n* = 53)	56.7±4.9a (*n* = 29)
Darker	42	3	93.5±2.8a (*n* = 39)	69.0±6.2ab (*n* = 27)
Normal (control)	87	10	91.0±4.1a (*n* = 77)	73.7±4.2b (*n* = 58)

a,b Within a column means without a common superscript differed ($P < 0.05$); *n* = total number of oocytes used.

Table 3. Embryo development (mean±SEM) of *in vitro* matured goat oocytes microinjected with frozen-thawed sperm, according to ooplasmic morphology

Oocyte category	No. injected	Survival rate (%)	Fertilization rate (%)	Cleavage rate (%)	Morula (%)
Paler	22	87.9±7.2a (*n* = 19)	58.5±3.5a (*n* = 11)	60.4±6.3a (*n* = 7)	0 (*n* = 0)
Big fat globules	31	86.6±5.6a (*n* = 26)	66.2±8.9a (*n* = 16)	50.0±13.7a (*n* = 9)	0 (*n* = 0)
Darker	27	83.0±7.7a (*n* = 22)	68.3±1.7a (*n* = 15)	65.0±18.7a (*n* = 10)	0 (*n* = 0)
Normal	58	83.8±5.5a (*n* = 47)	74.3±1.2a (*n* = 35)	74.5±3.3a (*n* = 26)	12.6±7.2 (*n* = 4)

a,b Within a column, means without a common superscript differed ($P < 0.05$); *n* = total number of oocytes or embryos.

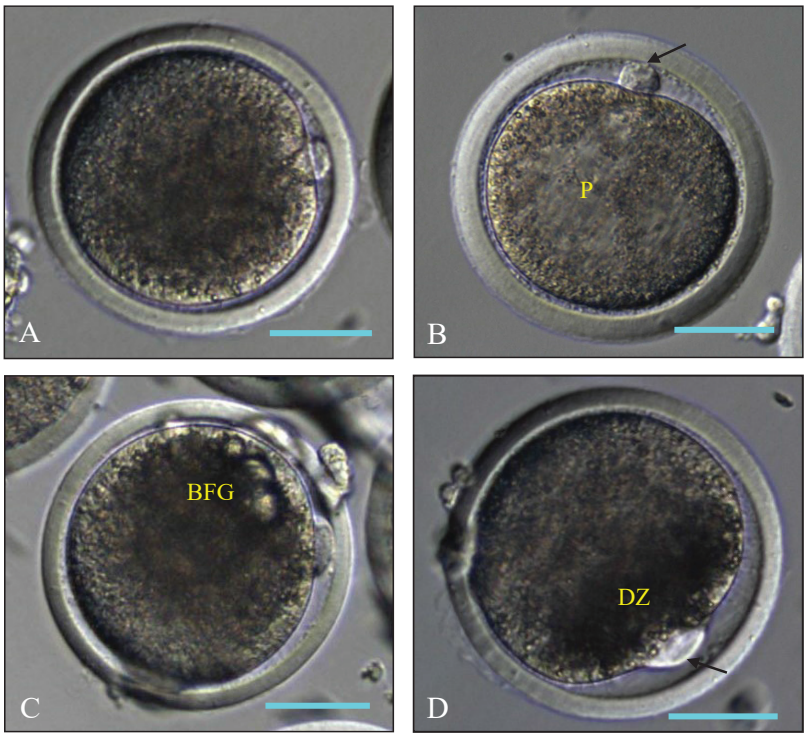


Figure 3. *In vitro* matured goat oocytes from different morphologic categories. A) normal oocyte, B) paler oocyte), C) oocyte with BFG and D) oocyte with DZ. Arrows indicate first polar body. P = paler oocyte, BFG = big fat globule, DZ = darker zone. Scale bar = 50 mm.

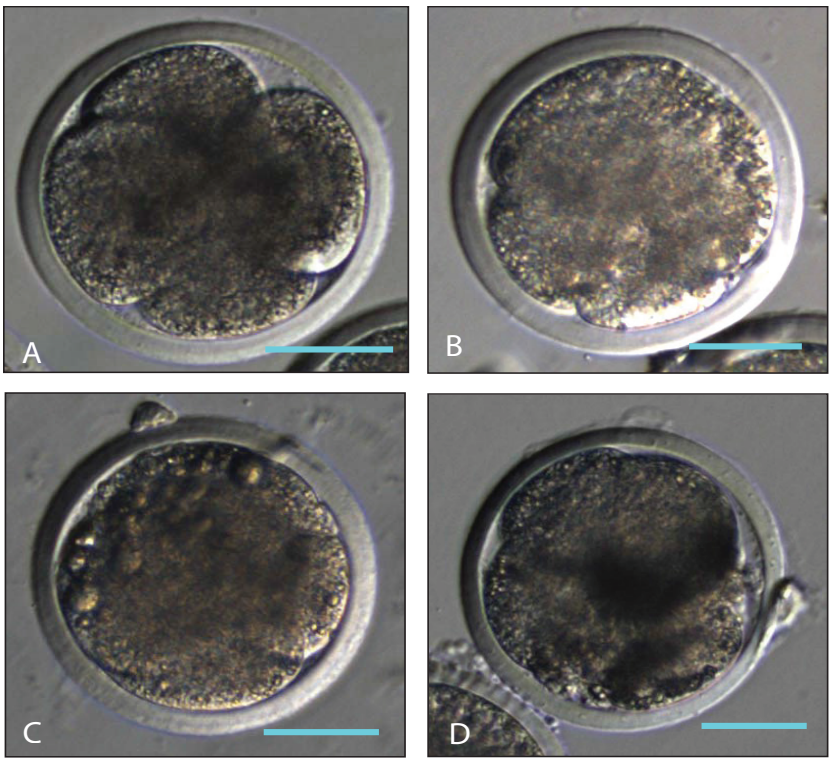


Figure 4. Goat 4-cell stage embryos developed following ICSI of oocytes from different morphologic categories. A) normal, B) paler, C) oocytes with BFG and D) oocytes with DZ. Scale bar = 50 mm.

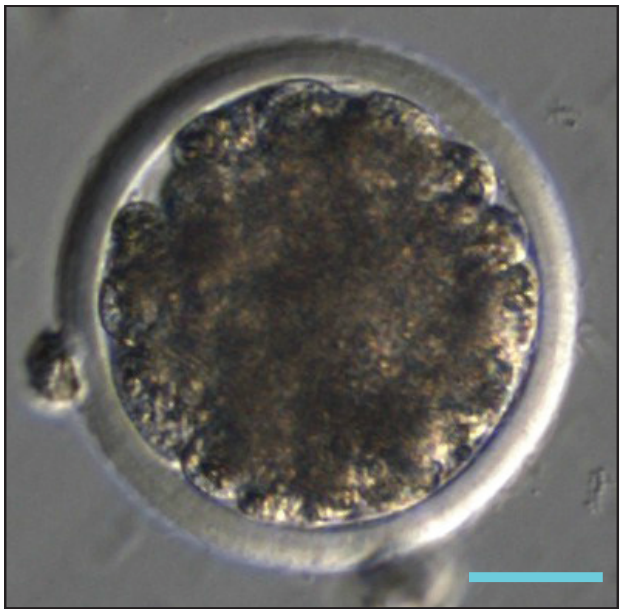


Figure 5. Goat morula stage embryos developed following ICSI of normal looking oocyte. Scale bar = 50 mm.

however, Islam *et al.* (2007) showed that ovaries with CL provided lower proportion of normal and higher proportion of abnormal oocytes than ovaries without CL. These authors indicated that oocyte abnormality and follicular degeneration might be associated with the effect of progesterone in the ovaries with CL. Although not reported in goat, polycystic ovarian syndrome in human is associated with high tonic LH (Balen *et al.* 1993), the concentration of which exceeds the serum FSH concentration (Ludwig *et al.* 1999). High LH has been claimed to decrease oocyte quality, lower conception and higher abortion rates (Adams *et al.* 1985, Homburg and Jacobs 1989, Regan *et al.* 1990). In human, it was speculated that the cause of ooplasmic morphological abnormalities might be multifactor. Firstly, ovarian stimulation is known to have an adverse effect on oocytes. Ovulation induction may result in the maturation of abnormal oocytes that would otherwise become atretic in the absence of stimulation (Sehral *et al.* 1997). Oocytes recovered from stimulated IVF cycles have been shown to have more than 40% incidence of numerical chromosomal abnormalities (Wramsby 1988, Van Blerkom and Henry 1992). Secondly, oocyte quality may be directly affected by the hormonal environment. Ovarian steroids, particularly estrogen and progesterone, are intimately involved in the initiation of ooplasmic maturation and the final stage of nuclear maturation of the oocytes (Thibault 1977). It was demonstrated that a high degree of morphological and nuclear anomalies in unfertilized oocytes in patients undergoing ovarian stimulation was associated with pure FSH treatment (Wojcik *et al.* 1995). However, in the present experiment, all the oocytes were retrieved from abattoir ovaries; therefore, the hormonal or physiological status of these goats was essentially unknown. In author's previous studies with laparoscopic ovum pick-up (LOPU) experiments, where goats were stimulated with hormones, recovery of dysmorphic oocytes was occasional and very few (Rahman *et al.* 2007, Rahman 2008, Abdullah *et al.* 2008, Rahman *et al.* 2009). Therefore, unlike human, in goats superovulation protocol may not be responsible for ooplasmic dysmorphism. Earlier studies in goat (Rajikin *et al.* 1994, Rajikin 1995) also revealed some abnormal or dysmorphic oocytes by ovary slicing, however, attention was not given on the *in vitro* developmental competence of those oocytes. As the physiological status of the goats was not known, therefore, the cause of ooplasmic dysmorphism in the present experiment could not be explained.

Except for the oocytes with DZ on the ooplasm, the IVM rates of paler oocytes or oocytes with BFG were significantly lower than that of the normal oocytes in the current experiment. Although data were not available for goat, the present results for oocytes with DZ was similar with the results of (Nagano *et al.* 1999) who reported bovine oocytes having heterogeneous ooplasm with dark cluster can be matured as normal oocytes with homogeneous ooplasm without significant difference. An IVF study in cattle reported no differences in oocyte maturation rates between normal looking and dysmorphic oocytes (oocytes with DZ on the periphery or dark cluster on the ooplasm and black or pale ooplasm) (Nagano *et al.* 2007). However, no IVM data were available for maturation rates of different types of human dysmorphic oocytes. This is because human oocytes are not subjected to IVM as they are already in MII stage when recovered, and ICSI is generally performed within 2-3 h of oocyte recovery.

Oocyte quality is an important prognostic factor as the nuclear and ooplasmic maturity of the oocyte may be directly related to the success rate of ICSI (Kahraman *et al.* 2000). The occurrence of specific ooplasmic dysmorphic phenotypes in oocytes has been suggested to reflect intrinsic defects that may negatively influence on oocyte competence (Xia 1997, Van Blerkom and Henry 1992).

In agreement with the previous studies of Momozawa and Fukuda (1995) and Hazeleger (1999) with bovine IVF, the present study also revealed no significant difference in fertilization rates between normal and oocytes having DZ. On the other hand, Nagano *et al.* (2006) reported a significantly higher fertilization, cleavage and blastocyst development rates with bovine IVF oocytes having normal morphology than pale or black oocytes. In spite of the lower cleavage rate, oocyte with dark clusters in the same study progressed to blastocyst in a similar rate as normal oocytes. In contrast to this report, they found significantly higher fertilization and cleavage rates with dysmorphic oocytes having dark clusters than normal oocyte in an earlier study, although no differences were seen in blastocyst development between the two types of oocytes (Nagano *et al.* 1999). In their later study, Nagano *et al.* (2006) revealed that an overall darker appearance of an oocyte indicates an accumulation of lipids and good developmental potential, while a pale or light-colored ooplasm indicates a low density of organelles and poor developmental potential. The present study is in support of this statement. In addition, paler oocyte and oocytes with BFG in the present experiment might also indicate degeneration process and lower developmental potential. This is supported by the finding that most of the dysmorphic oocytes degenerated rather than arrested in the present study.

In human, there is some debate on the effect of oocyte morphology on fertilization and embryo quality following ICSI (Ebner *et al.* 2000). The current results were in agreement with Lourtradis *et al.* (1999) who found human oocytes with dark ooplasm, ooplasmic vacuoles and fragments led to poor quality embryos. Although, normal fertilization and embryo development were achieved in oocytes with abnormal ooplasm, however, the resulting embryos failed to demonstrate the same implantation potential as those derived from normal oocytes (Sehral *et al.* 1997). In contrast, no significant differences in fertilization, embryo quality, implantation and pregnancy rates were found after ICSI with normal and abnormal oocytes (Balaban *et al.* 1998) or oocytes with abnormal morphology or centrally located granular cytoplasm (CLGC) (Kahraman *et al.* 2000). Although cause of CLGC or ooplasmic granulation was not clearly known, it was speculated that chromosomal abnormality or aneuploidy might be responsible for this (Van Blerkom and Henry 1992, Kahraman *et al.* 2000, Merviel *et al.* 2017). It was evidenced by a very high rate (52.2%) of aneuploidy in blastomere biopsy of embryos derived from CLGC oocytes in preimplantation genetic diagnosis (Kahraman *et al.* 2000). In addition, Alikani *et al.* (1995) observed a higher rate of miscarriage in women with dysmorphic oocytes.

Conclusion

The present study concluded that the dysmorphic goat oocytes are able to mature *in vitro* and following ICSI these oocytes can be fertilized and cleaved up to 8-cell stage like normal oocyte. However, irrespective of type of dysmorphism, these oocytes have less developmental potential than normal oocytes. To the knowledge, this is the first report of *in vitro* maturation and embryo development following ICSI of goat oocytes having ooplasmic dysmorphism.

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