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# Morphometric Assessment of the Bovine Ovary for *in vitro* Matured Oocyte Quality to Determine Developmental Competence

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## ABSTRACT

**Background:** Embryo production *in vitro* requires three steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). The first step in the *in vitro* maturation of oocytes is germinal vesicle breakdown (GVBD), followed by completion of the 1st meiotic division and formation of the 1st polar body. These parameters are critical during ovary and oocyte selection. This study aimed to evaluate bovine ovary and oocyte collection for IVM.

**Methods:** Ovaries collected from cows in a 0.9% NaCl saline solution at the central slaughterhouse were divided into two normal and abnormal ovaries according to their morphological appearance. The morphometric dimensions of bovine ovaries, such as weight and deamination (length, width and volume), were recorded. The oocyte was extruded and its deamination occurred before and after culture in maturation medium.

**Result:** The mean ovary weight, volume, length and width were not significantly different between the two ovary types. Additionally, the dominant and subordinate follicle diameters in both ovary types showed no significant differences. Furthermore, the oocyte number per ovary of the normal and abnormal oocytes showed no significant differences. The mean cumulus oocyte complex before maturation showed no significant difference (52.73±7.23 mm for the normal ovary vs. 43.015±5.41 mm for the abnormal ovary). However, after maturation, a highly significant difference was found (P<0.001) between the normal ovaries before and after maturation (178.10±15.36 µm) and abnormal ovaries (10.45±7.99 µm). Additionally, data analysis of oocytes with or without 1st polar bodies revealed a highly significant difference (P<0.001) between oocytes of the normal ovary with (32.15±4.19) and without (10.95±1.59) 1st polar bodies and oocytes of the abnormal ovary with (7.0±078) and without 1st polar bodies (3.3±0.32). Thus, the critical point at which the normal ovary produces better *in vitro* matured follicles with good oocyte quality and produces the 1st polar body determines developmental competence. Therefore, the best selection of normal ovaries will enhance *in vitro* maturation for subsequent experiments, such as *in vitro* fertilization or cloning and *in vitro* embryo development.


**Key words:** Abnormal, Bovine, *In vitro* maturation, Morphometric, Normal, Ovaries.

## INTRODUCTION

Embryo production *in vitro* requires three steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Oocyte developmental competence is a determining factor that influences the outcomes of an IVF cycle regarding the ability of a female gamete to reach maturation, be fertilized and uphold embryonic development until the blastocyst stage (Sirait *et al.*, 2021). The first step in the *in vitro* maturation of oocytes is germinal vesicle breakdown (GVBD), followed by completion of the 1st meiotic division and formation of the 1st polar body. The most important step of *in vitro* production (IVF) is selecting good-quality oocytes that can undergo nuclear and cytoplasmic maturation, which occurs during follicle growth (Sirard, 2001).

Nuclear maturation is the ability to resume meiotic division up to metaphase II by initiating the breakdown of germinal vesicles (GVBDs) and formation of the 1st polar body. Secondary oocytes are then extrused from Graafian follicles and the second metaphase plate of the oocytes appear (Watson, 2007).

Cytoplasmic maturation includes a succession of transformations, essentially of mitochondria, cortical granules and smooth and rough endoplasmic reticulum (Hyttel *et al.*, 1997), all of which are necessary for the maturation and blockade of polysperm. These events indicate that new protein synthesis occurs, such as the "factor of the growth of masculine pronucleus" MPGF and maturation-promoting factor MPF.

In addition to these phenomena, the mucification and expansion of the cells surrounding the oocyte and reduction in the number of intercellular junctions between the granulosa cells and oocytes begin, interrupting ionic

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transport between the cells of the cumulus and oocytes (Hyttel *et al*., 1997). Cytoskeletal proteins (Sun and Schatten, 2006) and organelle distribution (Brevini *et al*., 2007) are crucial for oocyte maturation and may affect oocyte competence. Kim *et al*. (1996, 2000) demonstrated the close relationship between nuclear maturation and cytoskeleton dynamics in pig and cattle oocytes. Cytoskeleton dynamics are also related to oocyte developmental competence (Brevini *et al*., 2007). Correct organelle positioning is also critical because low-quality bovine oocytes fail to translocate to the mitochondria from the cytoplasm periphery to the center (Stojkovic *et al*., 2001). Bilodeau-Goeseels, (2006) reported that glucose, pyruvate, lactate and glutamine were stimulatory to nuclear maturation in bovine oocytes. Insufficient cytoplasmic maturation of M II oocytes may be one reason for the low rate of embryo production *in vitro* (Marchal *et al*., 2001).

Dairy cattle have a variety of health issues, the most prevalent of which are mastitis, which may be either obviously or subclinical (Chouhan *et al*., 2021) and cystic ovarian disease, which is a widespread and economically important ailment in dairy cattle that affects fertility (Chauhan *et al*., 2021) and do not forget the role of the season on dairy cows and their production (Kumar *et al*., 2021).

Therefore, the crucial step in selecting bovine oocytes requires careful morphological measurement to predict subsequent developmental competence, particularly when the available oocytes are limited. The morphometric selection of *in vitro* matured oocytes has been reported in humans and several animal species (Kitagawa and Nimura, 2006; Yousaf and Chohan, 2003; Lasiene *et al*., 2011). Therefore, this study aimed to determine morphometric dimensions of bovine ovaries and oocytes, such as the ovary weight and diameter and different types of follicles, including the layer of follicle cells surrounding the oocytes and the thickness of the zona pellucida around the oocytes. Additionally, we evaluated the ooplasm and perivitelline space with or without the first polar body to increase the probability of oocytes that could support *in vitro* embryonic development.

## MATERIALS AND METHODS

Unless otherwise specified, all the chemicals and hormones used in this study were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Cow ovaries were obtained from a native slaughterhouse in Riyadh and were transported in 0.9% (v/v) NaCl solution at 30-33°C to the laboratory within 2-3 h. The contents of antral follicles were aspirated using a 19-gauge needle attached to a 10 mL disposable syringe containing 0.5 ml of tissue culture medium (TCM-199 with Hank's salts, L-glutamine, 25 mM HEPES and L-amino acids supplemented with 10% fetal calf serum (FCS), 0.3 mM sodium pyruvate, 25 mg/ml of gentamycin sulfate and 143 mg/ml of heparin sodium salt (Caisson Lab. Inc., Smithfield, UT, USA). Cumulus-oocyte complexes (COCs) with uniformly granulated cytoplasm and more than three layers of compact cumulus cells were chosen and evaluated (Al mutary *et al*., 2020).

COCs with more than 3 layers of cumulus cells and uniform cytoplasm (n ¼ 2641) were matured for 24 h in TCM-199 Earle's salts supplemented with 10% fetal bovine serum (FBS), 0.3 mM sodium pyruvate, 1 mg/ml of estradiol-17b, 0.023 IU/ml of luteinizing hormone (LH), 0.02 IU/ml of follicular stimulating hormone (FSH) and 25 mg/ml of gentamycin sulfate at 38.5°C and exposed to 5% carbon dioxide (CO2) in air at high humidity. The oocytes (10-15 per droplet) were cultured in 60 mm Petri dishes containing 10 drops, each 50 ml in size and were covered with embryo-tested mineral oil (Al mutary *et al*., 2020).

The cumulus cells were removed from the oocytes after IVM. Trituration was performed in HEPES-buffered TCM-199 with 0.1% (v/v) hyaluronidase and two washes in TCM-199 augmented with 10% (v/v) FBS. The morphometric parameters of the oocytes were assessed according to previous studies (Otoi *et al*., 1997; Griffin *et al*., 2006). Images were captured using a camera and the software of the microscope (Leica Application Suite, Version 4.0) and the morphometric parameters of the captured images were analyzed using Image 1.50i software (NIH, USA) and the scale bar as an arbitrary scale for pixel analysis by the software in the same oocyte image and all the results were recorded. The morphometric parameters described are as follows: oocyte outer diameter (ZPO), zona pellucida thickness (ZPT), inner oocyte diameter (ZPI) and ooplasm diameter (OD).

## RESULTS AND DISCUSSION

The collected ovaries were divided into normal and abnormal ovary types, according to their morphological structure with a corpus luteum or large vacuole follicles (Fig 1a). Statistical analysis of the mean ovary weight showed no significant difference (13.4±1.73 g and 18.45±1.38 g) between the normal and abnormal ovaries, respectively (Table 1). Additionally, the volume, length and width of the ovaries were not significantly different between the ovary types (Table 1). Furthermore, the dominant follicle diameter in both ovary types showed no significant differences (8.41±0.88 vs. 8.52±0.99 mm). Additionally, the subordinate follicles in both ovary types showed significant differences in the diameter (1.65±0.16 and 0.16 mm) and their number per ovary (9.5±1.4 vs. 8.55±1.58 mm) of abnormal subordinate follicles (Table 1).

Before maturation of the cumulus oocytes, the complex showed no significant differences in the diameter (normal: 52.73±7.23 mm; abnormal: 43.015±5.41 mm). However, after maturation, a highly significant difference ($P<0.001$) was found in the diameter between the normal (178.10±15.36 µm) and abnormal ovaries (10.45±7.99 µm). Additionally, maturation between the normal and abnormal conditions ($P<0.001$) (Table 2) will affect post maturation steps such as IVF and later embryo development *in vitro*.

Data analysis of the oocyte parameters of normal and abnormal ovaries revealed similar findings, such as those regarding the outer oocyte diameter of normal (189.016±2.24 µm) and abnormal (188.91±1.77 µm) ovaries. The oocyte zona pellucida thicknesses in normal and abnormal oocytes were 17.017±0.59 µm and 17.245±0.73 µm, respectively. The ooplasm diameters in normal and abnormal oocytes were 134.82±1.56 µm and 137.63±1.51 µm, respectively. The sizes of the perivitelline space in normal and abnormal oocytes were 55.09±2.26 µm and 51.22±1.30 µm, respectively (Table 2). Data analysis of the oocytes with or without 1st polar bodies showed a highly significant difference (P<0.001) between the oocytes with (32.15±4.19) or without (10.95±1.59) 1st polar bodies in normal ovaries and oocytes with (7.0±078) or without (3.3±0.32) 1st polar bodies in abnormal ovaries.

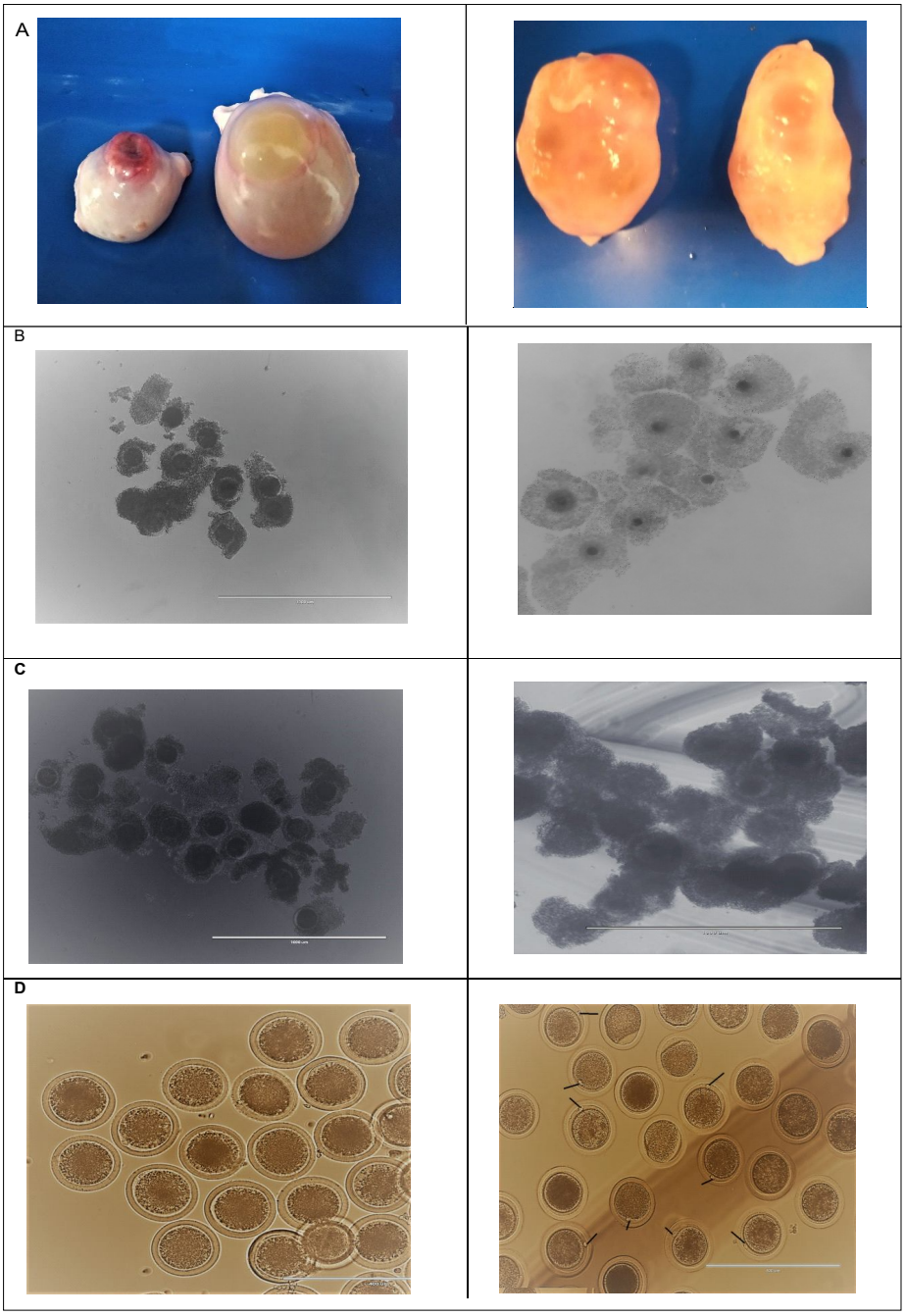


**Fig 1:** Normal and abnormal bovine ovaries. A: Normal and abnormal bovine ovaries. B: Cumulus ovary complex (COC) before and after maturation from normal and abnormal ovaries. C. Cumulus ovary complex (COC) before and after maturation from normal and abnormal ovaries. D: Oocytes with and without polar bodies.

**Table 1:** The morphometric means of normal and abnormal bovine ovary.

| Bovine ovary type and no. | Ovary weight (g±SEM) | Ovary volume ($mm^3$±SEM) | Ovary length (mm±SEM) | Ovary width (mm±SEM) | Diameter of dominant follicle (mm±SEM) | Dominant follicle no. | Diameter (mm±SEM) | Subordinate follicle no. | Oocyte no. per ovary |
|---|---|---|---|---|---|---|---|---|---|
| Normal no. 168 | 13.4±1.73 | 4.61±0.56 | 30.95±1.99 | 19.3±0.83 | 8.52±0.88 | 42 | 1.56±0.16 | 190 | 9005.38/ovary |
| Abnormal no. 40 | 18.45±1.38 | 6.51±0.94 | 36.19±1.75 | 22.47±0.82 | 8.52±0.99 | 31 | 1.55±0.17 | 167 | 2355.87/ovary |

**Table 2:** The morphometric means of normal and abnormal bovine Cumulus complex before and after maturation with oocytes parameters.

| Bovine oocyte Type and no. | Cumulus oocyte complex before maturation (mm±SEM) | Cumulus oocyte complex after maturation (mm±SEM) | Outer oocyte diameter (µm±SEM) | Zona Pellucida thickness (µm±SEM) | Ooplasm diameter (µm±SEM) | Perivitelline space (µm±SEM) |
|---|---|---|---|---|---|---|
| Normalno. 900 | 52.732a±7.25 | 178.40b*±2.34 | 189.016±2.24 | 17.017±0.59 | 134.82±1.56 | 55.09±2.26 |
| Abnormal no. 167 | 43.014a±5.41 | 101.45b*±7.99 | 188.91±1.77 | 17.245±0.73 | 137.63±1.51 | 51.22±1.30 |

a/b ($P<0.001$) highly significant between before and after maturation.

b*/b* ($P<0.001$) highly significant after maturation between normal and abnormal ovary cumulus oocytes complex (COC).

The best selection of oocytes *in vitro* from normal good ovaries will affect the subsequent steps in *in vitro* maturation, *in vitro* fertilization or cloning and *in vitro* embryo culture and increase embryo development quality. Some studies have shown that the corpus luteum in an ovary enhances the quality and developmental competence of oocytes derived from that ovary (Reis *et al.*, 2006; Moreno *et al.*, 1993; Penitente-Filhe *et al.*, 2015). However, our study findings contrast those of other studies (Shabankareh *et al.*, 2015; Quezada-Casasola *et al.*, 2018), as well as subsequent embryonic development (Gonzalez-Bulnes *et al.*, 2005; Manjunatha *et al.*, 2008; Boediono *et al.*, 1995; Pirestani *et al.*, 2011). Additionally, several studies have reported no effects (de Wit *et al.*, 2000; Sungulle, 2008). Bovine oocytes with a diameter of 110 mm can achieve complete meiotic competence, while smaller oocytes have significantly less transcriptional activity, indicating that they are still in the growing phase (Fair *et al.*, 1995).

Additionally, oocytes with diameters of 110-120 and 120 µm reached MII in similar proportions (76 and 81%, respectively) (Fair *et al.*, 1995). Otoi *et al. (*1997) indicated that meiotic competence was attained once oocytes reached 115 µm in diameter, while full developmental capacity was obtained when the diameter was at least 120 µm. Bovine oocytes with an inside-zona diameter smaller than 95 µm cannot resume meiosis *in vitro*; however, a high proportion of bovine oocytes can resume meiosis. Otoi *et al.* (1997) showed that the oocyte must measure 110 µm or higher to reach the MII stage. In the present study, all mature oocytes from both normal and abnormal ovaries had ZP thicknesses of 17.017±0.59 and 17.245 µm, respectively, values similar to those reported by Cavelia *et al.* (2008). Additionally, bovine oocytes with an inside-zone diameter smaller than 95 mm could not resume meiosis *in vitro* Sungulle *et al.* (2008).

No significant differences were found in the outer oocyte diameter, ooplasm diameter or oocyte periventricular space between bovine oocytes collected from normal and abnormal ovaries. Other studies have indicated that nuclear maturation, such as that in pigs (Luca *et al.*, 2002), buffalos (Yousaf and Cohan, 2003) and camels (Saadeldin *et al.*, 2017) and blastocyst production in cows were positively correlated with the oocyte diameter (Otoi *et al.*, 1997; de Wit and Kruip, 2001; Arlotto *et al.*, 1996). The same parameters employed in evaluating oocyte morphology in human oocytes by Lasiene *et al.* (2011) include the appearance of the structure of the cumulus-oocyte complex, oocyte cytoplasm, perivitelline space, zona pellucida, polar body and meiotic spindle.

The lower rate of *in vitro* fertilization and *in vitro* embryo development could be because most oocytes derived from the ovary for *in vitro* maturation originate from subordinate or growing follicles that are at least viewed days away from any possible ovulation. Although most of these oocytes complete their nuclear maturation, few develop to the blastocyst stage.

## CONCLUSION

The critical point at which the normal ovary produces better *in vitro* matured follicles with good oocyte quality and produces the 1st polar body determines developmental competence. Therefore, the best selection of normal ovaries will enhance *in vitro* maturation for subsequent experiments, such as *in vitro* fertilization or cloning and *in vitro* embryo development.

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