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# Effect of plasma progesterone on oocyte recovery, oocyte quality, and early *in-vitro* developmental competence of embryos in *Bos indicus* dairy cows

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ABSTRACT

The objective of present study was to determine the effect of plasma progesterone (P4) on oocyte recovery, oocyte quality, and early *in-vitro* developmental competence of embryos in *Bos indicus* dairy cows. The ovaries were collected in an abattoir. These ovaries ($n = 750$) were divided into two groups: 1) estrous CYCLIC ($n = 318$), and 2) estrous ACYCLIC ($n = 432$). Mean serum concentrations of P4 in a subset of ($n = 85$; 4.21 ± 0.4 ng/ml compared with 0.5 ± 0.2 ng/ml; $P < 0.05$) were greater in estrous CYCLIC as compared to ACYCLIC cows, respectively. The mean number of oocytes recovered per ovary (6.5 ± 0.5 compared with 4.0 ± 0.2; $P < 0.05$) was greater for estrous CYCLIC than ACYCLIC cows, respectively. The oocytes with grade I_+_II quality (55.3% compared with 47.6%; $P < 0.05$) were greater, whereas, there was lesser percentage with grade III_+_IV quality (44.5% compared with 52.4%; $P < 0.05$) from estrous CYCLIC as compared with ACYCLIC cows, respectively. Cleavage rate (70.9% compared with 52.8%; $P < 0.05$) was greater for embryos derived from estrous CYCLIC than ACYCLIC cows, respectively. Similarly, the embryo developmental rates to the 8- (38.5% compared with 20.8%; $P < 0.05$) and 16- (20.0% compared with 10.9%; $P < 0.05$) cell stage were greater for embryos derived from estrous CYCLIC as compared to ACYCLIC cows, respectively. In conclusion, the presence of greater plasma P4 has a beneficial effect on oocyte recovery, oocyte quality, and early IVEP outcomes in *Bos indicus* dairy cows.

## 1. Introduction

Assisted reproductive technologies have resulted in modernization of the dairy industry for the rapid multiplication of superior genetics (Moore and Hasler, 2017). The exploitation of genetics through *in-vitro* embryo production (IVEP) in farm animals, however, remained a challenge during the last 2 decades (Thompson, 1997). There are several factors that affect the outcome when there is use of IVEP, which include 1) ovarian follicular size in cows from which oocytes are collected (Pavlok et al., 1992; Lonergan et al.,

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1994a), 2) preparation method for sperm used for IVEP (Parrish et al., 1995), 3) lactation number of cows from which oocytes are collected (Snijders et al., 2000a), 4) ovarian phase of cows from which oocytes are collected (De Wit et al., 2000), 5) body condition score (BCS) of cows from which oocytes are collected (Snijders et al., 2000b), 6) heat stress of cows from which oocytes are collected (Wolfenson et al., 2000), 7) season of year when oocytes are collected (Al-Katanani et al., 2002), 8) origin of oocytes (Lonergan et al., 2003), 9) growth factors in follicle in which oocytes developed (Purohit et al., 2005), 10) presence of corpus luteum (CL) in cows from which oocytes are collected (Boediono et al., 1995), and 11) plasma P4 concentration in cows from which oocytes are collected (McEvoy et al., 1995). These factors, in addition to the laboratory procedures (Gordon, 2003) are the key determinants for the success of IVEP.

Results from previous *in-vivo* studies with cattle indicated the conception rates were less when there were relatively lesser P4 plasma concentrations during the growth phase of the follicle from which ovulation occurred in dairy cows (Pursley and Martins, 2011). It is now well established that the lesser conception rate is due to lesser concentrations of P4 that primarily alter the follicular development, leading to the oocytes that are released with ovulation having abnormalities, and resulting in the impaired early embryonic development (Mihm et al., 1994; Ahmad et al., 1995). The presence of greater plasma P4 concentrations was also associated with more than a two-fold increase in blastocyst size in recipient cows (Lonergan et al., 2007). Results of *in-vitro* studies indicate the presence of a CL at the time of oocyte collections is not only associated with an enhanced oocyte quality, but also an enhanced developmental competence of oocytes in *Bos taurus* cows (Pirestani et al., 2011). These results, however, are inconsistent with the results from other studies (Hajarian et al., 2016). In these studies estrous cyclic or acyclic status of cows at the time of oocyte collection was not considered when categorizing the treatment groups. This perhaps confounded the results from these previous studies where this variable was not considered (Neglia et al., 2003; Sugulle et al., 2008; Penitente et al., 2015). When considering results of these previous studies, it is assumed that recovery, quality and early *in vitro* developmental competence of oocytes collected from estrous cyclic cattle will be greater than when oocytes are collected from estrous acyclic *Bos indicus* dairy cows.

*Bos indicus* cows are known for heat tolerance and tick resistance in tropical and subtropical regions of Southeast of the USA and Asia (Glass et al., 2005; Khan et al., 2008). The reproductive physiology of *Bos taurus* is quite different than that of *Bos indicus* cows (Sartori et al., 2010). Bos indicus cows generally have a longer prepubertal period, have prolonged periods of postpartum anestrus, have a tendency for seasonal breeding, and a shorter duration of behavioral estrus with fewer overt signs of estrus (Bó et al., 2003). Most of the research with the ART has been conducted with *Bos taurus* cattle. Similar information is generally lacking in *Bos indicus* cows. The primary objective of the present study, therefore, was to determine the effect of plasma P4 at the time of oocyte collection on oocyte recovery rate, oocyte quality, as well as early *in-vitro* embryo development, and developmental competence of embryos derived from oocytes of *Bos indicus* dairy cows that were collected when cows were estrous cyclic or acyclic. It was hypothesized that for the oocytes recovered from the estrous cyclic cows there will be a greater recovery rate, oocyte quality, as well as an early *in-vitro* development, and developmental competence of embryos derived from oocytes of these cows compared with those from estrous acyclic *Bos indicus* dairy cows.

## 2. Materials and methods

This study was conducted from December 2017 to April 2018 in embryology laboratory of Central Laboratory Complex of University of Veterinary and Animal Sciences, Ravi Campus, Pattoki. The experiments were performed using ready-made commercially available IVF media (Minitüb GmbH, Germany) and reagents of Sigma Chemical Company (St. Louis, MO, USA).

### 2.1. Collection of oocytes

The ovaries were collected in a local abattoir (*Bos indicus;* 5–8 years old with differing numbers of previous parities) with clinically normal reproductive tracts. The ovaries from estrous cyclic and acyclic cows were transported separately within 2 h in a thermos flask to the embryology laboratory in a 0.9% normal saline solution (30–35 °C) comprised of sodium chloride (Sigma; cat # S5886), 50 mg/ml streptomycin (Sigma; cat # P4562), and 500 µg/ml penicillin (Sigma; cat # P3032). To minimize the contamination, the ovaries were washed three times with normal saline and one time with distilled water soon after delivery to the laboratory.

### 2.2. Experimental design

These ovaries ($n$ = 750) were divided into two groups: 1) CYCLIC ($n$ = 318; presence of CL on either left or right ovary), and 2) ACYCLIC ($n$ = 432; no CL on either ovary). Afterward, oocytes were aspirated from follicles using the 18-gauge needle attached to a 10 ml syringe. The aspirated oocytes were graded into four categories on the basis of the morphology of COCs, compactness of granulosal layers, and homogeneous nature of the ooplasm (Kastrop et al., 1990). Thereafter, COCs were washed three times in TL HEPES solution (Minitüb GmbH, Germany) supplemented with 6 mg/ml bovine serum albumin (BSA; Sigma fraction V, cat # A6003).

### 2.3. In vitro maturation (IVM)

The COCs were initially washed three times using the IVM media. Oocytes were subsequently transferred in the form groups (10/group) in four-well plates (SPL, Life Sciences, Korea) containing 100 µl droplets of IVM culture medium consisting of (TCM 199 stock solution; Minitüb GmbH, Germany) supplemented with oestrus cow serum (OCS, own production), 0.5 µg/ml follicle stimulating

hormone (FSH; Sigma, cat # F2293), and 0.25 µg/ml luteinizing hormone (LH; Sigma, cat # L5269). The droplets with oocytes were covered with pre-warmed mineral oil and incubated for 24 h at 38.5 ºC in a $CO_2$ incubator (Shell lab®) with 5% $CO_2$ in air and 90%–95% relative humidity.

### 2.4. In vitro fertilization (IVF)

After the IVM, the COCs were washed three times in the fertilization medium before being transferred, in groups, to four-well plates containing 100 µl fertilization medium droplets consisting of TL stock solution (Minitüb GmbH, Germany) supplemented with 6 mg/ml bovine serum albumin (BSA; Sigma fraction V, cat # A6003), 10 µl/ml sodium-pyruvate (Sigma; cat # P4562) and 20 µl/ml heparin (Sigma; cat # H3149). The IVF media droplets were pre-warmed and covered with mineral oil. Frozen semen that had been evaluated and categorized to be of good quality of a Sahiwal bull was thawed and prepared using the sperm swim up procedure as described previously (Lonergan et al., 1994b). Thawed sperm (0.5 ml) was layered under 1 ml of TL stock solution (Minitüb GmbH, Germany) supplemented bovine serum albumin (BSA; Sigma fraction V, cat # A6003), 50 µl/ml sodium-pyruvate (Sigma; cat # P4562), and 1 µl/ml gentamicin (Sigma; cat # 345815) in four separate tubes and these tubes were placed in an incubator at a tilted angle. After 1 h of culturing at 38.5 °C, 0.85 ml of media that was at the top of each tube was removed and pooled. Separated sperm were centrifuged at 328 × g for 10 min. The sperm pellet was re-suspended in 0.5 ml of TL stock solution (Minitüb GmbH, Germany) supplemented with bovine serum albumin (BSA; Sigma fraction V, cat # A6003), 50 µl/ml sodium-pyruvate (Sigma; cat # P4562), and 1 µl/ml gentamicin (Sigma; cat # 345815) and this mixture was allowed to equilibrate at 22 °C for 10 min to make 2 x $l0^6$ sperm/ml, the final concentration. Afterward, a droplet of capacitated semen was added in IVF media containing COCs. Subsequently, sperm and COCs were incubated together for a period of 18 h at 38.5 °C in 5% $CO_2$ with maximal conditions of 90%–95% relative humidity.

### 2.5. In vitro culture (IVC)

After 18 h of IVF, presumptive zygotes were washed three times in the washing media consisted of TL HEPES (Minitüb GmbH, Germany) and supplemented with 6 mg/ml bovine serum albumin (BSA; Sigma fraction V, cat # A6003). Meanwhile, denuding was performed meticulously with gentle pipetting. After removing the cumulus complex, presumptive zygotes were washed three times using IVC media composed of the SOF stock solution (Minitüb GmbH, Germany) supplemented with 1 ml oestrous cow serum (own production), 40 µl/ml amino acids essential (Sigma; cat # M5550), and 10 µl/ml amino acids non-essential (Sigma; cat # M7145). The 100 µl IVC droplets were subsequently covered with pre-warmed mineral oil and incubated for 24 h at 38.5 °C in a $CO_2$ incubator in conditions of 5% $CO_2$ in the atmosphere and 90%–95% relative humidity. To determine every cell stage, *in vitro* embryonic development was recorded on days 3, 4 and 5 after the day of insemination.

### 2.6. Blood samples and progesterone assay

Blood samples from each cow were collected from the jugular vein immediately preceding the time of slaughter and the animal identification label was placed on the sample tube. When the ovaries were collected from the same (earlier tagged) cows concentrations of P4 in the blood samples were used to classify cows as being either CYCLIC or ACYCLIC based on the presence or absence of a CL and P4 concentrations in a subset representing both groups ($n$ = 42 CYCLIC and $n$ = 43, from ACYCLIC) of cows. An 18-gauge, 3.8 cm hypodermic needle attached to a 10 ml syringe without inclusion of anticoagulant was used to collect the blood. The serum was separated within 30 min and stored at -20 °C until assayed. Serum concentrations of P4 were quantified using double-antibody radioimmunoassay using commercially available kits (ImmunoTech, Prague, Czech Republic) using $^{125}$I-labelled tracer. Hormonal assay was performed in the endocrinology laboratory of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad. The inter-assay and intra-assay coefficients of variations were 6.2% and 3.5%, respectively.

### 2.7. Statistical analysis

Cows were classified as CYCLIC if any portion of either ovary contained a corpus luteum on the day of slaughter otherwise cows were classified as being ACYCLIC. Continuous response variables such as mean number of oocytes recovered per ovary and serum P4 concentrations of CYCLIC and ACYCLIC cows were analyzed using PROC TTEST. Other response variables such as oocyte quality, oocyte maturation, cleavage, embryo development to the 8-, and 16-cell stages when oocytes were derived from either CYCLIC or ACYCLIC cows were analyzed using the GLIMMIX procedure of SAS. All models included the fixed effect of estrous cyclicity and the random effect of replicate (day of collection of ovary). The contemporary group on a specific slaughter day was considered as the experimental unit. The data were of a binomial distribution. All these data were analyzed using the Statistical Analysis System (SAS ver. 9.4 Institute, Inc., Cary, NC, USA). The level of significance to reject the null hypotheses (HO) was 5%, and values for a variable were considered to be different when $P \leq 0.05$.

## 3. Results

The data for effect of CL on the serum P4 concentrations between estrous CYCLIC and ACYCLIC *Bos indicus* cows are presented in Table 1. Results indicate that the mean serum P4 concentrations (4.21 ± 0.4 ng/ml compared with 0.5 ± 0.2 ng/ml; $P < 0.05$) were greater for estrous CYCLIC as compared with ACYCLIC cows, respectively. Data for the effect of plasma P4 on the number of

**Table 1**
Effect of corpus luteum on serum concentrations of progesterone (mean ± SEM) in *Bos indicus* dairy cows.

| Groups | No. of cows (*n*) | Progesterone (ng/ml) |
|---|---|---|
| CYCLIC | 42 | 4.2 ± 0.4[a] |
| ACYCLIC | 43 | 0.5 ± 0.2[b] |

[a] Values with different superscripts differ within a column ($P < 0.05$).
[b] Values with different superscripts differ within a column ($P < 0.05$).

oocytes recovered per ovary are depicted in Fig. 1. The mean number of oocytes recovered per ovary (6.5 ± 0.5 compared with 4.0 ± 0.2; $P < 0.05$) was greater for estrous CYCLIC than ACYCLIC cows. The data for the effect of plasma P4 concentration on quality, *in-vitro* oocytes maturation, and embryonic development are included in Table 2 and the data for morphological assessments are depicted in Fig. 2. The oocytes with grade I_+_II quality (55.3% compared with 47.6%; $P < 0.05$) were greater, whereas, oocytes with grade III_+_IV quality (44.5% compared with 52.4 $P < 0.05$) were less from ovaries of estrous CYCLIC as compared with ACYCLIC cows, respectively (Table 2). *In-vitro* maturation rate (92.0% compared with 92.9%; $P < 0.05$) for embryos did not differ between two groups. In contrast, cleavage rate (70.9% compared with 52.8%; $P < 0.05$) of embryos was greater when oocytes were derived from estrous CYCLIC as compared with ACYCLIC cows, respectively. Similarly, the embryo development to the 8- (38.5% compared with 20.8%; $P < 0.05$) and 16- (20.0% compared with 10.9%; $P < 0.05$) cell stage embryos was greater when oocytes were derived from estrous CYCLIC as compared with ACYCLIC cows, respectively.

## 4. Discussion

To the best of our knowledge, this is the first study in which there were determinations of the effect of plasma P4 on the oocyte recovery rate, oocyte quality, and early IVEP outcomes when oocytes were derived from estrous CYCLIC and ACYCLIC *Bos indicus* dairy cows. It was hypothesized that with oocyte recovery from ovaries of estrous cyclic (greater P4) cows there would be a greater recovery rate, oocyte quality, and early *in-vitro* developmental competence of embryos when oocytes were derived from estrous cyclic compared with acyclic cows (lesser P4). In the current study, the most salient findings were manifested in differences in cleavage rate, 8-, and 16-cell embryo development which were 20%, 15%, and 10% greater when oocytes were derived from estrous CYCLIC as compared to ACYCLIC cows. These findings are supported by results of previous studies where progesterone was found to be required for the early embryonic development and its survival in dairy cows (Green et al., 2005). When there was use of oocytes from ovaries containing a CL, there was a greater rate of cleavage and embryo production with use of these oocytes as compared with oocytes from ovaries that did not contain a CL (Manjunatha et al., 2007). Similarly, the COCs recovered from ewes having a CL (cyclic) had an enhanced developmental competence, and there were greater rates of fertilization and blastocyst production as compared to embryos derived from COCs collected from ewes without a CL (Gonzalez-Bulnes et al., 2005). Furthermore, it was reported that the pig oocytes recovered from the ovaries containing a CL were highly competent when used for *in vitro* maturation and embryonic development

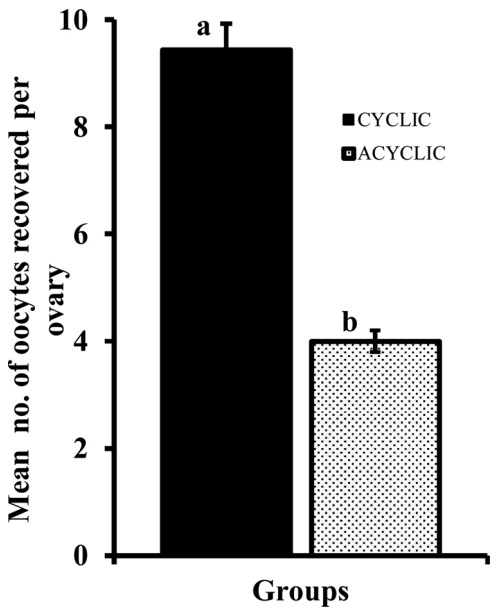


**Fig. 1.** Effect of plasma progesterone on the mean number of recovered oocytes per ovary in estrous CYCLIC and ACYCLIC Bos indicus cows; [ab]Superscripts indicate differences (P < 0.05) between groups.

**Table 2**
Results of effect of plasma progesterone (Least square means ± SEM)[1] on oocyte recovery rate, oocyte quality, and early *in-vitro* developmental competence of embryos derived with use of oocytes from estrous CYCLIC and ACYCLIC *Bos indicus* dairy cows.

| Variables | CYCLIC[2] | ACYCLIC[3] | *P*-value | Odds ratio |
|---|---|---|---|---|
| Grade I_+_II oocyte[4]; % | 55.3 ± 1.0 | 47.6 ± 1.0 | < 0.0001 | 1.363 |
| Grade III_+_IV oocyte[5]; % | 44.5 ± 1.0 | 52.4 ± 1.0 | < 0.0001 | 0.734 |
| Maturation rate[6]; % | 92.0 ± 1.0 | 92.9 ± 1.0 | 0.6766 | 0.874 |
| Cleavage[7]; % | 70.9 ± 2.0 | 52.8 ± 2.0 | < 0.0001 | 2.167 |
| 8-cell stage embryos[8]; % | 38.5 ± 3.0 | 20.8 ± 3.0 | < 0.0001 | 2.403 |
| 16-cell stage embryos[9]; % | 20.0 ± 2.0 | 10.9 ± 2.0 | 0.0046 | 2.062 |

GLIMMIX procedure was applied to test the difference between the groups.
Grade I: Oocyte having more than 5 layers of granulosa cells, Grade II: Oocyte having more 3–5 layers of granulosa cells, Grade III: Partially denuded oocytes, Grade IV: Completely denuded oocytes.

[1] All variables are represented with LSM ± SEM generated from the model.
[2] CYCLIC; Cow having active CL on either left or right ovary.
[3] ACYCLIC Cow without CL on either ovary.
[4] Grade I_+_II oocyte %; No. of oocytes with grade I_+_II/ total no. of oocytes recovered * 100.
[5] Grade III_+_IV oocyte %; No. of oocytes with grade III_+_IV/ total no. of oocytes recovered * 100.
[6] Matured rate; No. of oocytes matured/ total no. of oocytes cultured for IVM * 100.
[7] Cleavage %; No. of oocytes cleaved/ total no. of oocytes cultured for IVM * 100.
[8] 8-cell stage embryo %; No. of 8-cell stage embryos/ total no. of oocytes cleaved * 100.
[9] 16-cell stage embryo %; No. of 16-cell stage embryos/ total no. of oocytes cleaved * 100.

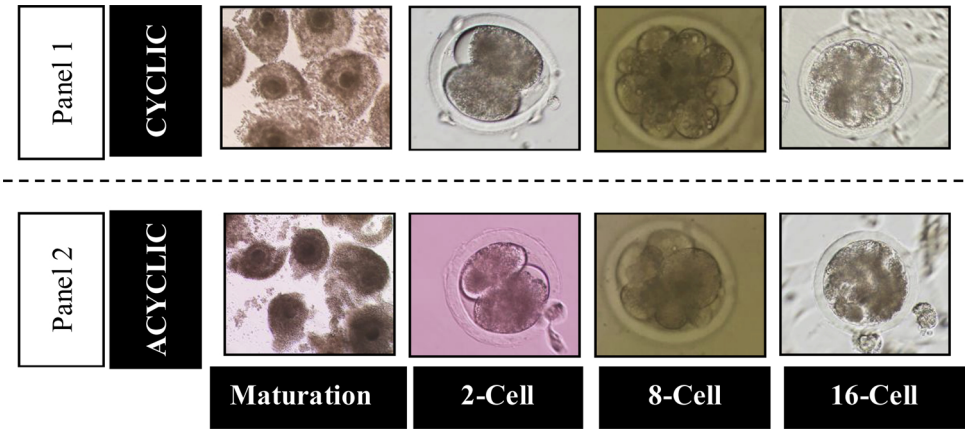


**Fig. 2.** Effect of plasma progesterone on the quality, *in-vitro* maturation of oocytes and early developmental competence of embryos derived from oocytes collected from estrous CYCLIC and ACYCLIC *Bos indicus* dairy cows; These images were observed using an inverted microscope (Olympus IX73) with an attached DP74 camera (400X); Maturation rate did not differ ($P < 0.05$) between two groups; Percentage of 2-cell, 8-cell, and 16-cell stage embryos were greater ($P < 0.05$) in estrous CYCLIC as compared to the ACYCLIC group.

studies (Yoshizawa et al., 2009). Consistent with results from previous *in-vivo* studies, there was a markedly greater pregnancy rate in dairy cows with greater compared with lesser P4 prior to AI (Inskeep, 2004; Wiltbank et al., 2011). Similarly, if there is a dominant follicle that develops in cows during the second wave of ovarian follicular development occurring during the estrous cycle from which ovulation occurs (relatively greater progesterone), there is a greater pregnancy rate than in cows having ovulations from the dominant follicle that develops during the first wave of ovarian follicular development (relatively lesser progesterone) (Bisinotto et al., 2010). The most plausible reason for the lesser IVEP rate in estrous acyclic cows (Santos et al., 2016) could be the lesser concentration of P4 which is associated with a lesser feedback of this hormone at the hypothalamus resulting in a relatively greater frequency of release of LH pulses (Callesen et al., 1986) as a result of the regulatory effect on the hypothalamus-pituitary-gonadal axis (Clarke and Pompolo, 2005). Furthermore, the lesser P4 may result in the differences in follicular fluid composition (Cerri et al., 2011), cumulus expansion and oocyte competence (Fair and Lonergan, 2012). The relatively lesser P4 concentrations leads to disruption of the oocyte nuclear maturation (Rajamahendran and Manikkam, 1994), and ultimately compromised embryo quality when there is a fertilization process involving oocytes derived from animals with lesser P4 concentrations (Rivera et al., 2011). It, therefore, would be interesting to ascertain the effect of P4 added to the IVEP media in future studies.

In the present study, the percentage of good quality oocytes was greater when oocytes were collected from estrous CYCLIC as compared with ACYCLIC cows. Likewise, in a previous study the percentage of good quality COCs was greater in pregnant as compared to non-pregnant Holstein Friesian cows (Moreno et al., 1993).Furthermore, not only is the presence of CL (Pfeifer et al., 2009a) but also its diameter (Penitente-Filho et al., 2014) is associated with a greater quality of COCs. The most plausible reason for

these outcomes is that there is an increased percentage of good quality COCs when there is greater concentrations of P4 (Ferguson et al., 2012), resulting in an optimal pattern of cyclic follicular atresia and development during waves of follicular development (Savio et al., 1993), and inhibition of oocyte apoptosis (Salhab et al., 2011) in estrous cyclic cows. Taken together, these results indicate that IVEP studies when there is use of the OPU technique might be conducted and there be more desirable outcomes when oocytes are collected from estrous cyclic as compared to acyclic cows.

The results of the present study indicate that recovery of oocytes was 1.6 times greater in estrous CYCLICc as compared to ACYCLIC cows. Similarly, the mean number of oocytes per ovary was 1.5 times greater in estrous CYCLIC than ACYCLIC cows (Moreno et al., 1993). In another previous study, the mean number of oocytes per ovary, however, was not different (15 compared with 15) between CL bearing and CL non-bearing ovaries in dairy cows (Boediono et al., 1995). The mechanism of how progesterone is involved in the regulation of follicle recruitment is still unclear (Pfeifer et al., 2009b). Suboptimal P4 concentrations during follicular growth may alter uterine functions (Shaham-Albalancy et al., 1997), endometrial release of PGF2α, subsequent luteal lifespan (Shaham-Albalancy et al., 2001; Cerri et al., 2011) and impair oocyte competence (Bisinotto et al., 2010). The variability of outcomes among studies might be attributed to number of parities of cows before the time of oocyte collection (Grimard et al., 2013), BCS at the time of oocyte collection (Siddiqui et al., 2002), hormonal imbalance at the time of oocyte collection (Pfeifer et al., 2009b), and aspiration technique used for oocyte collections (Mahesh et al., 2014) from cows. It would be interesting to determine the effect of the use of a CIDR (intra-vaginal inserted device containing progesterone) on the recruitment of COCs in estrous acyclic dairy cows.

It is concluded from results of the present study that the mean number of oocytes per ovary, percentage of the grade I_ + _II (good quality) oocytes, as well as cleavage rate, and development rate of embryos to the 8-, and 16-cell stage were greater when oocytes were derived from estrous CYCLIC as compared with ACYCLIC *Bos indicus* dairy cows.

## Conflict of interest statement

The authors intend to submit manuscript entitled as “Effect of plasma progesterone on oocyte recovery, oocyte quality, and early *in-vitro* developmental competence of embryos in *Bos indicus* dairy cows” in Animal Reproduction Science and have no conflicts of interest.

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