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Supplementation of Cattle Follicular Fluid Improves Developmental Competence of Buffalo Oocytes

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Abstract

DURING in vitro embryo production (IVEP), follicular fluid acts as a natural supplement to the maturation medium; however, species differences in its effectiveness remain unclear. The study aimed to compare progesterone and estradiol concentrations in cattle and buffalo follicular fluid from the dominant follicle and to assess the effect of cattle follicular fluid on the developmental competence of buffalo oocytes. Buffaloes and cows were synchronized using CIDR-based protocols, and follicular fluid was aspirated from the dominant follicle (≥ 12 mm) of slaughtered ovaries. Estradiol and progesterone concentrations were quantified in the follicular fluid, which was then incorporated into the IVM medium, divided into three groups: Cattle follicular fluid (cFF), Buffalo follicular fluid (buFF), and control containing oocytes (n = 1073) retrieved from 668 ovaries of slaughtered buffaloes. The results indicated that cFF exhibited higher estradiol (E2), progesterone (P4), and estradiol/progesterone ratio compared to buFF aspirated from dominant follicles. Buffalo oocytes supplemented with cFF exhibited higher maturation rates based on cumulus expansion, compared to other treatments. Supplementation of cFF into the maturation media improved the cleavage, morula, blastocyst rates, as well as the cell count of hatched blastocysts, compared to the other treatments. In conclusion, incorporating cFF into the maturation medium enhanced cumulus expansion and improved the developmental competence of buffalo oocytes, leading to higher blastocyst yield.

Keywords: Cattle follicular fluid, Cumulus expansion, Buffalo, Synchronization, Developmental competence.

Introduction

During the in vitro embryo production (IVEP) process, the transformation of the oocyte into an embryo is a complex mechanism, which is particularly associated with the health and quality of the oocyte. The oocyte maturation is the first and most crucial step toward its success [1], which is influenced by multifold factors, linked to oocyte donor conditions (age, metabolic status, stage of estrous cycle, follicle size, seasonality etc.), in vitro cultures conditions (recovery method, composition of media, culture system, etc.) or oocyte quality (nuclear or cytoplasmic maturation, cumulus cells integrity, oocyte metabolomics etc.) [2]. In vivo oocyte maturation is superior to the in vitro process because the follicular microenvironment provides an ideal condition for oocyte maturation and developmental competence. Keeping this idea of follicular fluid incorporation to maturation media as a natural bio fluid for oocyte and an economical source of complex matrix, numerous studies reported

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the beneficial impact of bovine follicular fluid in cattle IVEP [3, 4]. Also similar phenomenon was replicated in buffalo IVEP by adding buffalo follicular fluid to the maturation medium optimizes buffalo oocyte maturation and embryo development [5-8], but the outcomes were inconsistent. Interestingly, supplementation of bovine follicular fluid was graded as a superior bio fluid and used to improve the embryo quality in cattle [4, 9]. Previously, the beneficial impact of follicular fluid supplementation has been investigated inter-species for the enhancement of oocyte maturation [10-14]. However, there is no literature available about the use of cFF in buffalo oocytes. Incorporation of bovine follicular fluids was also linked to its natural properties, which are helpful in IVEP with less epigenetic and gene expression aberrations than standard protocols [3].

Buffalo show low reproductive efficiency compared with cow, due differences in follicular dynamics and hormonal milieu. However, comparative data on follicular fluid composition between the two species at the same physiological stage and their effect on the developmental competence of buffalo oocytes *in vitro* limited. Therefore, we hypothesized that buffalo exhibit low estradiol and progesterone concentrations in the follicular fluid than cows at the preovulatory stage, and that supplementation of cFF from dominant follicle improves oocyte cumulus expansion and subsequent developmental competence compared to buFF. The present study aimed to compare the progesterone and estradiol concentrations between cattle and buffalo follicular fluid and to assess the effect of cFF from dominant follicle on cumulus expansion and developmental competence of buffalo oocytes.

Material and Methods

All the experimental procedures were performed in accordance with the Ethics Review Committee for Animal Experiments of Guangxi University, Nanning, China. Unless stated otherwise, all media and chemicals were purchased from Sigma (St. Louis, MO, USA). The filtered solutions were stored at 4°C or -20°C until use.

Follicular fluid recovery

For this purpose, a total of multiparous cows (n = 11) and buffaloes (n = 12) with body condition score (BCS) 3.5 ± 0.1 and parity 2.1 ± 0.2 were synchronized through a CIDR-based protocol [15]. The synchronized animals were selected at the early stage of the estrus period by monitoring follicle size ≥12 mm) through ultrasonography and were labeled before slaughter. Following slaughtering, the paired ovaries were collected immediately and transferred to the laboratory in normal saline in separate thermos for each cattle and buffalo category. Follicular fluids were aspirated from the ovaries of buffaloes and cows containing ≥12 mm size follicles using an 18-gauge needle. A portion of follicular fluid from each individual follicle was reserved for hormonal analysis. The remaining follicular fluid from all follicles of the same species was pooled, samples were centrifuged at 3000 rpm for 30 minutes at 4ºC, and supernatant was filtered through 0.22 µm filters and frozen at -80ºC until further use. A schematic illustration of the experimental design was presented in Figure 1.

Hormonal assay

The concentrations of E_2 and P_4 in the individual follicular fluid samples were determined to confirm the status of the follicle dominance or atresia [16]. Follicles were classified as pre-ovulatory or non-ovulatory when the E2/P4 ratio was > 1 or < 1, respectively [17]. E2 and P4 levels in the follicular fluid were determined using a BioTek Epoch micro plate spectrophotometer and ELISA Kits (E2; Fine Test EB0081 China, and P4; Fine Test Cat # EB0016 China), following the manufacturer's instructions. The variation coefficients within and between assays were under 15% for both hormones. This experiment was repeated three times.

Cumulus-Oocyte Complex collection and in vitro maturation

The buffalo ovaries (n = 668) were collected from a local abattoir in Nanning (23.003° N, 108.571°E) China, and transferred to the laboratory within 3-4 hours in normal saline. Ovaries were washed with normal saline to remove the blood and foreign materials [18]. This experiment was replicated fifteen times. Cumulus-oocyte complex (COCs) was aspirated from ≥ 6 mm follicles through a 10-mL needle containing 1 ml HEPES-buffered tissue culture medium (TCM)-199 supplemented with 10% fetal bovine serum (FBS). The COCs (n = 1073) were selected based on the COCs morphology, ooplasm homogeneity, and number/compactness of layers of cumulus cells using a stereomicroscope (SMZ645; Nikon, Tokyo, Japan). Later, the selected COCs were washed thrice in maturation medium and placed in a group culture condition, presented 20-25 COCs in the four-well plate containing 100µl volume *in vitro* maturation media: 10% cFF (v/v), 10% buff (v/v), or 10% FBS (control: v/v) into TCM-199; Earle's salts, Gibco; L-Glutamine, 2.2g/L sodium bicarbonate, 25 mM Hepes, 0.5 mg/ml FSH and 5 gm/ml LH. The oocytes were incubated in IVM media at 38.5 °C, 5% CO2, and 90-95% humidity for 22-24 hours. The rate of oocyte maturation was assessed by observing the expansion of the accumulation after the incubation period. The

maturation rate based on cumulus expansion: Grade A, full expansion and even cytoplasm; Grade B, partial expansion and uneven cytoplasm; and Grade C, non-expansion. A subgroup of COCs placed in maturation medium containing cFF (n = 115), buFF (n = 150), or control (n = 162), was assessed for maturation based on polar body extrusion. Firstly, the cumulus cells were manually pipetted with 2.5 mg/mL hyaluronidase and fixed in 4% paraformaldehyde [19]. Later, the oocytes were stained for 5 min at room temperature with 1 g/mL Hoechst (33342) after three washes with DPBS. Oocyte polar body extrusion was observed under a fluorescence microscope on glycerin-sealed slides (IX73; Olympus).

In vitro fertilization and culture

The mature COCs of grades A and B (cumulus fully and partially expanded) selected under a stereomicroscope (SMZ645; Nikon, Japan) were later processed for the separation of cumulus cells and in vitro fertilization. Thawed buffalo semen was added to 3 mL of fertilization medium (Tyrode's medium with 0.6% BSA, 2.5 mM caffeine sodium benzoate, and 20 µg/mL heparin) in a sterile centrifuge tube. The sperm were allowed 30-40 min to float to the top in a humidified incubator (150i; Heraeus, Germany) at 38.5°C with 5% CO_2. A final concentration of 1–2 × 10^6 sperm/mL was added to the medium drop (10-15 oocytes per drop) and incubated at 38.5°C with 5% CO_2 for 6-8 hours [18]. The presumptive zygotes were cultured in an embryo culture medium (BO-IVC, Falmouth, UK) in an incubator (150i; Heraeus, Germany) at 38.5°C with 5% CO_2 and 5% O_2, in humidified air for 7 d. The cleavage rate was assessed 48 h after insemination, whereas development rates were monitored at days 4, 5, 6, and 7 post-inseminations. The rate of maturation, cleavage, morula, and blastocyst formation (%) were determined by dividing the number of matured oocytes, cleaved oocytes, morulae, and blastocysts formed by the respective total number of oocytes cultured for IVM, inseminated, or cleaved, and then multiplying by 100.

Cell count

A subgroup of embryos at day 8 of culture was stained in TCM-199 with 1 µg/mL Hoechst 33342 for 30 min in the dark at room temperature to count the number of cells. DPBS (Dulbecco's phosphate buffered saline) was used with glycerol and Hochest (ratio of 1:1:0.001) as the mounting media and sealed the slides with nail polish. Blastocyst cells were counted using an epifluorescence microscope at 200X magnification (50i; Nikon, Japan) after washing three times with DPBS. Data were collected from fifteen replicates.

Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests determined data normality. PROC TTEST was used to compare estradiol, progesterone, and their ratio (EPR). Oocyte maturation, cleavage, morula, and blastocyst stages were analyzed using a generalized linear mixed effects model using SAS's GLIMMIX technique. Drop was considered an experimental unit. Continuous cell count data were analyzed using a linear mixed-effects model with the Mixed procedure in SAS. A value of $P \leq 0.05$ was considered significant. All data were analyzed by a statistical method (SAS ver. 9.4, Institute, Inc., Cary, NC, USA).

Results

Table 1 indicates that the concentrations of E_2, P_4, and the E_2/P_4 ratio in follicular fluid aspirated from follicles (size ≥12 mm) were higher ($P < 0.05$) in the ovaries collected from cows compared to buffaloes.

Table 2 depicts that the cumulus expansion-based in vitro maturation rate supplemented with cFF was higher (350/374 vs 304/360 vs 289/339; $P < 0.01$) compared to other treatments; albeit oocyte maturation rate based on polar body extrusion was the same (65/115 vs 84/150 vs 89/162; $P > 0.05$) among the treatments. The cleavage rate was greater (199/339 vs 148/304 vs 143/290; $P < 0.02$) when supplemented with cFF compared to the other treatments. The proportion of embryo development at morula stages was greater (112/199 vs 60/148 vs 59/143; $P < 0.01$) supplemented with cFF than in other treatments. Likewise, the blastocyst rate was higher (62/199 vs 28/148 vs 29/143; $P < 0.02$) when supplemented with cFF compared to other treatments (Figure 2). The average number of blastocyst cell was greater ($P < 0.01$) in the treatment supplemented with cFF compared to other treatments.

Discussion

Under in vivo conditions, follicular fluid surrounds the oocyte, as a natural bio fluid that promotes oocyte maturation, fertilization, and embryo development. The current document is one of the few that has examined the effect of cFF on in vitro maturation, subsequent embryo development, and embryo quality in buffalo oocytes.

The obtained results of high oocyte maturation rate, based on cumulus expansion, indicate the effective support by cFF compared to buFF. This might have happened due to the higher estradiol and E_2/P_4 ratio in cFF compared to buFF from the dominant follicle. The probable reason for the less potent effect of steroids and their ratio in buFF might be due to variations in ovarian size [20], high incidence of deep atresia [21], and a smaller

population of recruit able follicles compared to the bovine species. The follicular fluid from the dominant follicle in cattle likely creates a more robust estrogenic environment by enhancing gap-junction communication and aromatase activity, thus maintaining estrogen synthesis during dominance [22]. Conversely, buffalo follicular fluid, characterized by lower estradiol levels and increased atresia, may offer a less conducive setting for oocyte developmental competence, which could account for interspecies differences in in vitro maturation outcomes [22]. It is also noteworthy that optimal steroid levels in the dominant follicle have the potential to enhance cumulus expansion by regulating EGF expression from P_4-producing granulosa cells [23]. The follicular fluid from the dominant follicle plays a regulatory role in stimulating factors, cumulus expansion, and oocyte meiotic maturation [16]. Current data suggest that cumulus expansion might be directly linked to embryonic development when supplementing cFF into maturation media. It has been reported that the expansion of cumulus cells in bovines enhances the uptake of glucose by granulosa cells [24]. Bovine ovaries that had a dominant follicle with higher concentrations of E_2 were more likely to develop into the blastocyst [25]. During the recruitment and selection stages, the E_2 concentration in the follicles is low, as the follicle approaches dominance; when this E_2-rich follicular fluid is added to the maturation medium, it enhances cytoplasmic maturation and increases oocyte developmental competence [26]. The absence of glycoprotein analysis presents a limitation in this study, as only steroids were quantified. Moreover, the oocyte quality and maturity correlate with E_2 levels in follicular fluid, as well as their ratio [27], which aligns with findings from previous studies [28]. In the present study, we have collected the follicular fluid from dominant follicles of both species and determined the E_2 concentration and E_2/P_4 ratio; however, based on polar body extrusion, oocyte maturation results remained the same among the treatments.

To investigate the impact of cFF on oocyte developmental competence, the embryo development and quality after fertilization were analyzed, and the results revealed a discernible effect of cFF on cleavage, morula, and blastocyst development compared to the other treatments. Such variations in embryo production might be linked to the dominant follicle environment because it contains molecules such as sterols, purines, and growth factors, which act as mediators in various species [29]. The blastocyst cell numbers in buffalo oocytes were higher when supplemented with cFF compared to other treatments, which indicated that cFF has higher and more potent steroid concentrations for embryo development. These events likely explain the improvement achieved by cFF supplementation during IVM and embryo development in buffalo oocytes.

Conclusion

In conclusion, buffalo showed lower estradiol and progesterone levels in follicular fluid than cows in dominant follicle, and incorporating cFF from dominant follicle alone or in combination with serum in a TCM medium enhanced cumulus expansion and developmental competence of buffalo oocytes, resulting in higher blastocyst yields. Future studies are warranted to explore the comparative transcriptomic, proteomic, and metabolomics analysis of oocytes matured with cFF versus those with buFF may determine which supplement in the cattle follicular fluid most effectively promotes developmental competence of buffalo oocytes.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

TABLE 1. Comparison of estradiol (E_2) and progesterone (P_4) concentrations, alongside E_2/P_4 ratio, in cattle (cFF) and buffalo follicular fluid (buFF) derived from ovaries of follicular size ≥ 12 mm.

	Follicular fluid			
Characteristics	**cFF**	**buFF**	**SEM**	***P-value***
Progesterone; ng/ml	34.23	29.83	0.69	<0.01
Estradiol; ng/ml	133.92	103.05	2.1	<0.01
E2/P4 ratio	3.92	3.44	0.1	0.03

Values $P \leq 0.05$ considered statistically significant. Data expressed as the least square means ± SEM.
Experiment was repeated for three times.

TABLE 2. Comparative effect of cattle and buffalo follicular fluid on in vitro maturation and developmental competence of buffalo oocytes.

	Follicular fluid				
Characteristics	**cFF**	**buFF**	**Control**	**SEM**	***P-value***
Oocytes examined (n)	374	360	339		
Maturation rate; %	93 [a]	82 [b]	85 [b]	0.01	<0.01
[+]Maturation rate; %	56	56	54	0.04	0.96
Cleavage rate; %	58 [a]	48 [b]	49 [b]	0.02	0.02
Morula; %	56 [a]	40 [b]	41 [b]	0.04	<0.01
Blastocyst; %	31 [a]	18 [b]	20 [b]	0.03	0.02
Cell count/blastocyst	124.3 (18) [a]	97.8 (16) [b]	100.6 (19) [b]	1.1	<0.01

Values of $P \leq 0.05$ were considered statistically significant.
[+] indicates maturation rate based on the first polar body extrusion in the subgroup from three replications.

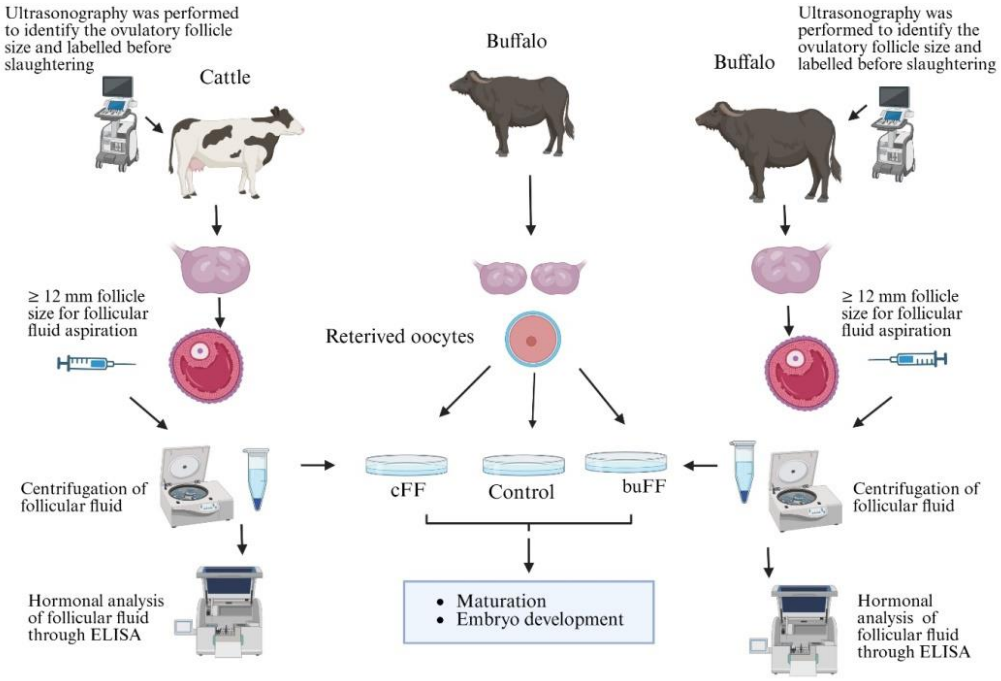

Fig. 1. Experimental concept schematic diagram.

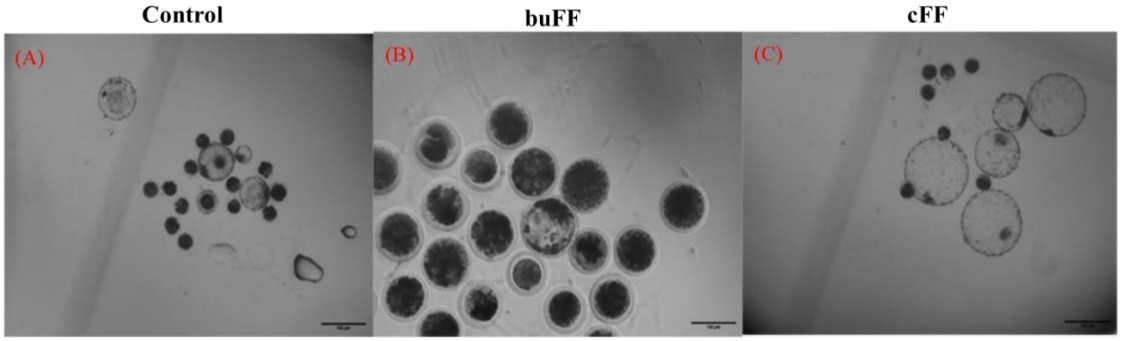

Fig. 2. Representative images of in vitro blastocyst development, either treated with control (A) or buffalo follicular fluid (buFF; B) or cattle follicular fluid (cFF; C) derived from a dominant follicle. The images were captured with a microscope (50i; Nikon, Japan) at 100X resolution.

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