Evaluations of Ovarian and Luteal Blood Flow Waveform Patterns in Buffalos Subjected to OvSynch Protocol in Cold and Hot Seasons

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Abstract: This current study aimed to determine ovarian and luteal blood flow waveform patterns in buffalos synchronized using OvSynch protocol in cold and hot seasons. Six cyclic buffalo cows aged 6±0.5 years old, having a weight of 400 ± 50 kg, were scanned daily along three successive estrous cycles transrectally by Doppler ultrasonography to evaluate the normal ovarian hemodynamic during the normal spontaneous ovulation and before the start of experiments. Buffaloes were synchronized with gonadotropin[GnRH] –prostaglandin[P] –gonadotropin (GPG) protocol in which animals received 10µg of GnRH on day ??, 0.250µg of PGF2α on day 7, and another dose of 10µg of GnRH was administered 48h after the PGF2α injection. Blood sampling and ovarian ultrasound examinations (color and spectral Doppler modes) were conducted on the day of the estrous and luteal phases. Results revealed that peak systolic velocity waveform (PSV) was significantly ($P<0.05$) increased in the cold season compared to the hot season. The Luteal blood flow after the end of OvSynch protocol on days (5,7,9, and 11) was significantly increased in the cold season than that in the hot one. The serum levels of estradiol (E2) and nitric oxide (NO) after the second GnRH injection in the OvSynch protocol were significantly ($P<0.05$) elevated in the cold season as compared to the hot one.

Moreover, the progesterone (P4) levels had risen in OvSynch-treated buffaloes on days 5,7,9, and 11 of the cycle in the cold season compared to the hot one. Conclusion: In the cold season, ovarian hemodynamics was significantly improved compared to the hot one; this may influence the reproductive efficiency of buffaloes. Further studies were needed to prove it.

Keywords: Buffalos, Doppler, season, follicles, luteal blood flow.

1. INTRODUCTION

The buffalo (Bubalus bubalis) has an important role in the agricultural economy in Egypt, providing about 10.90% and 1.97% of the world's buffalo's milk and meat, respectively [1]. Besides, buffalo can adapt to harsh environments and live in poor-quality forage [2]. Many factors hampered the reproductive efficiency in buffalo cows, such as; late puberty, silent heat, long calving intervals, and postpartum anestrous with a low response to super stimulation and embryo transfer [3]. Heat stress negatively affects fertility as temperatures rise [4]. Summer heat stress lowers fertility in cattle in hot environments by influencing oocyte quality, follicular activity, and blood plasma progesterone (P4) levels [4,5]. Silent estrus is a severe problem in large herds during periods of heat stress [6]. To improve reproductive efficiency, many estrus synchronization treatments have been used to improve the reproductive efficiency in buffaloes with the successful application of artificial insemination (AI) [7,8]. Although the OvSynch (OvS) program increases fertility performance during heat stress, it cannot prevent embryonic deaths [9]. Doppler ultrasonography is considered a non-invasive method to determine the blood velocity within the ovarian follicle and the developed corpus luteum (CL) [10, 11]. The blood flow of the follicles and/or CL correlated with their dimensions in heifers [12] and mares [13]. Furthermore, the changes in the follicular hemodynamics provided an indicator of ovulation in heifers [14] and mares [15]. Studies of follicular and luteal hemodynamics along the estrous synchronization with GPG in correlation with the hormonal levels of progesterone and estrogen are not available for Egyptian buffaloes. Therefore, this study aimed to evaluate the effect of Ovsynch- protocols on follicles population and ovarian hemodynamics ascertained by color Doppler ultrasonography and hormonal evaluation during cold and hot seasons.

2. MATERIALS AND METHODS

The protocol for animal experiments was ethically approved by the Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Cairo University: (2018-07V)

2.1. Animals

The study was conducted during the hot and cold seasons; cold season (winter (from December 2020 to

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March 2021), average temperature, precipitation, and humidity for 3 months: respectively 20.5 ◦C (min 18–max 23◦C), 50.0%; n = 6), and Hot season (summer (from June 2021 to August 2021), average temperature, precipitation, and humidity for 3 months: respectively 35.5 ◦C (min 34.2–max 40.1 ◦C), 54.0%; n = 6). The buffaloes (6±0.5 years old) weighed 400 ± 50 kg, and the average body condition score was 3.0 ± 0.59. Buffaloes were raised on the farm belonging to the Faculty of Veterinary Medicine, Theriogenology, Cairo University, Egypt (latitude 30'01" N; longitude 31'21" E). Buffaloes were housed under natural temperature and daylight. Each animal received the maintenance requirement composed of commercial concentrate ration (16% crude protein/) and the green clover (Trifolium alexandrinum) ad libitum. Physical examination was performed to confirm that none of the animals had any evidence of disease.

2.2. Estrous Synchronization and Experimental Design

The OvS protocols s, including treatments, time of Doppler scanning, and a blood sample, were illustrated in Table **1**. Synchronization with Gonadotropin-releasing hormone-prostaglandin (OPG OvSynch) and PRID: Buffaloes received 10μg of GnRH (gonadotropin-releasing hormone analog; 5.0 ml Receptal, MSD Animal Health, Intervet International GnBH, Germany) at Day 0 accompanied by 250μg of PGF2α at Day 7 and another 10μg of GnRH was administered 48h after the PGF2α injection [16]. One to three days after removal of the PRID device, Buffalo cows were checked for estrous.

2.3. Doppler Ultrasonography

All animals were subjected to Transrectal Doppler ultrasonographic scanning to determine follicle growth and ovulation by monitoring the corpus luteum. The ovarian structures (Figures **1a-f**) were monitored using a Real-Time B-mode 12 MHz linear array transducer (SonoAceR3, Samsung, Medison, South Korea). Ovarian follicles were counted and classified into recruited small follicles (≤5 mm), selected medium-sized (>5- to <10 mm,), and deviated dominant follicles (≥10mm, Figure **1**) according to their diameter [17]. For blood flow mapping, the color flow mode (CFM) was activated, and the blood flow of follicles and developing CL was recorded. All the scans were performed by the same analyzer. The images and video clips of B-mode, color, and spectral Doppler were saved until further analysis. The disappearance of the F1 and the formation of CL at its site one to two days later was considered the time of ovulation (Day 0), as previously reported by [18]. The measurement of CL diameter was recorded throughout the estrous cycle [19]. The direction of blood flow within the ovarian artery was determined by color mode. The peak systolic velocity (PSV), end-diastolic velocity (EDV), and time-average mean velocity (TAMV) were recorded for the ipsilateral and the contralateral artery of the ovary and uterus by the spectral Doppler [20,21]. The resistance index (RI), the pulsatility index (PI), and the systolic/diastolic (S/D) Doppler indices were also automatically determined by the Doppler scanner. The blood flow volume (BFV) was estimated by the scanner depending on either the diameter or the area (Figures **1e-f**) of the blood vessel, and those depending on the diameter (VFD) were included in the analysis.

2.5. Image Analysis

The red and blue areas of Doppler images of color blood flow per pixel were determined and analyzed at each examination by using Adobe Photoshop CC software (1990-2013, Adobe Systems) as previously mentioned in dairy cows [22].

2.6. Blood Sampling and Hormone Assaying

Progesterone (P4, EIA-1561) and estradiol 17-β (E2 EIA-2693) were analyzed using ELISA commercial kits (DRG, Germany). The blood samples were collected

Table 1: Schematic OvS Protocol for Treatment in the Cold and Hot Season

Examination protocol in hot and cold seasons							
0th Day (OvS)begin	**7th day**	**9th day**	**10th day**	**15th day after OvSynch(D 5)**	**17th day after OvSynch(D 7)**	**19th day after OvSynch(D 9)**	**21st day after OvSynch(D 11)**
First GnRH injection	Prostaglandin injection	Second GnRH injection	Blood sampling	Blood sampling	Blood sampling	Blood sampling	Blood sampling
Inner and outer examination		Estrus occurs	Follicular dynamic measurement	Doppler investigation	Doppler investigation	Doppler investigation	Doppler investigation

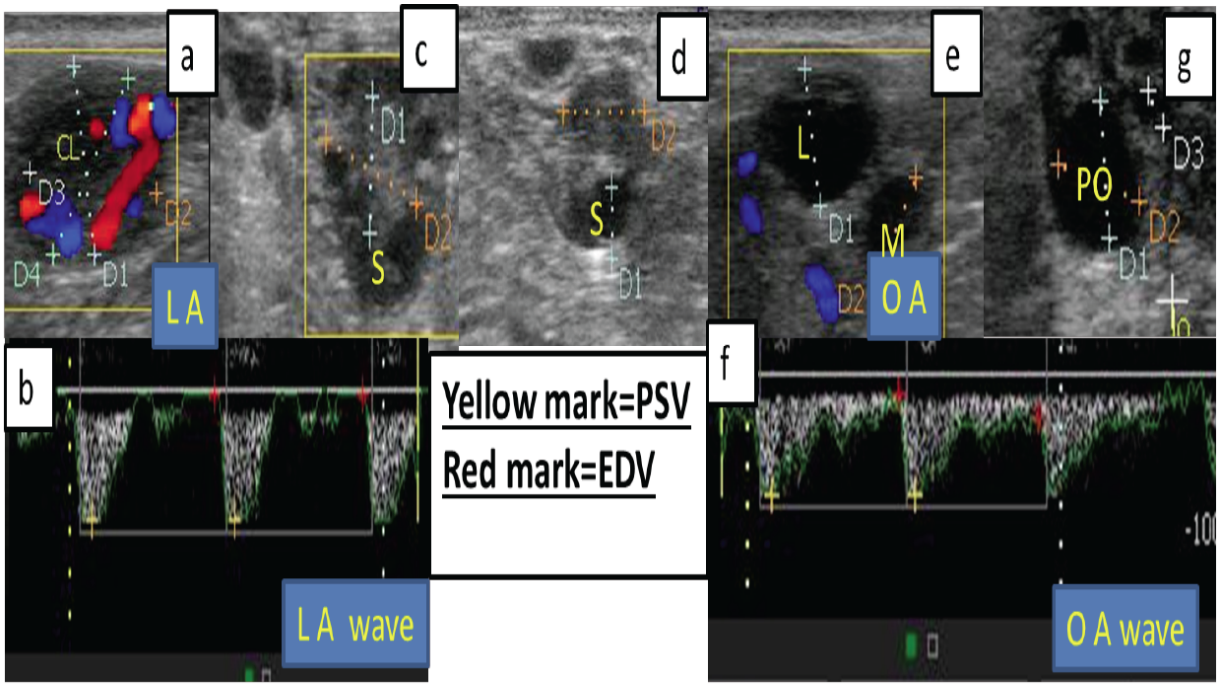

Figure 1: Ultrosograms reveled luteal artery (**a**,**b**), different follicle classes (**c**,**d**,**g**) and ovarian arteries (**e**,**f**) in buffalos at 15th day after OvSynch(D 5) in cold season.

following each ultrasound Doppler examination and centrifuged at 3000 rpm for 10 minutes, and sera were harvested and stored at -20ºC till further assay. The assay's sensitivity was 0.045 ng/ml, and the test intra- and inter-precisions were 5.4 and 9.96 for P4. The sensitivity of the assay was 9.7pg/ml, and the test intra- and inter-precisions were 6.81 and 7.25 for E2, as previously measured by [23].

2.7. Statistical Analysis

Middle uterine arteries' blood flow pattern and antioxidant levels were measured by one-way ANOVA. Also, the Duncan range test was used. The obtained data were analyzed with SPSS (Version 18.0, SPSS Inc., Chicago, Illinois, 2010). P values ≤ 0.001 were considered highly significant, and P values ≤ 0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1. Effect of GPG Protocol Estrous Synchronization Treatments on the Follicular and Luteal Blood Flow in Buffaloes During the Hot and Cold Seasons

It has been determined that heat stress negatively affects mostly the follicular fluid, the oocyte, and the granulosa cells [24]. Heat stress affects follicle selection and extends the follicle wave duration, resulting in a decrease in the quality of oocytes [25]. These results show that follicle content and structure are affected rather than the follicle size, which supports our findings. The follicular diameter of the buffalo ovary did not vary between the hot and cold seasons (Table **2**).

The peak systolic velocity waveform (PSV) significantly increased in the cold season than that observed in the hot season. The reverse trend was observed in P1 and R1 after the second GnRH injection in OvSynch protocol in hot and cold seasons (Table **2**). There is a great reduction in ovarian hemodynamics in rabbits and poultry by about 20-30% [26]. In cattle, fertility is reduced during Summer heat stress; this may be attributed to influencing oocyte quality, follicular activity, and blood plasma progesterone (P4) levels. The impact of heat stress was influenced mainly by a follicular dynamic which involves the duration of the follicular wave, follicular fluid, and oocyte quality [24,25]. The explanation of this effect may be due to lower levels of IGF-1 values in the summer than in the thermoneutral period [27,28]. However, the assay of IGF-1 is not recorded herein. IGF-1 plays a role in developing follicles in synergy with follicle-stimulating hormone (FSH) and LH and supports steroidogenesis by enhancing LH receptor induction and inhibin synthesis [29]. IGF-1 also increases the sensitivity of follicular cells to FSH and LH [30].

The Luteal blood flow after the end of the OvSynch protocol on days (5,7,9, and 11) is significantly increased in the cold season than in the hot one (Table **3**). In this respect, It has been demonstrated that heat stress causes a 20–30% reduction in blood flow in the ovaries of rabbits and poultry [31,32]. To our knowledge, there are no publications to date in which the differences between the LBF or the CL sizes according to the season in buffaloes were examined with color Doppler data. In addition, the buffaloes had the highest number Luteal total area (CLTA) and blood flow rate (CLBFR), luteal echotexture after the end of OvSynch protocol on days (5,7,9, and 11) in hot and cold seasons (Table **4**). In this respect, there is a decrease in blood flow shortly after ovulation, but with angiogenesis on the 2nd–5th days of the cycle, progesterone also increases with an increase in CL volume [33]. However, in this study, the CL measurements were made on the 7th and 9th days after the OvS protocol. Many factors support the function of CL. Luteinizing Hormone (LH) and Growth Hormone (GH) are necessary for the development and function of CL. In addition, angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are also effective [34].

3.2. Effect of GPG Protocol Estrous Synchronization Treatments on Steroid Hormone (P4 and E2) and No Metabolites Levels in Buffaloes during the Hot and Cold Season

Estrogen plays an important role in controlling the blood flow of the genital tract due to its vasodilator role [17]. The serum levels of E2 and No after the second GnRH injection in OvSynch protocol are significantly elevated in the cold season compared to the hot one (Table **2**). E2 levels and follicular blood flow were markedly increased after administering GnRH. These will lead to increases in metabolic function in follicular cells [35]. Moreover, the progesterone levels had risen

Table 2: Follicular Morphometry, Ovarian Hemodynamics, Serum Estradiol, and Nitric Oxide Metabolites Values 24 h after Second GnRH Injection in OvSynch Protocol in Hot and Cold Seasons. Data are Presented as Mean ±SEM

Season	Follicles (cm^2)	Ovarian A.PI	Ovarian A.RI	Ovarian A.PSV (cm/sec)	Estradiol(pg/mL)	Nitric oxide (µmol/L)
Hot season	1.66±0.65	1.37±0.02[b]	0.68±0.01[b]	13.25±0.69[a]	155.32±61.21[a]	21.58±3.02[a]
Cold Season	1.53±0.14	0.84±0.01[a]	0.44±0.01[a]	16.68±1.02[b]	241.14±77.22[b]	44.23±2.12[b]
P-Value	0.551	0.02	0.02	0.01	0.01	0.01

RI=resistance index, PI=pulstility index, PSV=peak velocity point.
[a,b]Superscripts values differ within the same day (P < 0.05).

Table 3: Luteal Blood Flow and Progesterone Levels after the End of OvSynch Protocol on Days (5,7,9, and 11) in Hot and Cold Seasons. Data are Presented as Mean ±SEM

	Hot season	Cold Season	P-Value
Day 5 after Ovsynch			
LBFV mL/min	1325±21.74[a]	2658±23.55[b]	0.01
Progesterone	0.87±0.01	0.97±0.01	0.52
Day 7 after Ovsynch			
LBFV mL/min	2031±21.74[a]	3215±22.85[b]	0.01
Progesterone	0.99±0.02	1.02±0.01	0.25
Day 9 after Ovsynch			
LBFV mL/min	2254±22.31[a]	3362±25.14[b]	0.01
Progesterone	0.84±0.02[a]	1.21±0.01[b]	0.02
Day 11 after Ovsynch			
LBFV mL/min	2569±21.45[a]	3542±23.65[b]	0.01
Progesterone	0.88±0.02[a]	1.33±0.21[b]	0.04

[a,b]Superscripts values differ within the same day (P < 0.05).

Table 4: Luteal Total Area (CLTA) and Blood Flow Rate (CLBFR), Luteal Echotexture after the End of OvSynch Protocol at Days (5,7,9, and 11) in Hot and Cold Seasons. Data are Presented as Mean ±SEM

	Hot season	Cold Season	P-Value
Day 5 after Ovsynch			
CLTA cm2	2.12±0.22 [a]	4.36±0.66 [b]	0.04
CLBFR bpm	63.21±5.21 [a]	75.21±8.21 [b]	0.02
TE (NPVs)	78.65±2.95 [b]	65.24±7.65 [a]	0.02
PH (SdNPVs)	23.54±1.65 [b]	18.66±0.74 [a]	0.02
Day 7 after Ovsynch			
CLTA cm2	2.65±0.54 [a]	4.87±0.05 [b]	0.01
CLBFR bpm	74.32±2.36 [a]	81.32±7.55 [b]	0.01
TE (NPVs)	74.12±5.69 [b]	61.25±8.55 [a]	0.02
PH (SdNPVs)	20.12±0.74 [b]	16.32±0.25 [a]	0.02
Day 9 after Ovsynch			
CLTA cm2	3.11±0.36 [a]	5.12±0.01 [b]	0.01
CLBFR bpm	78.33±2.77 [a]	88.32±0.32 [b]	0.01
TE (NPVs)	69.25±5.74 [b]	55.24±8.65 [a]	0.02
PH (SdNPVs)	19.25±0.71 [b]	15.22±3.25 [a]	0.02
Day 11 after Ovsynch			
CLTA cm2	3.21±0.24 [a]	5.32±0.01 [b]	0.01
CLBFR bpm	79.32±4.32 [a]	89.66±5.21 [b]	0.01
TE (NPVs)	61.25±5.69 [b]	52.31±2.54 [a]	0.02
PH (SdNPVs)	17.58±3.21 [b]	14.62±0.32 [a]	0.02

[a,b]Superscripts values differ within the same day ($P < 0.05$).

in OvSynch-treated buffaloes on days 5,7,9, and 11 of the cycle in the cold season compared to the hot one (Table **3**). In cows, when OvS protocol is initiated with high P4 concentration, a significant difference favoring CP in terms of LBF and CL size in the comfort period (CP) compared to the summer period (HP; Table **4**). LBF of developing CL is higher when starting OvS with high P4 values in cows [36].

CONCLUSION

In the cold season, the ovarian hemodynamics was greatly improved compared to the hot one, which may influence the reproductive efficiency of buffaloes. Further studies were needed to prove it.

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