Ovarian response and conception rate following oestrus synchronization using three protocols in Egyptian buffalo heifers

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Keywords
Reproduction, CIDR, Ovsynch, PGF2α

Summary
Objective: The aim of this study was to monitor the ovarian response and conception rate following estrous synchronization using CIDR, Ovsynch and double prostaglandin F2α protocols in Egyptian buffalo heifers. Material and methods: A total of 80 cyclic buffalo heifers were divided into four equal groups: CIDR (intravaginal progesterone releasing device, EAZI-BREED® CIDR®), Ovsynch (GnRH, PGF2α, GnRH injections), PGF (double PGF2α doses) and control. Timed artificial insemination (TAI) was performed in all heifers. All animals were examined using ultrasound and blood samples were collected for measurement of progesterone. Results: A new follicular wave occurred earlier in the Ovsynch and PGF groups than in the CIDR group (p < 0.05). The mean diameter of the ovulatory follicle was smaller in the CIDR group than in the Ovsynch and PGF groups (p < 0.05). The ovulation rate was 100% in the CIDR group, 75% in the Ovsynch group and 70% in the PGF group. In the control group a lower pregnancy rate (20%) was determined in than in the CIDR (35%), Ovsynch (40%) and PGF (35%) groups. Progesterone concentrations were numerically higher in pregnant heifers of the CIDR group but the difference was not significantly compared to the Ovsynch, PGF and control groups (p > 0.05). Conclusion and clinical relevance: EAZI-BREED® CIDR®, Ovsynch-based TAI and PGF protocols were effective in synchronizing oestrus and resulted in nearly similar pregnancy rates in Egyptian buffalo heifers.

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Introduction
Buffaloes are an integral part of the agricultural economy and livestock production in Egypt. The total number of buffaloes in Egypt reaches about 3.950 millions, of which 42% are dairy cows, 6% buffalo bulls, 32% heifers less than 2 years old and 20% male calves less than 2 years old. The aggregate share of buffalo milk from all types of production systems is about 81% of the total milk production in Egypt (10). In buffalo herds, late attainment of puberty, lower intensity of oestrus expression, seasonality of calving and...
long subsequent postpartum anoestrus periods are all factors that can affect reproductive efficiency much more than in cows (5, 20, 41). Thus, difficulty in oestrus detection is one of the main causes for low reproductive performance in herds where reproductive biotechnologies such as artificial insemination have been applied. In these cases, the low incidence of homosexual behaviour during oestrus and the wide variation in the duration of oestrus are aspects that limit the use of this technology.

Recently, oestrus synchronization and the synchronization of ovulation have been studied with the aim of making this a viable technique for buffalo herds. Progesterone and prostaglandin F$_2$α (PGF$_2$α) were administered to synchronize oestrus in swamp buffaloes (15), Mediterranean buffaloes (18, 32), Murrah buffaloes (33), Mediterranean x Murrah buffaloes (8), Egyptian buffaloes (2, 24, 26), and beef cattle (30). It was reported that a sequence of GnRH and PGF$_2$α could synchronize ovulation using fixed timed artificial insemination (TAI). The CIDR device is successful in synchronizing oestrus and resulted in higher pregnancy rates in cattle (16). The use of CIDR in reproductive management to increase fertility is gaining popularity because of the short treatment period (7 days) and the reduced incidence of persistent follicles (1). In Egypt, there are no data on the use of this protocol in swamp buffaloes (45). Despite the number of studies that have been published in the last few years on oestrus synchronization in buffaloes (12, 18, 19), not many studies have described follicular dynamics during these hormonal protocols; this knowledge is of great importance for TAI (7). Furthermore, limited work has been carried out on the application of oestrus synchronization in buffalo heifers (14, 32, 36).

This study evaluated the possibility of controlling oestrus and ovulation synchronization in cyclic buffalo heifers and compared three treatments (Eazi-Breed™ CIDR®, Ovsynch and PGF$_2$α regimens) used to facilitate artificial insemination (AI) and improve conception rates in Egyptian buffalo heifers in Upper Egypt.

Materials and methods

Animals and management

A total of 80 apparently healthy Egyptian female buffalo heifers (Bubalus bubalis) were included in this study. The mean age ± SEM was 28.7 ± 0.9 months and mean weight was 365 ± 2.5 kg; body condition scores were ≥ 3.5 (scale: 1 = thin, 5 = fat [9]). The animals were housed in groups in open yards with shelters that correspond to 50% of the total area in the commercial buffalo farm (Abnoob, Assiut province). Buffalo heifers were fed forage dry matter (Egyptian clover, alfalfa) and a concentrate mixture. Wheat straw was available ad libitum. These rations provided 16% crude protein (CP) and 67% total digestible nutrient (TDN). The experiment was conducted between January and April 2013 (57.29 ± 2.3% relative humidity and 18.45 ± 0.9 °C maximum atmospheric temperatures). Before starting the synchronization program, the reproductive tracts of all females were examined rectally and by ultrasound to record ovarian and uterine variables. All heifers were cyclic and none exhibited congenital anomalies or clinical endometritis.

Experimental design and treatments

The 80 heifers were allocated to one of four groups. Heifers in the first group (CIDR, n = 20, two replicates) received a controlled internal prostaglandin releasing device (Eazi-Breed™ CIDR®, Pfizer, USA) at random stages of the oestrous cycle (designated as day 0). Thereafter, heifers in this group received 25 mg PGF$_2$α intramuscularly (Dimoprost, Lutalyse®, Pfizer, Pharmacia and Upjohn Company, NY, USA) on day 6 of the CIDR insert. The insert was removed on day 7. The animals were artificially inseminated 48 and 72 hours after CIDR removal using thawed semen from the same origin.

Heifers in the second group (Ovsynch, n = 20, two replicates) were synchronized using a 10 µg GnRH treatment on day 0 (Buserelin, Receptal®, Intervet International B.V., Boxmeer, Holland). Seven days later, 25 mg PGF$_2$α (5 ml) were administered intramuscularly and 48 hours later (day 9) the animals received a second intramuscular dose of 10 µg GnRH. All animals were artificially inseminated 16–28 hours after the second GnRH treatment.

Heifers in the third group (PGF, n = 20, two replicates) were synchronized using double PGF$_2$α injections. All animals received 25 mg PGF$_2$α intramuscularly on day 0. Animals exhibiting oestrous symptoms were artificially inseminated. The other animals were injected with a second PGF$_2$α dose on day 11 and insemination took place 3 days after the second treatment.

Heifers in the fourth group (control, n = 20) were inseminated during natural oestrus; these animals served only as a reference for the pregnancy rates. Follicular dynamic and ovulation rate could not be recorded in this group.

Ultrasound examination

All heifers were examined rectally and by ultrasound (Pie Medical, 100 LC, Maastricht, the Netherlands) with a sonar device connected to a 6/8 MHz changeable transducer. Examinations were performed once daily from day 0 to day 8 in both CIDR and Ovsynch groups and from day 0 to 11 in the PGF group, then every 12 hours until ovulation was observed. The number, diameter and relative position of all follicles were recorded. Follicles were categorized as either small (3–5 mm), medium (5–8 mm) or large (≥ 9 mm). Corpora lutea diameters were also measured and sketched separately for each heifer. Ovulation was considered to have occurred when a large, growing antral follicle that had been observed for several days was no longer detectable (22). If the large antral follicle persisted for a long time without ovulation and with increasing wall thickness, it was considered luteinized. The corpus luteum diameter was measured at the widest part; the mean of two measurements was used if the corpus luteum was elongated. Regression of the corpus luteum was considered to have oc-
curred when its diameter decreased markedly and this was later confirmed by hormonal analysis (progesterone concentration < 1 ng/ml).

The following ovarian characteristics were identified and compared between groups: emergence of the new follicular wave; emergence is defined as the first day in which the dominant follicle is retrospectively identified and has a diameter of 3–5 mm (21); the interval between the beginning of treatment and the emergence of a new follicular wave; the diameter of the ovulatory follicles; ovulation, defined as occurring on the day when the large follicle (≥ 10 mm) was no longer observed. Pregnancy was diagnosed sonographically 40 days after AI. The pregnancy rate per TAI was defined as the percentage of cows that were confirmed pregnant at the single pregnancy diagnosis after one TAI (37).

**Blood sampling**

In animals of the treatment groups blood samples were collected from the jugular veins into non-heparinised tubes twice weekly for 2 weeks prior to the program start and on days 0, 2, 4, 7, 9, 10 and 11 of the program. The blood samples were then taken twice weekly from all groups until pregnancy diagnosis on day 40. The samples were transported on ice to the laboratory within 20–30 minutes, centrifuged at 3000 rounds/minute for 20 minutes and the harvested serum was stored at –20 °C until analyzed for progesterone (P4). The P4 concentration was determined using a direct ELISA technique. Kits were provided by Diagnostic System Laboratories Co. (Bio Check, Foster City, CA 94404, USA) and use a micro-well method. This kit has a sensitivity of 0.0625 ng/ml with inter- and intra-run precision coefficients of variation of 4.5% and 2.6%, respectively.

**Statistical analysis**

The data were presented as mean ± SEM. Statistical analysis was carried out using the statistical package for social sciences for Windows (43). Differences among groups were evaluated by one-way ANOVA for the replicated parameters. Changes in the progesterone concentration were tested with repeated measure analysis. Means were compared by the least significance difference at 5% level of probability.

**Results**

At the beginning of the experiment, 50 heifers had corpora lutea and 35 heifers had a follicle ≥ 10 mm (Table 1, only the data of the treatment groups are presented). All heifers in the CIDR group did not show ovulation during the insertion period but ovulation occurred after the CIDR removal. After CIDR insertion in the same group, 10 animals showed regression of the follicles (atresia), five heifers had a persistent follicle and the other five showed no reaction (neither ovulation nor atresia). In the Ovsynch group after the first GnRH injection, ovulation was recorded in seven heifers, follicular regression was noticed in eight heifers, while one had a persistent follicle and the other four heifers showed no reaction. In the PGF group after the first dose of PGF, ovulation was recorded in seven heifers, follicular atresia was reported in eight heifers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CIDR</td>
</tr>
<tr>
<td>Heifers with a CL</td>
<td>14/20 (70%)</td>
</tr>
<tr>
<td>Heifers with a follicle ≥ 10 mm</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>Heifers with ovulation of the DF after CIDR insertion, GnRH-1, PGF-1</td>
<td>–</td>
</tr>
<tr>
<td>Heifers with follicle atresia</td>
<td>10</td>
</tr>
<tr>
<td>Heifers with a persistent follicle</td>
<td>5</td>
</tr>
<tr>
<td>Heifers with no response (no ovulation, no atresia)</td>
<td>5</td>
</tr>
<tr>
<td>Diameter of the dominant follicle on day 0 (mm)</td>
<td>11.2 ± 0.7a</td>
</tr>
<tr>
<td>Diameter of the CL on day 0 (mm)</td>
<td>13.7 ± 0.3a</td>
</tr>
<tr>
<td>CL regression after CIDR insertion, GnRH-1, PGF-1</td>
<td>4/12 (33.3%)</td>
</tr>
</tbody>
</table>

Values in the same superscripts in the same row do not differ significantly.

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heifers, while one heifer had a persistent follicle and the other four heifers showed no reaction (Table 1). The diameters of both follicles and corpora lutea on day 0 (day of treatment begin) did not differ significantly among groups (p > 0.05; Table 1). Regression of the corpus luteum was recorded in four, seven and 11 heifers after CIDR insertion, first GnRH dose and first dose of PGF₂₀, respectively (Table 1).

The proportion of heifers with emergence of the follicular wave within 7 days after the beginning of treatment differed among the CIDR (60.0%), Ovsynch (90.0%) and PGF (80.0%) groups (Table 2). The interval from beginning of treatment to follicular wave emergence was longer in the CIDR group than in the two other groups (p < 0.05). There was no significant difference in the time of follicular wave emergence between the Ovsynch and PGF groups (p > 0.05, Table 2).

The diameter of the dominant follicle on day 7 was significantly smaller in the CIDR group than in the Ovsynch and PGF groups (p < 0.05). Mean CL diameter on day 7 was significantly different (p < 0.05) between the three protocols. The mean diameters of ovulatory follicles on days 9–11 were significantly smaller in the CIDR group than in the Ovsynch and PGF groups (p < 0.05). Ovulation was recorded in all heifers (100%) of the CIDR group, 75% of the Ovsynch group and 70% of the PGF group. The interval between CIDR removal and last treatment until ovulation was the shortest in the Ovsynch group (p < 0.05) but did not significantly differ between the CIDR and PGF groups (Table 2).

All heifers showed oestrus after removal of the CIDR; this rate was higher than in the Ovsynch and PGF groups. The proportion of cows with synchronized oestrus (by 48 hours after CIDR removal in the CIDR group and by 18 hours after the last dose of GnRH in the Ovsynch group) differed from the PGF group (Table 2).

Table 2
Effects of treatment protocols with CIDR, Ovsynch and PGF on follicular dynamics, ovulation rate and time of ovulation in buffalo heifers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CIDR</th>
<th>Ovsynch</th>
<th>PGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of heifers with the emergence of a new follicular wave after treatment</td>
<td>12/20 (60%)</td>
<td>18/20 (90%)</td>
<td>16/20 (80%)</td>
</tr>
<tr>
<td>Interval between beginning of the protocol and emergence of the new follicular wave (range in days)</td>
<td>4.9 ± 0.7ᵃ</td>
<td>2.5 ± 0.6ᵇ</td>
<td>2.9 ± 0.5ᵇ</td>
</tr>
<tr>
<td>Diameter of the dominant follicle on day 7 (mm)</td>
<td>9.1 ± 0.6ᵃ</td>
<td>11.3 ± 0.9ᵇ</td>
<td>11.0 ± 0.7ᵇ</td>
</tr>
<tr>
<td>Diameter of the CL on day 7 (mm)</td>
<td>14.9 ± 0.3ᵃ</td>
<td>11.1 ± 1.7ᵇ</td>
<td>12.4 ± 0.2ᶜ</td>
</tr>
<tr>
<td>CL regression on day 9 (n [%])</td>
<td>10/10 (100%)</td>
<td>8/12 (66.7%)</td>
<td>0/7 (0.0%)</td>
</tr>
<tr>
<td>Diameter of the ovulatory follicle (mm)</td>
<td>11.3 ± 0.3ᵃ</td>
<td>14.9 ± 0.1ᵇ</td>
<td>15.3 ± 0.4ᵇ</td>
</tr>
<tr>
<td>Follicular growth rate (mm/day)</td>
<td>0.73 ± 0.04ᵃ</td>
<td>1.2 ± 0.07ᵇ</td>
<td>1.43 ± 0.1ᵇ</td>
</tr>
<tr>
<td>Ovulation rate (n [%])</td>
<td>20/20 (100%)</td>
<td>15/20 (75%)</td>
<td>14/20 (70%)</td>
</tr>
<tr>
<td>Interval to ovulation after CIDR removal and last treatment (hours)</td>
<td>59.7 ± 12.9ᵃ</td>
<td>20.5 ± 5.4ᵇ</td>
<td>68.2 ± 6.4ᵃ</td>
</tr>
</tbody>
</table>

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Fig. 1 Time of oestrus occurrence in buffalo heifers in the treatment groups CIDR (EAZI-BREED™ CIDR⁰), Ovsynch (protocol using GnRH – PGF₂₀ – GnRH) and PGF (double prostaglandin F₂₀ protocol).
The pregnancy rate was lower in the control group (20%) than in the CIDR (35.0%), Ovsynch (40.0%) and PGF (35.0%) groups (▶Table 3).

The progesterone level in the Ovsynch group increased gradually up to the 6th day after the first GnRH treatment (p < 0.05). The average P4 concentration in the CIDR group decreased sharply after administration of PGF$_{2\alpha}$ on days 6 and 7 after the CIDR was removed (p < 0.05; ▶Fig. 2). Progesterone concentrations in the pregnant heifers in all groups did not differ up to the 3rd week after insemination. Between the 4th and 7th weeks post insemination the progesterone concentrations in pregnant heifers in the CIDR group were numerically higher but not significantly different (p > 0.05) from those in the Ovsynch, PGF and control groups (▶Fig. 3).

**Discussion**

The emergence of a new follicular wave was observed in all treatment groups; the highest percentage occurred in the Ovsynch group, followed by the PGF group, while the lowest percentage was observed in the CIDR group. A possible explanation for this result is that progesterone levels in the CIDR group were higher than in the other two groups; this in turn led to decreased numbers of heifers showing emergence of a follicular wave. The emergence of a new follicular wave appears to be critical for high pregnancy rates in a progestin-based TAI protocol. This event is necessary to prevent the development of persistent follicles and must take place early enough to allow for sufficient growth prior to the induction of ovulation (16, 27, 37, 39).

Interestingly, the mean diameter of the dominant follicles on day 7 (day of CIDR removal and PGF$_{2\alpha}$ injection in the Ovsynch group) was significantly smaller in the CIDR group than in the other groups. Differences are presumed to be the consequence of dominant follicles emerging from a new follicular wave in the CIDR group, where progesterone levels were high. Consequently, the proportion of heifers with pre-ovulatory follicles > 13 mm on day 9, a condition which may reduce pregnancy rates following TAI, was greater in the Ovsynch and PGF groups than in the CIDR group. Similar results have been reported in cattle (27, 28).

In this study, subsequent follicular development was synchronous among treatment groups of buffalo heifers. In the CIDR group, all heifers experienced ovulations due to very low levels of proges-

![Fig. 2](image_url)

**Fig. 2** Progesterone concentration (mean ± SEM) from beginning of treatment (day 0) to day 11 in serum of buffalo heifers in the treatment groups CIDR (EAZI-BREED™ CIDR®), Ovsynch (protocol using GnRH – PGF$_{2\alpha}$ – GnRH) and PGF (double prostaglandin F$_{2\alpha}$ protocol).

**Table 3**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy rate</td>
<td>7/20 (35%)</td>
<td>4/20 (20%)</td>
</tr>
<tr>
<td>CIDR</td>
<td>8/20 (40%)</td>
<td></td>
</tr>
<tr>
<td>Ovsynch</td>
<td>7/20 (35%)</td>
<td></td>
</tr>
<tr>
<td>PGF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Treatment groups: CIDR = controlled internal drug release (EAZI-BREED™ CIDR®), Ovsynch = protocol using GnRH – PGF$_{2\alpha}$ – GnRH, PGF = double prostaglandin F$_{2\alpha}$ protocol.
terone, especially following PGF$_{2a}$ treatment; hence, all dominant follicles had undergone ovulation within 72 hours. Oestrus with subsequent ovulation (indicated by the presence of a CL) in heifers following CIDR removal suggests that progesterone (P4) released from the intravaginal CIDR in these animals was absorbed through the vaginal wall and entered circulation (3, 4, 45). This increased P4 concentration exerted negative feedback on the hypothalamus and anterior pituitary, which in turn promoted storage of GnRH, FSH and LH. Once P4 therapy had stopped (after CIDR removal by day 7), PGF$_{2a}$ treatment led to a rapid decline in P4 concentrations. This decrease promoted the release of GnRH, as the negative feedback of P4 is followed by the release of FSH and LH, with subsequent controlled ovulation and good synchronisation (3, 4, 46).

After the second dose of GnRH in the Ovsynch group, ovulation was precisely synchronized and was observed within 18 hours in 16 of 20 buffalo heifers. The precision of synchrony of oestrus depends on the synchrony of follicle development (35). The diameters of the largest follicle in heifers that ovulated were larger than those in animals that showed no ovulation. In the present study, ovulation time in heifers was earlier than that reported in the previous studies. In crossbred (Murrah X Mediterranean) buffaloes, ovulation occurred an average of 26.5 and 24.4 hours after the second GnRH or LH treatments, respectively (8, 33). In cattle, ovulation occurred 24–32 hours after the second GnRH treatment (38). This difference in response to the same treatment in different studies may be related to parity and species.

Pregnancy rates in the present study were higher in the CIDR and Ovsynch groups than in the control group. In crossbred buffaloes (Murrah X Mediterranean [8]) and Brazilian buffaloes (6), conception rates after TAI were reported as 55.6–64.2%; these rates are higher than we documented in the present study. In contrast, a pregnancy rate of only 15% has been reported in swamp buffalo heifers and of only 42.9% in buffalo cows (14). Possible reasons for this marked difference may include differences in parity, breed, management or environmental conditions. The low pregnancy rates we documented may result from the fact that with the Ovsynch TAI protocol, the second injection of GnRH may induce ovulation of the dominant follicle before development is complete and therefore the quality of the follicle, oocyte and/or the subsequent corpus luteum may be compromised and plasma concentrations of progesterone are too low to maintain pregnancy. Reduced secretion of progesterone to below a critical threshold is one cause for embryonic loss in cattle (25, 29, 40) and a similar explanation has been proposed for buffaloes (11–13).

To control the timing of oestrus with exogenous hormones, it is necessary to ensure that either induced regression of the CL or termination of a progesterone treatment coincides with the selection of the dominant follicle during the wave; this method ensures precise onset of oestrus and high fertility (25, 34). In the present study, all heifers were inseminated approximately 72 hours after the second PGF$_{2a}$ dose in the PGF group, an interval that should be appropriate (oestrus at 60 + 12 hours, according to the a.m./p.m. rule). The resulting pregnancy rate was only 35% but when other significant variables and interactions are considered, pregnancy rates after TAI improved. 100% of the heifers in the CIDR group exhibited oestrus but only 45% of the treated heifers became pregnant; the reason remains unclear. Early embryonic death is a possible factor but further study is needed. Similar results have been reported in cattle (25) and buffaloes (11).

Progesterone levels in the control group were not comparable because there was wide variation in the timing of ovulation (asynchronous); in the PGF and Ovsynch groups, progesterone concentrations decreased to basal levels in response to the PGF$_{2a}$ dose and then increased linearly. This pattern appears to be associated with ovulation of the dominant follicle present at the time of GnRH injection and subsequent CL formation (2, 25–27). In contrast, progesterone levels were high in the CIDR up to day 7 after CIDR insertion and then decreased markedly after the PGF$_{2a}$ dose (Fig. 2). Progesterone concentrations during the late luteal phase but before insemination have been shown to positively influence conception rates (44). In this study, progesterone concentrations during the period when the CIDR was in place may have influenced pregnancy rates but there is no direct evidence to support this statement. Progesterone levels after conception were high and similar in all groups up to the 3rd week of pregnancy. After the
3rd week of pregnancy, the level of progesterone in the pregnant heifers in the CIDR group was higher than in the other groups; this may be because some heifers ovulated, even if they were already pregnant. It was reported that the CL diameter and maternal serum P4 concentration of pregnant ewes were higher than in non-pregnant ewes, and ewes bearing a single fetus had a lower P4 concentration than that found in ewes bearing twin fetuses (23).

Conflict of interest
The authors declare that they have no conflict of interest.

Acknowledgements
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43. SPSS for Windows. Personal package. 2007 version 16.0.