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Reproductive management in buffalo by artificial insemination

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ABSTRACT

Artificial insemination (AI) is important for genetic improvement and to control the period of breeding in buffalo and has increased significantly over the past 20 years. AI is more difficult in buffalo compared with cattle due to variable estrous cycles, reduced estrous behavior, and reproductive seasonality. The latter is associated with a higher incidence of anestrus and increased embryonic mortality during the nonbreeding season. Protocols to control the stage of the estrous cycle have undergone recent development in buffalo. These protocols are based on the control of both the luteal phase of the cycle, mainly by prostaglandins and progesterone, and follicle development and ovulation, by prostaglandins, progesterone, GnRH, hCG, eCG and estradiol. Protocols that synchronize the time of ovulation enable fixed timed AI, avoiding estrous detection. Factors to consider when selecting an AI protocol include animal category (heifers, primiparous or pluriparous), reproductive status (cyclic or anestrus), and season. This review looks at the current status of estrus synchronization and AI in buffalo and provides some practical suggestions for application of AI in the field.

1. Introduction

The world buffalo population has increased significantly over the last 30 years and in 2017 exceeded 200 million animals [1]. More than 97% of buffalo are in developing countries. Their adaptability to tropical environments and disease resistance makes buffalo very important to many rural economies [2]. In developed economies such as Italy, buffalo have been bred for high-value milk production. Only 0.2% of world buffalo are in Europe and about 93% of these are in Italy. The Italian Mediterranean river buffalo is used to produce Mozzarella di Bufala Campana which has protected designation of origin status (DOP) [3]. The economic and social importance of buffalo in Italy is reflected by the increase in numbers from 182,000 in 2000 to 400,000 in 2017 [1].

Natural mating remains the predominant form of breeding in buffalo in the world, although the use AI has steadily increased [2,4]. The use of AI is made possible by increased understanding of the reproductive physiology of buffalo [5–8] and improvements in semen cryopreservation [9–12]. A number of estrus synchronization protocols have been developed in buffalo and applied with some success [4]. The aim of the present review is to illustrate how estrus synchronization protocols in buffalo have taken into consideration the particular features of ovarian function and seasonal breeding in buffalo.

2. Reproductive characteristics

2.1. Seasonality

The buffalo is a short-day breeder and shows increased reproductive activity with decreasing day length [5,13]. This reflects the origins of buffalo domestication in the Indo Valley where births were synchronized with optimal climatic conditions and food availability [14]. In the equatorial belt, where day length remains similar throughout the year, the reproductive season is determined mainly by the availability and quality of feed [15]. Buffaloes become increasingly seasonally polyestrus with distance from the equator [5,13]. Females that calve out of the breeding season have an extended postpartum anestrous period with a proportion not resuming ovulation until the following breeding season [8]. The period of decreasing day length in Italy is from July to December. Buffalo have a 10-month gestation and, if they are bred in the period of decreasing day length, milk production for mozzarella would occur mainly during autumn-winter (November to March),

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when demand for mozzarella cheese is very low [13]. Thus, in order to link milk production with peak demand for mozzarella (March to August), buffalo are bred using estrus synchronization and AI from February/March to September [13,16].

The number of oocytes recovered from slaughterhouse ovaries was not influenced by season in buffalo but there was an increase in IVF cleavage (71.7 vs 58.0%) and blastocyst rate (26.5 vs 18.8%) with oocytes obtained in autumn compared to spring [17]. Similar IVF outcomes were observed with ovum pick-up (OPU) [18,19]. These findings suggested that IVF should be avoided during increasing day length to optimize benefits/costs. An influence of season on oviductal fluid production was demonstrated in buffalo and may impact oocyte quality and IVF outcome [20]. Season additionally has a strong influence on corpus luteum (CL) function [21–23] and this impacts both embryonic mortality [24,25] and anestrous [5]. Several studies have assessed CL activity in buffalo by using the eco-color Doppler technique [21–23,26,27]. The main advantage of this approach is that blood flow can be objectively evaluated by specific parameters such as time average medium velocity (TAMV), resistive index (RI), and pulsatility index (PI) [26]. A strong relationship was found between blood flow in the CL (TAMV in particular) and blood progesterone (P_4) levels [23]. This makes eco-color Doppler a valuable diagnostic tool for reproductive management in buffalo (see below). Season can also affect male reproductive activity [28] and AI with semen collected during the breeding season should replace natural mating to increase herd fertility during the non-breeding season.

2.2. Estrous behavior

The difficulty in detecting estrus in buffalo limits the use of AI in natural cycles. Signs of estrus such as vulvar edema, frequent urination, vaginal discharge and vaginal congestion are less intense in buffaloes compared with cattle, regardless of season [5]. Behavioral signs of estrus including restlessness, bellowing, tail raising and homosexual mounting behavior are present only in a small proportion of buffalo and are typically exhibited during the night [29]. Only 3.4% of female buffalo show homosexual behavior and more than 60% have a silent estrus [30]. The reduced intensity of estrus in buffalo may be partially related to low circulating concentrations of 17β-estradiol in comparison with dairy cattle (from approximately 6.7 to 13,0 pg/ml compared to 10.0–14.0 pg/ml in buffalo and cattle, respectively) [6,31,32]. The dimensions (about 13–14 mm compared to 15–16 mm, in buffalo and cattle, respectively) and the volume (about 11–12 ml compared to 16–17 ml, in buffalo and cattle, respectively) of the pre-ovulatory follicle in buffaloes is smaller than in dairy cattle and may explain lower estradiol in buffalo [32,33]. Differences in estradiol metabolism and clearance from circulation may occur in buffaloes [5].

2.3. Other reproductive characteristics

The average length of the estrous cycle in buffalo is 23.7 days, although it can vary from 16 to 28 days [33,35]. Buffalo typically have two follicular waves (60.7%) and about 36% have three follicular waves [34]. Estrous duration in cyclic buffaloes is influenced by season and lasts 10–20 h in the breeding season and 2–72 h in the non-breeding season [5]. The interval between the onset of estrus and the LH surge can vary from 1 to 12 h and ovulation occurs between 26 and 33 h after the LH surge [5,35]. These parameters can be influenced by the biostimulating effects of a bull, such as reducing the incidence of anestrus [36].

3. Application of AI

A number of strategies have been evaluated to deal with the particular reproductive biology of female buffalo. These include the use of teaser bulls [36], radiotelemetry [35], pedometers [37], and the development of several estrus synchronization protocols [2,4,38,39]. Radiotelemetry (Heatwatch®; DDx Inc., Boulder, Colorado, USA) was successfully used to detect estrous signs in prostaglandin-induced estrus in buffalo, with 100% accuracy [35]. Pedometers are routinely used in dairy cows to record higher motor activity of females in estrus [40]. In buffalo cows, pedometers detected estrous status in 80% of animals, with an accuracy of 75% [37]. If a vasectomized bull is present in the herd, pedometers record around 90% of females in estrus [37].

The most practical approach to utilizing AI is after estrus synchronization. Synchronized heats are more regular and a lower incidence of double ovulations is recorded compared to natural estrus [5]. A number of protocols have been developed in buffalo to manipulate the estrous cycle and, in some cases, to control the timing of ovulation [39]. These are based on the utilization of hormones that can act at different points in the hypothalamic – pituitary – ovarian axis. The protocols can be classified into two categories:

- ✓ treatments that control the luteal phase of the cycle, where prostaglandins and progesterone (P_4) analogues are primarily used;
- ✓ treatments that control follicle development and ovulation, by using prostaglandins, P_4, gonadotrophin releasing hormone (GnRH), human chorionic gonadotrophin (hCG), equine chorionic gonadotrophin (eCG) and estradiol (E_2).

3.1. Prostaglandins

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its analogues are used for their luteolytic action on the CL, which causes a rapid decline in blood P_4 within 24 h. $PGF_{2\alpha}$-induced luteolysis occurs from day 5 to day 18 of a normal estrous cycle in both cattle [41] and buffalo [42]. $PGF_{2\alpha}$ increases the intra-luteal production of vasoactive substances such as endothelin-1 and angiotensin II, both of which have important roles in the luteolytic cascade, decreasing luteal blood flow [43]. After the assessment of CL presence, a single $PGF_{2\alpha}$ injection will induce estrus after 60 h on average (***One shot protocol***). Synchronization with $PGF_{2\alpha}$, without assessment of CL presence, involves two injections, 11–14 days apart (***Two shots protocol***). This maximizes the number of females in estrus after the second $PGF_{2\alpha}$ injection [44]. Natural (lutalyse) and synthetic prostaglandins (cloprostenol, luprostiol, dinoprost, etc.) have essentially the same efficacy in buffalo [21,45].

Several factors may affect the response to $PGF_{2\alpha}$. The beginning of estrus/ovulation occurs earlier (about 15 h) in buffaloes that receive $PGF_{2\alpha}$ in the early luteal phase (6–9 days after estrus) compared to those in the late (11–14 days) luteal phase [35]. A large variability in estrous behavior, estrous duration, and ovulation, has also been recorded according to the follicular population when the treatment is carried out [30]; i.e. the absence of a dominant follicle can prolong the onset of estrus for 5 days, whereas it is reduced to 2–3 days in the presence of a dominant follicle [38].

The efficiency of the two $PGF_{2\alpha}$ protocol is high if animals are cyclic. About 85–90% of buffaloes are in estrus after treatment and a 50% pregnancy rate is recorded during the breeding season [45]. A pregnancy rate higher than 25–30% is unusual in the non-breeding season [46]. Hussein et al. [47] reported similar efficiency in buffalo heifers treated by double $PGF_{2\alpha}$, Ovsynch-TAI or P4 treatments,

while a slight increase was recorded in other studies compared to the Ovsynch-TAI protocol (see below), in particular if live weight of the animals is considered [44]. Double $PGF_{2\alpha}$ administration has also been used to carry out a preliminary screening (Pre-synch Protocol) to identify females that can be subsequently synchronized. Only cyclic animals with a CL will respond to $PGF_{2\alpha}$ administration and are selected for further synchronization. Although this approach should theoretically result in a higher pregnancy rate, no significant differences were recorded in one study with pluriparous buffaloes (45% and 35% pregnancy with and without pre-synchronization, respectively) [48].

3.2. Progesterone and progestagens

The utilization of progesterone or progestagens can be carried out by different routes of administration such as subcutaneous usually in the ear, oral, injectable or intravaginal. Only the last method is registered in Italy, whereas i.e. oral administration of melengestrol acetate is registered i.e. in North and South America and Asia [4,39,49–51]. Treatments using progesterone and progestagens are particularly utilized in buffaloes in the non-breeding season, as P_4 can act at the hypothalamus to initiate ovarian cyclicity in anestrous animals [49,52,53]. Treatment with exogenous P_4 in anestrous cattle, in which P_4 concentrations were at subluteal levels, augmented LH pulse-frequency and E_2 synthesis, together with the number of follicular LH receptors [52,54]. Therefore, the sensitivity of the hypothalamus to the negative feedback effects of E_2 is reduced, follicular growth is stimulated, and the largest ovarian follicle matures and responds to exogenous E_2 or a gonadotrophin [52]. The latter has led to the incorporation of E_2 (benzoate or valerate) and/or eCG in P_4-based protocols [49]. However, E_2 has not been registered in Europe since 2006 and it has been replaced by a GnRH injection before P_4 implant insertion [55]. High P_4 levels decrease the frequency of GnRH pulses in the hypothalamic–hypophyseal portal circulation, leading to a reduced synthesis of GnRH receptors and, consequently, a lower responsiveness of the pituitary to GnRH [56]. It is likely that exogenous P_4 increases LH storage in the pituitary gland and augments GnRH-induced LH release [56]. After the regression, or ovulation, of the dominant follicle, its inhibitory effect on the follicular population is removed allowing the development of a new follicular wave and the growth of a new dominant follicle, which will acquire ovulatory capability after P_4 withdrawal [4].

In some countries, ear implants containing norgestomet (17a-acetoxi-11b-metil-19-norpreg-4-en-3,30-diona) a progesterone analogue, were previously used [51], since it is no longer available. Since norgestomet is more potent than natural P_4, lower doses are required [4]. Oral administration of melengoestrol acetate has produced variable responses, most likely because of the difficulty in ensuring correct consumption [39]. In Italy, P_4 treatment is only performed by an intravaginal device (containing 1.4–1.6 g of natural P_4) for 9–10 days, and blood levels of 4–5 ng/ml are achieved [38,49]. Treatment with P_4 is associated with PGF injection to remove any CL and eCG injection. The latter improves follicular growth and P_4 production in the subsequent cycle, increasing the pregnancy rate after treatment [57]. Both drugs can be administered on day 7 after P_4 device insertion [39] or when the P_4 device is withdrawn [49]. A pregnancy rate between 30 and 50% has been recorded in adult buffaloes regardless of the day of prostaglandin and eCG administration [39,49]. Timed AI can be performed by administering GnRH 48 h after P_4 device withdrawal [58]. GnRH can be replaced with E_2 benzoate, 24 or 36 h after P_4 withdrawal, and results in a similar pregnancy rate (43–49%) in pluriparous buffalo cows and heifers [59].

The influence of P_4 blood concentrations on the follicular response and pregnancy rate is still unclear. Buffaloes with a CL were reported to show reduced development of the dominant follicle during synchronization with a E_2/P_4 protocol [60]. In another study, there was no difference in follicular growth when buffaloes were treated with either a new or previously used P_4 device which released different amounts of P_4 [61]. Circulating concentrations of P_4 result from the balance between endogenous P_4 production (or exogenous administration) and P_4 catabolism by the liver. The latter is highly correlated with dry matter intake [62]. Thus, it is likely that the physiological status, dry matter intake, BCS, and other factors. may explain the differences among studies. Further studies are needed to assess the influence of high endogenous or exogenous P_4 on the response to synchronization treatment in buffalo.

3.3. Control of follicular development and ovulation

Due to the difficulty of estrous detection in buffalo [6,35], the most commonly used protocols for estrus synchronization are based on the control of follicular development and ovulation. This enables fixed-timed AI, avoids the need for estrous observation, and is more efficient reproductive management of the herd. GnRH or GnRH agonists such as gonadorelin, buserelin or lecirelin are commonly used to control follicular development and ovulation. GnRH and its agonists induce LH, FSH and estradiol surges at any stage of the estrous cycle, promoting the ovulation of a dominant follicle or the luteinization and/or atresia of pre-dominant follicles. Plasma LH levels are elevated 6- to 8-fold 30 min and 3 h after GnRH injection [63]. The magnitude of the LH response is affected by GnRH dose and dominant follicle diameter. In one study, 100 μg of natural sequence GnRH (gonadorelin) induced ovulation in 100% of treated buffaloes compared to 33% with 50 μg GnRH [64]. The follicular diameter required for ovulation in response to GnRH appears variable [63,64]. In one study, follicles of 6.7 ± 2.4 mm diameter did not ovulate [53]. In other studies, follicles ranging from 4.0 to 12.0 diameter ovulated [63,64]. Studies which look at follicular LH and FSH receptors may shed some light on the optimal follicular size required for a response to GnRH in buffalo. A new follicular wave emerges two or three days after treatment with GnRH [65]. The type of GnRH agonist did not affect the ovulatory response or pregnancy rate, and similar results were obtained in buffalo with buserelin acetate and lecirelin [53]. In summary, GnRH induces ovulation in 60–70% of buffaloes [49,53,66,67] and the interval between GnRH administration and ovulation is 33 ± 8 h [38].

3.3.1. Ovsynch-TAI protocol

The most utilized estrus synchronization protocol in buffalo is Ovsynch-TAI [65]. This involves GnRH agonist injection (10 μg) on Day 0, PGF on Day 7, and GnRH agonist (10 μg) on day 9. AI is performed on day 10, at 60 and 16–20 h after PGF and second GnRH, respectively. Ovulation or the luteinization of the dominant follicle following the first GnRH results in the presence of luteal tissue which gives elevated P_4. Around 60% of buffaloes ovulate in response to the first GnRH and this is not influenced by P_4 levels at the time of treatment [63,67]. Increasing the dose of GnRH to 20 μg gave a 10% higher ovulation rate in buffaloes during the postpartum period [53]. Because of the relatively high ovulation rate after the first GnRH, only a few animals show estrous behavior between day 0 and day 7, whereas more than 80% of buffaloes are considered in estrus 48–72 h after PGF [67]. Buffaloes that ovulate after the first GnRH have greater P_4 on day 7 compared with buffalo that do not ovulate (2.56 ± 1.02 vs. 1.26 ± 0.82 ng/ml), but all buffaloes have P_4 below 1 ng/ml 48 h after PGF [67]. The second GnRH 48 h after PGF is intended to synchronize ovulation. The response to the first

GnRH also influences the ovulation rate. Buffaloes that do not ovulate after the first GnRH have a shorter interval from the second GnRH to ovulation (around 22 h vs 34 h) [53]. Replacement of the second GnRH with LH did not improve synchronization of estrus and pregnancy rate [66].

Between 78 and 90% of buffaloes show synchronization of ovulation in the Ovsynch-TAI protocol and the pregnancy rate ranges between 33 and 60% [49,53,68]. The pregnancy rate is dramatically decreased if Ovsynch-TAI is used in buffalo during transition to the non-breeding season because of high embryonic mortality (20–40%) [24,25] and during the non-breeding season because of anestrus [5,53]. A higher pregnancy rate is usually achieved in pluriparous buffalo compared with primiparous buffaloes [53]. Ovsynch-TAI is not recommended in heifers which show a low ovulatory response to the first GnRH and the development of the new follicular wave is not synchronized [53].

3.3.2. *Ovsynch-TAI derived protocols*

Several modifications have been applied to the Ovsynch-TAI protocol (Fig. 1). Progesterone supplementation from Day 0 (first GnRH) to Day 7 gave a 25% higher pregnancy rate in non-cyclic buffaloes but did not influence the response in cyclic buffaloes [38]. A decision on whether to include P_4 can therefore be made based on the cyclicity status. As noted above, synchronization and pregnancy rate are increased by the presence of a dominant follicle at the first GnRH [67]. Follicular status can be determined by ultrasound examination at the start of treatment [38] or animals can be pre-synchronized. A practical approach is the "G6G protocol", which consists of PGF and GnRH, respectively, 8 and 6 days before the start of the Ovsynch-TAI protocol [69]. This gives a greater ovulation rate after the first GnRH (84 vs 56% in G6G and Ovsynch, respectively), higher levels of circulating P_4 on day 7 (2.5 ± 0.4 vs. 1.3 ± 0.3 ng/ml in G6G and Ovsynch, respectively), and a larger dominant follicle diameter on the day of AI (11.7 ± 0.2 vs 10.9 ± 0.2 mm in G6G and Ovsynch, respectively) [69]. Pregnancy rate tended to be higher in the G6G protocol (56 vs 32% in G6G and Ovsynch, respectively) suggesting further studies are warranted.

An enhancement of the Ovsynch-TAI protocol is the Ovsynch-plus protocol [70]. The latter involves the administration of 500 IU eCG 3 days before the first GnRH [70]. When applied in anovulatory buffaloes, this treatment gave a higher ovulation rate after the first GnRH (61.5 vs. 96.6% in Ovsynch and Ovsynch-plus, respectively) and higher pregnancy rate (23.1 vs. 34.5% in Ovsynch and Ovsynch-plus, respectively) compared to the Ovsynch-TAI protocol [70].

The administration of cloprostenol ($PGF_{2\alpha}$ analogue) on the day of AI in the Ovsynch-TAI protocol increased pregnancy rate in buffalo from 31 to 48% [71]. In the latter, $PGF_{2\alpha}$ was associated with increased P_4 levels on Day 10 after TAI, which was presumed to favor embryo development and attachment. Replacing the second GnRH in the Ovsynch-TAI protocol with 1 mg 17β-estradiol 24 h after PGF injection (Heatsynch protocol), with AI at 48 and 72 h after E_2 injection, did not increase the pregnancy rate in anestrous buffaloes [72].

4. Reproductive management by AI

Male buffalo have reduced fertility during the nonbreeding season at higher latitudes and this can be managed with the use of AI [73]. Even so, around 40–50% of females that undergo estrus synchronization and AI conceive [67]. The proportion of females that conceive can be increased if the females that do not conceive are re-synchronized and again AI'd (Fig. 2) [50,74,75]. If this is repeated, approximately 95% of females can be pregnant after 93 days and three AI's (Fig. 2). Re-synchronization strategies have been successfully applied in pluriparous buffalo [74] and buffalo heifers [50]. The monitoring of females for ovarian status and pregnancy is incorporated into the cycles of re-synchronization and AI (Fig. 2). Observing for the presence of a functional CL by eco-color Doppler is an important component of re-synchronization strategies [74]. Also, the protocols chosen for synchronization and re-synchronization can be different based on the category of female and breeding or non-breeding season [50].

Re-synchronization is a powerful strategy to optimize the number of breeding females that conceive during a defined breeding period. It can be successfully used during the breeding and nonbreeding seasons [50,76] which is particularly important in Italy as it allows peak milk production to be linked to the annual

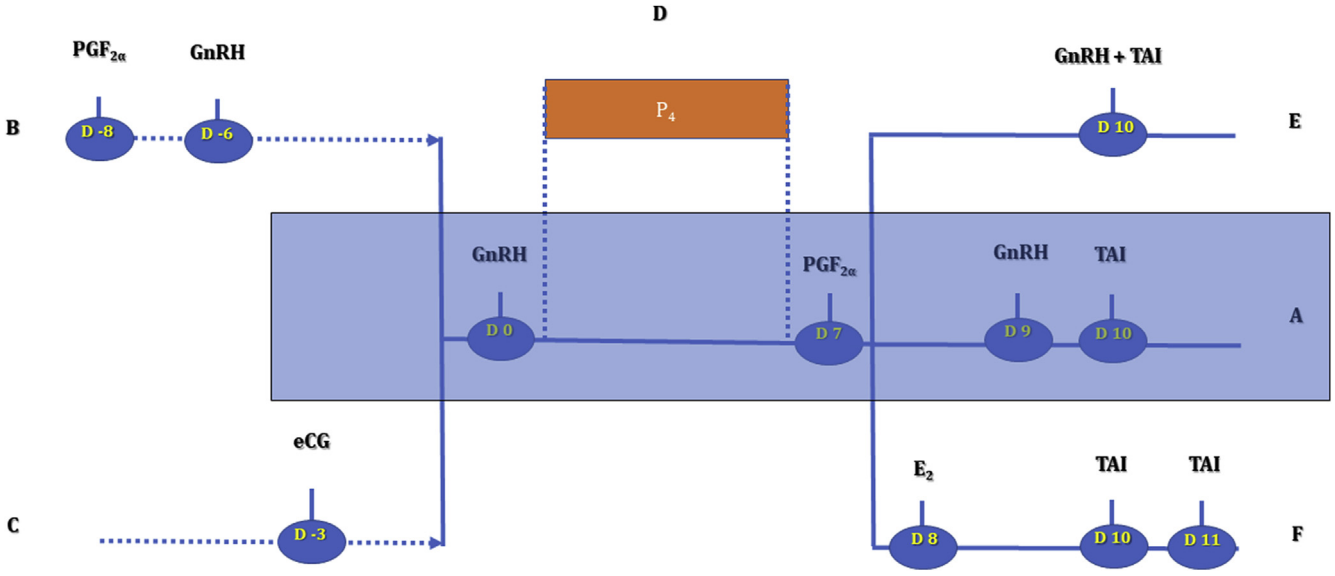

Fig. 1. Modified Ovsynch. The standard (A) Ovsynch-TAI Protocol (GnRH on Day 0, $PGF_{2\alpha}$ on day 7 and GnRH on day 9, with timed AI on day 10) can be modified with some pre-treatments, such as the G6G protocol (B) or the Ovsynch-Plus (C), by administering progesterone during the treatment (D), or by some post-treatments, such as the Co-synch protocol (E) or replacing the last GnRH with estradiol benzoate (F).

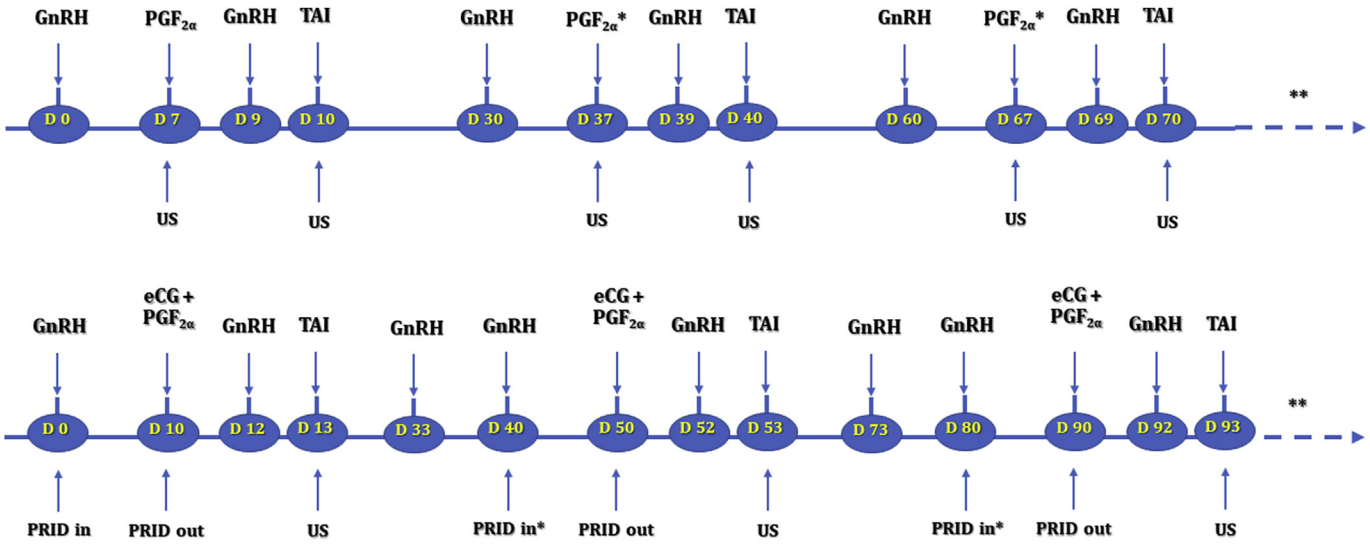

Fig. 2. Re-synch Protocols. Resynchronization protocols based on the utilization of a standard Ovsynch-TAI Protocol (A) or a treatment by progesterone (B).
TAI = Timed Artificial Insemination.
US = Ultrasound examination.
PRID in = Insertion of progesterone releasing intravaginal device.
PRID out = Removal of progesterone releasing intravaginal device.
* = Only for not pregnant animals and with functional corpus luteum.
** = The same schedule can be repeated.

peak demand for mozzarella [13]. The premium price for mozzarella justifies the investment in re-synchronization and AI. The latter illustrates the importance of undertaking a benefit/cost analysis when investing in assisted reproductive technology.

5. Sexed semen

The use of sexed semen adds an additional dimension to estrus synchronization and AI. Sexed semen can be used to generate a higher proportion of dairy females which can undergo more intensive selection for economically important milk production traits [77]. Sexed semen can also be used to preferentially generate either males or females in systems that are geared to meat production [78]. The potential of conventional and sexed semen to improve buffalo globally is noted below. This includes greater dispersal of germplasm from genetically improved buffalo in developed countries to developing countries.

In buffalo, AI with sexed semen has produced pregnancy rates comparable to conventional semen [79–83]. The first use of sexed semen in buffalo was performed in Italy in 2005 [79]. A special device (Ghent device) was tested for semen deposition near the utero-tubal junction in order to deliver a significantly reduced number of sperm, compared with the number of conventional frozen-thawed sperm deposited in the body of the uterus. With this approach a dose of 4 x 10^6 sexed sperm gave a pregnancy rate similar to 20 x 10^6 conventional sperm [79]. Sexed semen and IVF were combined to produce the first birth of buffalo calves using complementary assisted reproductive technologies [84].

The first large study on the use of sexed semen in Mediterranean buffalo heifers produced similar pregnancy rates as conventional semen [80]. A subsequent study with sexed semen in pluriparous buffaloes achieved a pregnancy rate of around 45% [81]. It was also shown that similar pregnancy rates could be achieved with the deposition of sexed semen in the body of the uterus or deep in the uterine horns. Deposition in the body of the uterus makes the use of sexed semen more practical and will facilitate adoption for genetic improvement. More recently, sexed semen of River buffalo gave good pregnancy rates in Swanp buffalo and F1 River x Swamp buffalo inseminated at spontaneous estrus [82,83]. This demonstrated the potential for accelerating genetic improvement in Swamp buffalo with sexed semen of highly selected River buffalo.

6. Conclusions

A deeper understanding of ovarian physiology and improvements in semen cryopreservation has led to the development of tailored estrus synchronization and AI protocols in buffalo. These protocols now achieve comparable levels of efficiency as protocols in beef and dairy cattle. The opportunity has been created for greater dispersal of germplasm using buffalo that have undergone genetic improvement in Europe, the Americas, and Asia. An integrated global approach to genetic improvement in buffalo will help meet the growing demand for buffalo milk and meat products.

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