CARIBBEAN FOOD CROPS SOCIETY

49

Forty-ninth Annual Meeting 2013

Port of Spain, Trinidad and Tobago
Vol. XLIX

PROCEEDINGS

OF THE

49TH ANNUAL MEETING

Caribbean Food Crops Society
49TH Annual Meeting
June 30 – July 6, 2013

Hyatt Regency Hotel
Port of Spain, Trinidad and Tobago

"Agribusiness Essential for Food Security: Empowering Youth and Enhancing Quality Products"

Edited
by
Wanda I. Lugo, Héctor L. Santiago, Rohanie Maharaj, and Wilfredo Colón

Published by the Caribbean Food Crops Society

ISSN 95-07-0410

Copies of this publication may be obtained from:

Secretariat CFCS
P.O. Box 40108
San Juan, Puerto Rico, 00940

or from:

CFCS Treasurer
Agricultural Experiment Station
Jardín Botánico Sur
1193 Calle Guayacán
San Juan, Puerto Rico 00936-1118

Proceedings of the Caribbean Food Crops Society. 49:322-331. 2013

EVALUATION OF TIMED ARTIFICIAL INSEMINATION PROTOCOLS IN WATER BUFFALOES IN THE TROPICS

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ABSTRACT: Three experiments were conducted to evaluate the effect of different timed artificial insemination (TAI) protocols on pregnancy rate (PR) in water buffaloes (*Bubalus bubalis*). In experiment 1 the efficacy of a modified ovsynch/GnRH and progesterone-based TAI protocols was evaluated. Buffalo cows and heifers (N = 38) assigned to Treatment 1 (n=19; ovsynch/GnRH), received an injection of progesterone (200 mg, i.m., day-3), three (3) days later (day 0) these animals received an injection of GnRH (Cystorelin, 100 µg, i.m.). Buffaloes assigned to Treatment 2 (n=19; progesterone/CIDR-based), received a CIDR device (1.8 g of progesterone, Pfizer, Mexico) on Day 0. On Day 7, the CIDR device was removed and all buffaloes (N=38) were injected with $PGF_{2\alpha}$ (Lutalyse, 25 mg, i.m, 5 ml/animal i.m; Pfizer Corporation, New York, NY). Thirty (30) hours after injection with $PGF_{2\alpha}$ all animals were injected with estrogen (estradiol benzoate, 500 ug, i.m.), and 30 h later (60 h post $PGF_{2\alpha}$) all animals were inseminated. Mean pregnancy rate was numerically higher in progesterone based (21%) than in GnRH-based (10.5%), though differences did not attain statistical significance. Experiment 2 evaluated the effectiveness of different ovulation synchronizing treatments. Buffaloes (N=32) were fitted with a controlled internal drug releasing (CIDR; 1.8g progesterone, Pfizer) devices for seven days and injected with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Forty eight (48) hours later (Day 9) animals were randomly assigned to receive an injection of either estrogen (estradiol benzoate 500 µg, i.m.; n = 16) or GnRH (Cystorelin 100 µg, im; n=16). All animals were inseminated 24 h later (72 h post $PGF_{2\alpha}$). The mean pregnancy rate obtained was 25% and was not affected type of ovulation synchronizing treatment or parity. Experiment 3 evaluated the necessity of using an ovulation synchronizing agent in TAI protocols, and the effect of time of insemination on the PR. Water buffaloes (N=34) were treated as in experiment 1 except 48 h after insert removal animals were randomly assigned to receive either saline (n=21) or GnRH (n=13). Within treatment groups buffaloes were randomly assigned to be inseminated at 60, 72 or both 60 and 72 h after progesterone withdrawal. The mean pregnancy rate (41.2%) was not affected by the use of GnRH as an ovulation synchronizing agent. The PR of animals inseminated 72 h after CIDR removal tended to be higher (P =.10, 67%) than animals inseminated at 60 or at both 60 and 72 h after progesterone removal. PR also varied significantly with inseminator. The use of CIDR-based TAI protocols with insemination at 72 h after insert removal may be practical tool in the management of reproduction in water buffaloes in the tropics.

Introduction

Water buffaloes (*Bubalus bubalis*) are well adapted to the climatic and nutritional environment and are tolerant to many endo- and ecto-parasites that exist making them

ideally suited for the production of meat and milk in developing countries (Reviewed by Rastogi and Rastogi, 2004). The water buffalo that exist in Trinidad is considered a valuable gene pool and have been exported to several countries including countries in the Caribbean, Central and South America, Italy, the USA and Taiwan (Rastogi and Rastogi, 2004). Despite the great potential of this animal resource the water buffalo sub-sector has shown significant contraction over the last two decades in part due to out-breaks of brucellosis (Fosgate et al., 2002, 2011) associated with uncontrolled natural mating and the failure to evaluate and utilize improved genetics.

In cattle, artificial insemination (AI) has been successfully used instead of natural mating to aid in the rapid dissemination of improved genetics and to reduce the risk of transmission of sexually transmitted diseases such as brucellosis. However, the use of traditional AI in water buffaloes is not as practical as in cattle since the behavioral signs of estrus in water buffaloes are less overt than in cattle, homosexual behavior among females is rare, and, signs of estrus are predominantly exhibited during the night or early morning, and are of short duration under hot and humid conditions (reviewed by Perera, 2011).

Timed artificial insemination (TAI) is a reproductive management technique that utilizes exogenous hormones to induce and synchronize the time of ovulation. Therefore, TAI negates the need for detection of estrus. Several TAI protocols have been evaluated for use in beef and dairy cattle (Stevenson et al., 1999; Macmillan, 2010; Dickson et al., 2012). The original TAI protocols (Pursely et al., 1995) were based on "turning over" the existing dominant follicle through atresia or ovulation with an initial injection of gonadotropin releasing hormone (GnRH) followed 7 days later by regression of any existing corpus luteum with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), followed 48 h later with a second injection of GnRH to induce and synchronize ovulation with insemination of all treated animals 12-24h after the second GnRH injection. Variation in the pregnancy rate due to parity (Dickson et al., 2012) and ovulatory status (Chebel et al., 2006) and concerns over the cost of establishing a pregnancy using TAI have resulted in alterations in the standard protocol including the application of exogenous progesterone during the time from the first GnRH injection and injection of $PGF_{2\alpha}$ (Stevenson et al., 2006); replacement of the second GnRH injection with various estrogen analogues (Stevenson et al., 2004; Dickson et al., 2012), changes in the timing of insemination as well as the inclusion of pre-synchronization treatments.

TAI has great potential to facilitate the wider use of AI in water buffaloes by obviating the need for detection of their less pronounced estrus. Despite significant differences in the reproductive cycle of bubaline and bovine animals (Drost, 2007; Perera, 2011) the few studies evaluating TAI in water buffaloes have used the general ovsynch/GnRH-based TAI protocols developed for cattle, and have reported variable pregnancy rates. Additionally, only one study have evaluated a progesterone-based TAI protocol. Therefore, the objective of this study was to further evaluate the pregnancy rate in water buffaloes reared under a humid tropical environment using different TAI protocols.

Materials and Methods

Location and Animals

The experiments in this study were conducted at the Aripo Livestock Station, located at the foothills of the Northern Range in Wallerfield, Trinidad (10^0N, $61^0$31'W). The water buffaloes used in this study (N = 104) were in fair to good body condition (BCS 3-6) of varying parity, postpartum interval, parity and ovulatory status. Animals used in the study were reared semi-intensively, grazed on native pastures, with access to wallowing ponds. The herd is routinely tested for Brucellosis, Johne's, Tuberculosis and Bovine Leukosis Virus (BLV). The Aripo herd of buffaloes is free of Tuberculosis and Brucellosis and no clinical cases of Johne's or BLV was detected. Prior to the start of experiments pregnancy diagnosis by rectal palpation or ultrasonography was conducted but the ovulatory status of non-pregnant animals was not determined.

Inseminators

Three technicians employed with the AI service attached to the Ministry of Agriculture performed the insemination procedures in all experiments. All but one (1) technician had little or no prior experience inseminating buffaloes.

Experimental Design

Experiment 1, was conducted to compare the PR/AI in buffaloes following modified TAI protocols in which exogenous progesterone was provided by a controlled intravaginal drug releasing (CIDR) device or endogenous progesterone secretion was induced from CL formed after injection with GnRH. Buffalo cows and heifers (N = 38) assigned to Treatment 1 (n=19), received a modified ovsynch TAI protocol which included an injection of progesterone (200 mg, i.m., day-3), and an injection of GnRH (Cystorelin, 100 µg, i.m.) three (3) days later (day 0). Buffaloes assigned to Treatment 2 (n=19), received a CIDR device (1.8 g of progesterone, Pfizer, Mexico) on Day 0. On Day 7, the CIDR device was removed and all buffaloes (N=38) were injected with $PGF_{2\alpha}$ (Lutalyse, 25 mg, i.m, 5 ml/animal i.m; Pfizer Corporation, New York, NY). Thirty (30) hours after injection with $PGF_{2\alpha}$ all animals were injected with estrogen (estradiol benzoate, 500 ug, i.m.), and 30 h later (60 h post $PGF_{2\alpha}$; progesterone withdrawal) all animals were inseminated. On day 60 post insemination pregnancy diagnosis was conducted by ultrasonography.

The pregnancy rate in experiment 1 was higher in the progesterone than in the ovsynch/GnRH-based treated buffaloes. Therefore, experiment 2 used a progesterone/CIDR-based TAI protocol to compare estrogen and GnRH as ovulation synchronizing agents. CIDR devices were placed in buffalo cows and heifers (d0, N= 34). On Day 7 the device was removed and all animals were injected with $PGF_{2\alpha}$. On Day 9, 48 h after injection with $PGF_{2\alpha}$, cows and heifers were randomly assigned to receive either estrogen (estradiol benzoate, 500 µg, i.m; n =17) or GnRH (Cystorelin,

100 µg, i.m; n =17) to synchronize ovulation. All animals were inseminated 24 h later (72 h post $PGF_{2\alpha}$; progesterone withdrawal).

Experiment 3 was similar to experiment 2, except that saline and GnRH was used as ovulating synchronizing agents and, within these treatment groups, animals were randomly assigned to be inseminated at 60, 72 or both 60 and 72 h post $PGF_{2\alpha}$ injection. Pregnancy diagnosis was conducted by transrectal ultrasonography on day 60 post insemination.

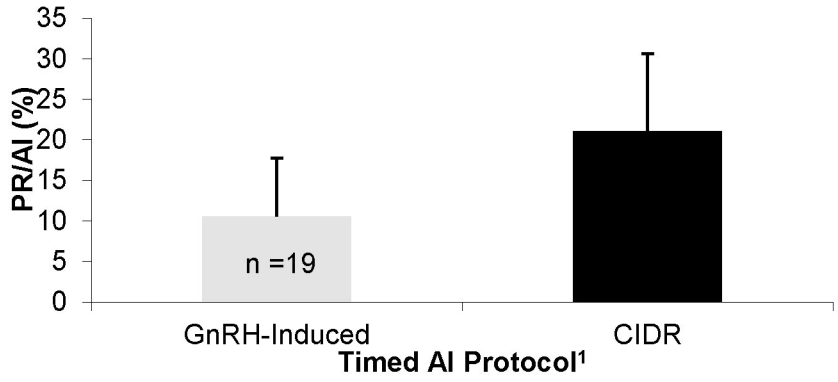
Statistical Analysis

The effect of treatments on pregnancy rate per AI (P/AI) was determined by logistic regression using the LOGISTIC procedure of SAS (SAS institute, Inc., NC, USA). The statistical model used included the fixed effects of source of progesterone (CIDR vs GnRH-induced, Experiment 1) type of ovulation-synchronizing agent (GnRH vs estrogen, Experiment 2; Saline vs GnRH, Experiment 3), time of insemination and inseminator (Experiment 3) and parity (Experiments 1, 2 and 3) and their interactions. No significant interactions were detected so experiments were reanalyzed with only main effects.

Results

Experiment 1

The mean PR/AI in experiment 1 was 15.8% (21 and 10.5% in buffaloes receiving the CIDR- and GnRH-based TAI protocols, respectively; Figure 1) and PR/AI did not differ with source of progesterone or parity.



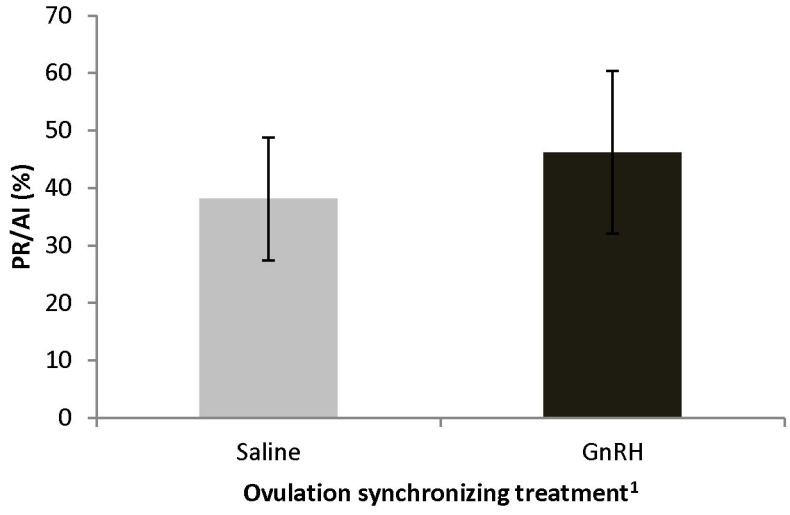
[1] Progesterone in the TAI protocol was either induced by an injection of GnRH (100 µg) at the beginning of treatment or provided from an exogenous source (controlled intravaginal drug releasing device, CIDR, 1.9 g progesterone) on day 0.

Figure 1. Pregnancy rate per AI (PR/AI) following ovsynch/GnRH and progesterone/CIDR-based timed artificial insemination (TAI) in water buffaloes

Experiments 2 and 3

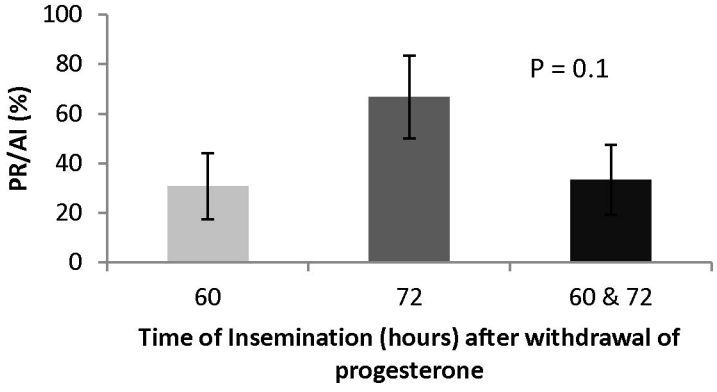
The mean PR/AI in experiment 2 (25%) was not affected by ovulation synchronizing agent or parity. The mean pregnancy rate in experiment 3 was 41.2% and was not affected by ovulation synchronizing agent (38.1 and 46.2% for animals treated with saline or GnRH as the ovulation synchronizing agent, respectively) or parity (54 and 33% for cows and heifers, respectively).

The PR/AI was 30.8, 66.7 and 33.3% for animals inseminated at 60, 72 and at both 60 and 72h after progesterone withdrawal, respectively. Insemination at 72h after progesterone withdrawal tended (P= 0.1) to result in higher pregnancy rates than insemination at either 60 or at 60 and 72h after progesterone withdrawal. PR/AI tended to vary among inseminators (P = 0.1; range 55 to 14.2%).



[1] The Water buffaloes were injected with either GnRH (cystorelin, 100 μg) or saline 48h after CIDR/progesterone removal.

Figure 2. The effect of gonadotropin releasing hormone (GnRH) when used as an ovulation synchronizing treatment in TAI on pregnancy rate per AI (PR/AI).



[1] Water buffaloes were inseminated at 60, 72 and 60 and 72 hours after withdrawal of progesterone (removal of CIDRs and injection with $PGF_{2\alpha}$)

Figure 3: Effect of time of insemination[1] on pregnancy rate per AI (PR/AI) following timed artificial insemination (TAI) in water buffaloes.

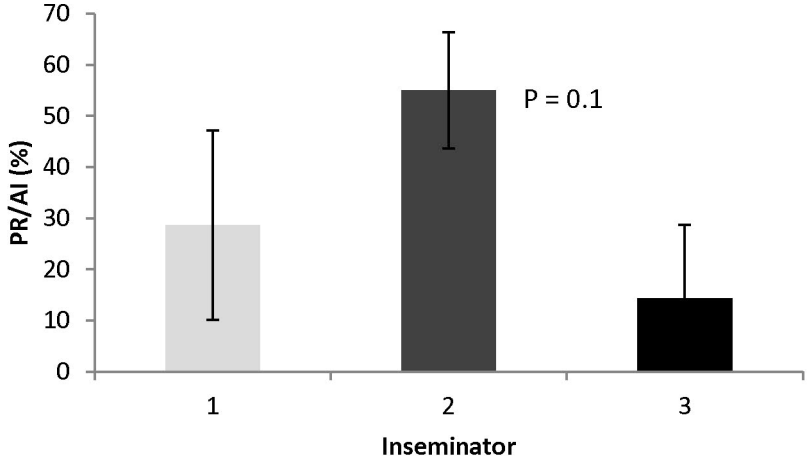


Figure 4. Effect of inseminator on pregnancy rate per AI (PR/AI) following timed artificial insemination (TAI) in water buffaloes

Discussion and Conclusion

The average pregnancy rate per AI (P/AI) in water buffaloes in this study varied between 11 and 46% across treatments, parity and experiments. The overall pregnancy rate for progesterone/CIDR-based TAI protocols (Experiments 2 and 3) ranged from 25-46% and was comparable to pregnancy rates previously reported for buffalo cows inseminated following progesterone/CIDR-based (Murugavel et al., 2009) or Ovsynch/GnRH-based TAI protocols (Chaikhun et al., 2010; Oropeza et al., 2010) or following AI at detected natural estrus (Mirahmoudi et al., 2013; Chaikun et al., 2010)

and higher than pregnancy rate observed in heifers following ovsynch TAI (Chaikun et al., 2010).

Overall pregnancy rate did not differ significantly between heifers and cows. Pregnancy rates tend to be lower in heifers following ovsynch/GnRH-based TAI in cattle (Macmillan, 2010) and buffaloes (Chaikhun et al., 2010). In experiment 3, pregnancy rate was 20% higher in cows than heifers, but this difference did not reach statistical significance. No previous study comparing fertility in buffalo cows and heifers following progesterone-based TAI has been reported in the literature.

Pregnancy rate was lowest in experiment 1 in which a modified GnRH-based "ovsynch" TAI protocol was compared to a progesterone/CIDR-based protocol. Pregnancy rate resulting from GnRH-based TAI protocols in anestrous animals is lower due to a reduced effectiveness in inducing ovulation and a failure to establish a fully functional CL. The ovulatory state of the water buffaloes used in these studies were not determined, but it is assumed some animals may have been anovulatory due to general body conditions scores (3-6), use of early postpartum cows and the use of nulliparous animals.

The modified ovsynch protocol used in this experiment included an initial injection of progesterone prior to injection of GnRH. In sheep and cattle the CL formed from induced ovulation during anestrous or from the first post anestrous ovulation can be short-lived or sub-functional. A single injection of progesterone prior to induced ovulation as used in experiment 1 ensures that any CL formed subsequently would have a normal lifespan. Therefore, it was assumed the pre-injection with progesterone three days prior to injection of GnRH should have increased the efficacy of the ovsynch/GnRH-based protocol.

In experiment 1 estrogen instead of GnRH was used as an ovulation synchronizing and inducing treatment due to its lower cost. Estrogen and estrogen analogues have successfully been used as a replacement for the second GnRH injection in TAI protocols in beef (Bridges et al., 1999; Sales et al., 2012) and dairy cows (Stevenson et al., 2004; Dickson et al., 2012), but have not been previously evaluated in water buffaloes.

The time of insemination was advanced to 60 instead of 72 h after withdrawal of progesterone (insert removal and injection with $PGF_{2\alpha}$) in experiment 1. In beef cattle the time from progestogen withdrawal to ovulation is shorter in heifers than cows. This finding has led to the recommendation that TAI should be performed by 48-60h after progestogen withdrawal in heifers (Sa Filho et al., 2013). Further, in beef cows the use of estrogen as the ovulation synchronizing agent resulted in tight synchrony of estrus and ovulation and, TAI performed at 58-60 h resulted in high pregnancy rates (Bridges et al., 1999). The duration from progestogen withdrawal to ovulation in water buffalo cows averaged 72 hours (range 62-80 h; Mirahmoudi et al., 2013) and 18-22h from injection of GnRH to ovulation (Mirahmoudi and Prakash, 2012). Although little data exist on the duration of progestogen withdrawal to ovulation or from injection with GnRH

to ovulation in water buffalo heifers, the findings in buffalo cows suggest that inseminations at 60 h following progesterone withdrawal might be too early and could have contributed to the low pregnancy rate in experiment 1.

Based on the findings in experiment 1, studies in experiment 2 used only progesterone-based TAI protocols with insemination at 72h after progesterone withdrawal to examine the effects of different ovulation synchronizing agents. The overall pregnancy rate of 25% was lower than that reported for TAI protocols in dairy and beef cattle (Pursely et al., 1995; Dickson et al., 2012) and in buffaloes in some studies (50-62%; Mirahmoudi and Prakash, 2012; Mirahmoudi et al., 2013), but was similar to values reported for water buffaloes in other studies (Chaikhun et al., 2010). Consistent with previous findings in cattle (Dickson et al., 2012) estrogen was as effective GnRH as an ovulation synchronizing agent (experiment 2) and the exclusion of an ovulation synchronizing treatment in the TAI protocol did not affect pregnancy rates (experiment 3). These findings suggest that the cost of establishing a pregnancy in progesterone-based TAI in water buffaloes might be reduced either by using estrogen, a cheaper ovulation synchronizing agent, or by completely eliminating the use of ovulation synchronizing agents. Further, evaluation of TAI protocols without ovulation synchronizing treatments with larger sample sizes is warranted.

In experiment 3, animals inseminated at 72 h had higher pregnancy rates than animals inseminated at 60 h or at both 60 and 72 h after withdrawal of progesterone. High pregnancy rates have also been reported in water buffalo cows inseminated at 72 and 84 h after progesterone withdrawal (Mirahmoudi et al., 2013). Since ovulation in water buffaloes occurs approximately 72 h after progesterone withdrawal (Mirahmoudi et al., 2013), insemination at 60 h post progesterone withdrawal might be too early to achieve a high level of fertility. In support of this suggestion Sales et al. (2011) reported lower pregnancy rates in dairy cows inseminated 12 h or more prior to ovulation.

Pregnancy rates of Thai swamp buffaloes inseminated at 60 and 72 h after progesterone withdrawal was similar (32%; Chaikun et al., 2010) to that obtained for water buffaloes inseminated at the same time points in experiment 3 (Figure 3) but lower than that obtained from a single insemination of water buffaloes at 72 h (Murugavel et al., 2009) or at 72 and 84h (Mirahmoudi et al., 2013). The reason for a lower fertility in animals inseminated at both 60 and 72 h is unclear. Insemination At both 60 and 72 h should theoretically span the time when the majority of cows and heifers should ovulate and thereby increase the probability of conception.

Pregnancy rates varied significantly with inseminator. As expected, inseminators with more experience working with buffaloes had higher pregnancy rates, suggesting that specific AI training or re-training with water buffaloes would increase pregnancy rates.

In conclusion, pregnancy rates of water buffalo cows and heifers of varying ovulatory status was higher following progesterone/CIDR-based TAI protocols than ovsynch/GnRH-based protocols in this study and was comparable to that previously reported for cyclic cows following TAI and of cows inseminated at natural estrus.

The use of ovulation synchronizing treatments in TAI following progesterone withdrawal may not be necessary and warrants further investigation. A single insemination at 72 hours following progesterone withdrawal resulted in the highest pregnancy rates and pregnancy rates tended to vary with inseminators. Progesterone-based TAI protocol with insemination at 72h following progesterone withdrawal is a cost effective approach for the management of reproduction and to increase utilization of improved genetics in water buffalo herds in the tropics.

Acknowledgements

This study was supported by The Government of The Republic of Trinidad and Tobago and British Petroleum Trinidad and Tobago. The authors also acknowledge the tremendous effort of the officers and staff of the Aripo Livestock Station and Artificial Insemination Unit of the Ministry of Food Production, Trinidad.

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