



Different times to perform timed artificial insemination when using a P4/E2/eCG-based protocol in buffalo

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ABSTRACT: *The aim of this study was to evaluate different times for timed artificial insemination (TAI) in buffalo submitted to a P4/E2/eCG-based protocol. In this study, 204 buffaloes were distributed into one of two groups (TAI56, n=103 and TAI64, n=101). At a random stage of the oestrous cycle (Day 0 = D0), in the morning (TAI56, a.m.) or afternoon (TAI64, p.m.), buffaloes received an intravaginal progesterone device (P4; 1.0 g) plus EB (2.0 mg i.m.). On D9 a.m. (TAI56) or p.m. (TAI64), the P4 was removed and buffaloes received PGF_{2α} (0.53 mg i.m. sodium cloprostenol) and eCG (400 IU i.m.). On D10 a.m. (TAI56) or p.m. (TAI64), 24 h after P4 removal, buffaloes were treated with EB (1.0 mg i.m.). Buffaloes from TAI56 and TAI64 were inseminated 56 and 64 h after P4 removal (D11, p.m. and D12, a.m., respectively). Ultrasound examinations were performed on D0 to ascertain ovarian follicular status, at TAI to measure the diameter of the dominant follicle (DF) and D42 for pregnancy diagnosis. The statistical analysis was performed using the GLIMMIX procedure of SAS[®]. There was no difference between TAI56 and TAI64 for the diameter of the DF at TAI and the pregnancy per TAI. It was concluded that TAI 56 or 64 h after P4 removal did not affect fertility in buffaloes submitted to the induction of ovulation with EB. The present research supports that is possible to perform TAI at any time throughout the day in buffalo synchronized during the non-breeding season.*

Key words: anoestrous, artificial insemination, oestradiol benzoate, follicle, induction of ovulation.

Momentos diferentes para executar a inseminação artificial em tempo fixo utilizando o protocolo à base de P4/E2/eCG em búfalas

RESUMO: *O objetivo deste estudo foi avaliar diferentes momentos para a realização da IATF em búfalas submetidas a um protocolo à base de P4/E2/eCG. Neste estudo, 204 búfalas foram distribuídas em um de dois grupos (IATF56, n=103 e IATF64, n=101). Em estágio aleatório do ciclo estral (Dia 0 = D0), pela manhã (IATF56, manhã) ou pela tarde (IATF64, tarde), as búfalas receberam um dispositivo intravaginal de progesterona (P4; 1,0 g) e BE (2,0 mg i.m.). No D9 pela manhã (IATF56) ou pela tarde (IATF64), a P4 foi removida e as búfalas receberam PGF_{2α} (0,53 mg i.m. cloprostenol sódico) e eCG (400 UI i.m.). No D10 pela manhã (IATF56) ou pela tarde (IATF64), 24 h após a remoção da P4, as búfalas foram tratadas com BE (1,0 mg i.m.). As búfalas dos grupos IATF56 e IATF64 foram inseminadas 56 e 64 h após a remoção da P4 (D11, pela tarde e D12, pela manhã, respectivamente). Avaliações ultrassonográficas foram realizadas no D0 para verificar o status folicular ovariano, na IATF para medir o diâmetro do folículo dominante (FD) e no D42 para o diagnóstico de gestação. A análise estatística foi realizada utilizando o procedimento GLIMMIX do SAS[®]. Não houve diferença entre os grupos IATF56 e IATF64 no diâmetro do FD na IATF e na prenhez por IATF. Conclui-se que a IATF 56 ou 64 h após a remoção da P4 não afeta a fertilidade de búfalas submetidas à indução da ovulação com BE. A presente pesquisa evidencia que é possível realizar a IATF durante todo o dia em búfalas sincronizadas durante a estação reprodutiva desfavorável.*

Palavras-chave: anestro, benzoato de oestradiol, folículo, indução da ovulação, inseminação artificial.

INTRODUCTION

TAI programs are used worldwide to increase reproductive efficiency and to enable genetic improvement in buffalo herds. P4/E2/eCG-based

protocols for the synchronization of ovulation and TAI (P4 + BE + PGF_{2α} + eCG/GnRH or EB) allow for the use of AI in buffalo cows and heifers during the breeding and non-breeding seasons (BARUSELLI et al., 2013; CARVALHO et al., 2013; CARVALHO et

al., 2014; MONTEIRO et al., 2016; CARVALHO et al., 2017; MONTEIRO et al., 2018). Currently, TAI can be used throughout the year with satisfactory ovarian responses and pregnancy outcomes in buffaloes (CARVALHO et al., 2016; CARVALHO et al., 2018). However, adjustments in the protocol and specific hormonal strategies to overcome seasonal anoestrus are necessary and important to permit a continuous enhance of the buffalo dairy and beef industry (CARVALHO et al., 2018).

The use of EB for ovulation induction in buffaloes led to ovulation occurrence ~70 hours after P4 device removal, close to TAI (64 hours after P4 device removal; (CARVALHO et al., 2017). Studies showed that inseminations should be performed within an optimal range before ovulation (SALES et al., 2015). The optimal AI time for the most desirable rate of fertilization in cattle is between 12 and 24 hours before ovulation (ROELOFS et al., 2006). The AI should occur near the time of ovulation to maximize sperm access to the ovum, but not so late that an aging ovum awaits sperm arrival and capacitation (DALTON et al., 2001). A lower P/AI had been associated with a reduced fertilization rate due to receiving AI close to or after ovulation in cattle (DRANSFIELD et al., 1998; ROELOFS et al., 2005; ROELOFS et al., 2006) and in buffaloes (MONTEIRO et al., 2018). However, an optimal pregnancy rate could be achieved when AI is performed 16.2 (MAATJE et al., 1997), 15.3 (AYRES et al., 2008), 14.7 (SÁ FILHO et al., 2013) and 21.8 (SALES et al., 2015) hours before ovulation in cattle. The aim of the present study was to evaluate different times for the performance of TAI in a P4/E2/eCG-based protocol in buffalo submitted to ovulation induction with oestradiol benzoate (EB) 24 h after P4 device removal during the non-breeding season. The hypothesis tested was that TAI performed earlier (56 hours after P4 device removal, ~14 hours before ovulation) would promote an optimal range for spermatid capacitation and viability of the oocyte, and increase the pregnancy rate in a P4/E2/eCG-based protocol in the non-breeding season for buffalo.

MATERIALS AND METHODS

Animals and management

The experiment was conducted at Santa Eliza Farm (Dourado, São Paulo, Brazil) during the non-breeding season which coincides with an increase in day length (spring to summer). Lactating crossbred Murrah x Mediterranean buffalo (*Bubalus bubalis*) cows (n = 204) at 3.2 ± 0.2 lactations (mean \pm standard error of the mean), 99.9 ± 3.7 days in milk,

6.7 ± 0.2 years old and with a body condition score (BCS) 3.9 ± 0.1 (scale 1–5, where 1 = very thin and 5 = very fat) were used. Cows were milked twice a day and had contact with their calves only during milking. Buffaloes grazed tropical grasses and were supplemented with corn silage, chopped sugar cane and a grain mix containing ground corn, soybean meal, citrus pulp, whole cottonseed, minerals and vitamins. Animals had free access to water.

Experimental design

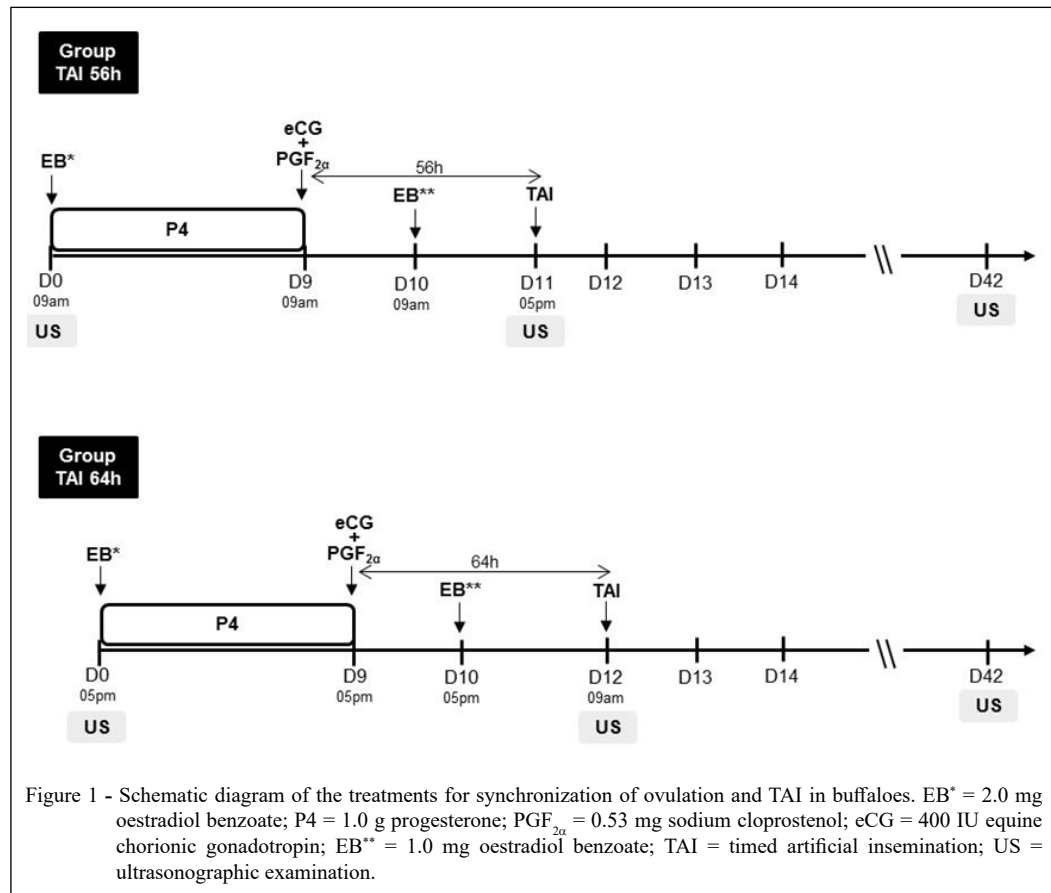
Buffaloes were distributed according to age, number of births, BCS and ovarian activity in one of two groups (TAI56, n = 103 and TAI64, n = 101). At a random stage of the oestrous cycle (Day 0), in the morning (TAI56) or afternoon (TAI64), buffaloes received an intravaginal progesterone device (P4; 1.0 g; Sincrogest®, Ourofino Agronegócio, Brazil) plus EB (2.0 mg i.m.; Sincrodiol®, Ourofino Agronegócio). On day 9, in the morning (TAI56) or afternoon (TAI64), the P4 device was removed and buffaloes received an injection of PGF_{2 α} (0.53 mg i.m.; sodium cloprostenol; Sincrocio®, Ourofino Agronegócio) plus eCG (equine chorionic gonadotropin; 400 IU i.m.; SincroeCG®, Ourofino Agronegócio). On Day 10, in the morning (TAI56) or afternoon (TAI64), 24 h after device removal, buffaloes received EB (1.0 mg i.m.). Buffaloes from TAI56 and TAI64 underwent TAI 56 and 64 h after P4 device removal (D11, afternoon and D12, morning, respectively; Figure 1). All inseminations were performed by the same technician. Frozen-thawed semen straws from two buffalo bulls of proven fertile were equally distributed between the treatment groups.

Ultrasonographic examinations

Ovaries were scanned by ultrasonography using a 7.5-MHz linear-array transrectal transducer (Mindray DP-2200Vet; Shenzhen, Guangdong, China). Ovarian ultrasonographic examinations were performed on D0 to ascertain ovarian follicular status, at TAI to measure the diameter of the DF, and D42 for pregnancy diagnosis (Figure 1). The pregnancy per TAI (P/TAI) was defined as the number of pregnant buffalo divided by the total number of buffalo mated by TAI. The detection of an embryonic vesicle with a viable embryo (presence of a heartbeat) was used as an indicator of pregnancy.

Statistical analyses

Statistical analyses were performed using Statistical Analysis System for Windows-SAS. The variables evaluated were diameter of the dominant



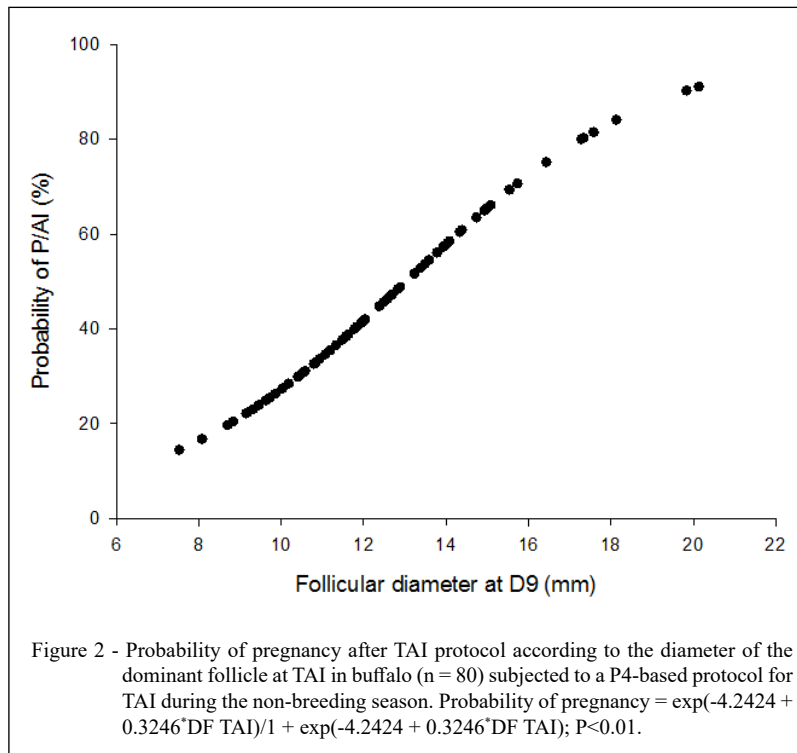
follicle and pregnancy rate. Statistical models created for continuous data were analyzed by Akaike's Information Criterion (AIC) and used the model with lower AIC. Then, the data were analyzed by the GLIMMIX procedure. All values are expressed as mean \pm SEM. The binomial variable P/TAI was analyzed using the PROC GLIMMIX procedure of SAS. Explanatory variables such as treatment and BCS at day 0 were included in the model as classes. The model included the fixed effects of treatment, number of births, sire and the random effects of buffaloes. All two-way interactions were tested in logistic regression models. Data were analysed by a multivariate logistic regression using the LOGISTIC procedure of SAS. Variables were removed by backward elimination, based on the Wald statistics criterion when $P > 0.20$ to form the final model. Variables included in the final model for analysis of P/TAI were treatment (TAI56 and TAI64), number of births and BCS at Day 0. The probability curves were obtained using the following formula: $Y = [\text{EXP}(\text{logit})/1 + \text{EXP}(\text{logit})]^*100$. Adjusted odds ratio (AOR) and 95% confidence

interval (CI) were generated during the logistic regression. Results are presented as proportions and AOR. The P/TAI was analysed using the GLIMMIX procedure of SAS. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

RESULTS

The diameter of the DF at TAI was similar between treatment groups (TAI56 = 12.9 ± 0.3 and TAI64 = 12.5 ± 0.5 ; $P = 0.45$). However, the diameter of the DF had an effect ($P = 0.02$) on the P/TAI [diameter < 10.0 mm = 8.3% (1/12)^b; diameter between 10.1 to 12.0 mm = 26.3% (5/19)^b; diameter > 12.1 mm 65.3% (32/49)^a]. An increase in the diameter of the DF increased the probability of P/TAI (Figure 2).

There were no significant interactions between treatments for explanatory variables such as BCS ($P = 0.79$) and sire ($P = 0.54$). No differences were reported ($P = 0.88$) between TAI56 and TAI64 for P/TAI (Figure 3).



DISCUSSION

The present study evaluated the flexibility of timing of TAI with regard to the pregnancy rate in buffalo submitted to the induction of ovulation with EB 24 hours after P4 device removal during the non-breeding season. It was reported that TAI performed 56 and 64 h after P4 device removal produced similar diameter of the DF at TAI and pregnancy rate. The initial hypothesis of the present study was rejected, since both treatments showed similar P/TAI. Nevertheless, this study provided novel approaches in a P4/E2/eCG-based protocol in buffalo. Based on these findings, currently is possible to synchronize all the buffaloes in the morning and perform TAI in the afternoon (56 hours) or synchronise all the buffaloes in the afternoon and perform TAI in the morning (64 hours), without compromising fertility. It confers greater flexibility and applicability of the P4/E2/eCG-based protocol in the non-breeding season for buffalo.

The use of EB for synchronizing ovulation in P4/E2/eCG protocols was previously reported with tight synchrony of ovulation and high fertility, showing a greater pregnancy rate for TAI in buffalo (NASEER et al., 2011; MIRMAHMOUDI et al., 2014; CARVALHO et al., 2017). Other evidence has

confirmed EB treatment efficiency for the induction of ovulation in buffalo. Studies have shown that EB treatment induces considerable release of LH (JACOMINI et al., 2014), has a low cost (BARROS et al., 2000; MANES et al., 2012) and maintains high circulating oestradiol concentrations, which likely creates a better uterine environment for embryonic development (BRIDGES et al., 2012).

In the present study, buffalo cows synchronized with EB for ovulation induction had a similar diameter of the DF at TAI between treatment groups. The ovulatory follicle diameter is important in TAI protocols as it is directly related to CL size in buffalo (VECCHIO et al., 2012; CARVALHO et al., 2013; MONTEIRO et al., 2016) and cattle (VASCONCELOS et al., 2001; DADARWAL et al., 2013). A larger CL secretes more P4 (PFEIFER et al., 2009; DADARWAL et al., 2013), which is related to the maintenance of pregnancy and improved fertility in buffalo (VECCHIO et al., 2012) and cattle (BINELLI et al., 2001; INSKEEP, 2004; BARUSELLI et al., 2009; LONERGAN, 2011). The similar diameter of the DF at TAI reported in this study may be associated with the similar P/TAI between treatments.

Although in the present study buffalo cows had similar diameter of the DF at TAI between

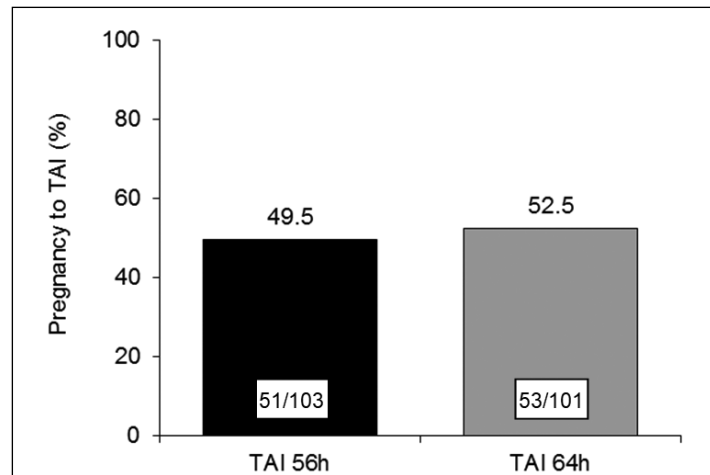


Figure 3 - Pregnancy to TAI in buffaloes submitted to a P4/E2/eCG-based protocol, according to the treatment* group (TAI56 or TAI64; P = 0.88).

*At random stage of the oestrous cycle (Day 0), in the morning (TAI56) or afternoon (TAI64), buffaloes received an intravaginal progesterone device (P4; 1.0 g) plus EB (2.0 mg i.m.). On day 9, in the morning (TAI56) or afternoon (TAI64), the P4 device was removed and buffaloes received an injection of PGF_{2α} (0.53 mg i.m.; sodium cloprostenol) plus eCG (equine chorionic gonadotropin; 400 IU i.m.). On Day 10, in the morning (TAI56) or afternoon (TAI64), 24 h after device removal, buffaloes received EB (1.0 mg i.m.). Buffaloes from TAI56 and TAI64 underwent TAI 56 and 64 h after P4 device removal (D11, afternoon and D12, morning, respectively).

treatment groups, it was verified that the diameter of the DF had an effect on the P/TAI. This result corroborated with a recent study that verified a positive correlation between diameter of the DF at TAI and ovulation and pregnancy rates in buffalo (MONTEIRO et al., 2018) and previous studies performed in cattle (VASCONCELOS et al., 2001; PERRY et al., 2007; SÁ FILHO et al., 2010). Also, it was demonstrated that the diameter of the DF at TAI showed a negative correlation with the occurrence of embryonic mortality in buffaloes (MONTEIRO et al., 2018). Furthermore, MONTEIRO et al. (2016) reported that the development of the synchronization of ovulation protocols for TAI in dairy buffalo should focus on establishing larger and healthy follicles at the end of the synchronization treatment because doing so increases the likelihood of ovulation and pregnancy following TAI.

In the present study, the similar pregnancy rates reported in buffalo that were submitted to different TAI timings during the synchronization protocol showed that the inseminations were performed within an optimal range before ovulation in both treatment groups (~6 to ~14 hours before ovulation in buffalo,

according to (CARVALHO et al., 2017). The optimal time at which insemination should take place relative to ovulation depends primarily on the lifespan of spermatozoa and on the viability of the oocyte in the female genital tract (HUNTER, 1994; AYRES et al., 2008). The sperm require time for capacitation and transport to the oviduct before fertilization (WILTBANK & PURSLEY, 2014). Previous studies demonstrated that 6 h is the minimum time needed for a viable sperm population capable of fertilization to pass through the oviduct; the number of progressive motile sperm peaks from 8 to 18 h after insemination (THIBAUT, 1973; HUNTER & WILMUT, 1984; HAWK, 1987; AYRES et al., 2008). The most desirable period for oocyte fertilization appears to be between 6 and 10 h after ovulation (BRACKETT et al., 1980).

CONCLUSION

The timing of TAI in buffaloes submitted to ovulation induction with EB during the non-breeding season did not change the fertility of these females. The findings of the present study lead to the conclusion

that is possible to perform TAI throughout the day (morning and afternoon) without compromising fertility in buffalo synchronized in a P4/E2/eCG-based protocol during the non-breeding season.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

AUTHOR CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

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