Butaphosphan and cyanocobalamin: effects on the aspiration of oocytes and in vitro embryo production in Jersey cows

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Butaphosphan and cyanocobalamin: effects on the aspiration of oocytes and in vitro embryo production in Jersey cows

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Abstract

The aim of this study was to evaluate butaphosphan and cyanocobalamin effects on production of oocytes in vivo and in vitro embryo production during a ovum pick up protocol. Thirty-six cows were homogeneously divided into two groups. The butaphosphan and cyanocobalamin group (CAT, n=17), received 4500 mg of butaphosphan and 2.25 mg of cyanocobalamin and the control group (CONT, n=19) received placebo (NaCl 0.9%). Treatment was performed at 14, 9, and 5 days prior the follicular aspiration sessions (FA) follicular and each cow was subjected to 3 sessions of ovum pick up (OPU) and in vitro embryo production. CAT group showed high (P<0.05) number of aspirated follicles between 3-6mm and number of oocytes grade 2 and a tendency (P = 0.10) of total number of follicles aspired. No difference (P>0.05) was found for the number of different sizes of follicles aspired, retrieved oocytes, recovery rate, viable oocytes, blastocysts and total
viable blastocysts. In conclusion, successive butaphosphan and cyanocobalamin administration can potentially increase in vitro embryo production and quality in Jersey cows during a ovum pick up protocol.

**Keywords:** Blastocyste, dairy, reproduction, vitamin B12

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**INTRODUCTION**

Regarding the female gamete donors, the oocyte numbers, quality and viability may be influenced by factors such as the breed group, health status, cyclicity and metabolic status (Thibier 2006). Considering the animal metabolism, it is known that nutrition can act through substrates and hormonal stimulation, affecting cattle reproductive activity at a profound level (Butler et al. 2003). These variations in herd reproductive performance can be observed in the ovarian tissue because it alters follicle development, granulosa cell proliferation and steroidogenic potential (Adamiak et al. 2005). Furthermore, most of the changes in the blood metabolites affect the follicular fluid composition and oocyte quality (Ali et al. 2008). Linking these factors, retrieved oocytes from cows with high genetic merit for milk production have a lower rate of cleavage and blastocyst development compared to cows of medium genetic merit (Snijders et al. 2000).

Butaphosphan is an organic molecule consisting of 17.3% of phosphorus and conventionally indicated for the treatment of metabolic disorders, including milk fever and hypomagnesemia, as well as for support in infertility treatment, tetany and paresis [European Agency for the Evaluation of Medicinal Products (EMEA) 2000]. Phosphorus plays an important role in buffering hydrogen in the blood system and is a critical component of nucleic acids, adenosine triphosphate (ATP) and adenosine monophosphate (AMP) (Cunningham 2002). Cyanocobalamin is a synthetic form of B12 vitamin and acts as a cofactor of the metilmalonil-CoA mutase, which is responsible for
converting methilmalonil-CoA in succinyl-CoA and is responsible for the speed of the Krebs cycle and gluconeogenesis (Taoka et al. 1994). In goats, vitamin B12 deficiency induced an irregular estrous cycle and reduced progesterone concentrations during diestrus (Mgongo et al. 1984), and in rats, the same deficiency during the growing period was related to lower uterus and ovary weight in the mature phase (Dryden and Hartman 1966).

The systemic effect of butaphosphan and cyanocobalamin combination was not completely elucidated. Within this context, butaphosphan and cyanocobalamin have been recommended during the postpartum period of dairy cows, with a proven effect of reducing the severity of a negative energy balance (NEB) (Rollin et al. 2010) and milk production increase (Kreipe et al. 2011) as a consequence of a decrease in NEFA and β-hidroxibutirate (Pereira et al. 2013a). In lambed sheep, it was possible to observe a decrease in the acetone concentration and an increase in dry matter intake (Pereira et al., 2013b). However, considering reproductive variables, only one trial with Holstein Gyr crossed lactating cows has identified some sort of benefits of butaphosphan and cyanocobalamin supplementation for in vitro embryo production (Reis et al. 2012).

We hypothesized that butaphosphan and cyanocobalamin supplementation in dairy cows increases the number and quality of oocytes, thereby providing greater in vitro embryo production (IVP) per donor. Until now, there has been no information in the scientific literature about the effects of butaphosphan and cyanocobalamin supplementation in Bos taurus cows on the IVP of bovine embryos. Thus, our aim was to evaluate butaphosphan and cyanocobalamin effects on follicles development and production of oocytes in vivo and on in vitro embryo production in Jersey cows.

MATERIALS AND METHODS

Animal welfare
The Committee for Ethics in Animal Experimentation from the Pelotas Federal University has approved all procedures performed in this experiment.

**Cows and experimental design**

The trial was conducted in a commercial farm in southern Brazil (lat. 32°16’S, long. 52°32’W). Thirty-six multiparous Jersey (14 were lactating and 22 were not lactating) cows with a body weight (BW) of 437.0 ± 49.8 kg and an average body condition score (BCS) of 3.5 were used in the study, based on a five-point scale, where obese equals 5 (Wildman et al., 1982). Lactating cows had an average of 208 ± 47 milking days and a daily milk production of 13.2 ± 1.9 kg. Animals were allocated homogeneously according to the lactating status (lactating or non-lactating) called as “Category”, and BCS into two groups: The CAT group (n=17; 6 lactating and 11 non-lactating) and CONT group (n=19; 8 lactating and 11 non-lactating). The experiment extended 42 days and it was divided into three trials (14 days each). Each trial consisted of three treatments of CAT (4500 mg butaphosphan and 2.25 mg cyanocobalamin; 45 ml of Catosal® B12, Bayer Health Animal, São Paulo, Brazil) or placebo solution (0.9% NaCl) in three different sites at semimembranosus and semitendinosus muscles area at 14, 9, and 5 days prior FA (Fig. 1). During the study, all cows were kept on a grazing system with ryegrass (*Lolium multi florum*) and white clover (*Trifolium repens*) offered ad libitum. After each milking (twice daily), lactating cows received supplementation with concentrate, corn silage and a mineral mixture.

**Follicular aspiration**

Four FA routines were performed, with a 14-day interval between sessions. The first FA (day 0) was considered to be the beginning of the experiment. Data from this collection was not considered in the statistical analysis. The collection of cumulus-oocyte complexes (COCs) was performed using a guided transvaginal probe for FA. All visible
follicles ≥ 2 mm were aspirated with the use of ultrasound equipment (Scaner B-mode, Aloka SSD 500, Tokyo, Japan) connected to a 7.5 MHz micro convex transducer. The diameter and ovarian structure distribution were recorded at the time of each FA and classified as the presence of corpus luteum and by the number of follicles up to 3 mm, 3-6 mm, 7-10 mm, 10-13 mm and larger than 13 mm. The FA, oocyte classification, and *in vitro* embryo production were always performed by the same technician. To perform FA, the animals received an epidural injection of 5 ml of 2% lidocaine hydrochloride (Xylestesin®, Cristália Ltda., Itapira, SP, Brazil). FA was performed using a disposable 20 G needle inserted into the guide puncture, which had a puncture line connected to a conical 50 ml tube, connected to a vacuum pump. The negative pressure created by the vacuum pump was approximately 80 mmHg, with a flow capacity of 13-15 ml of solution per minute. The COCs were selected under a stereomicroscope in petri dishes in accordance with the criteria established by De Loss et al. (1989) and were then kept in PBS and heparin to be sorted later. The COCs were identified and classified into four categories based on the morphological appearance and number of cumulus cells: *grade I*, cumulus cells multiple and compact, homogeneous ooplasm; *grade II*, two or three layers of cumulus cells, ooplasm with thicker and darker granulation; *grade III*, only one layer of cumulus cells and may present blackish spots; *grade IV*, expanded cumulus, denuded or degenerated oocytes.

**In vitro production of embryos**

Only oocytes classified as *grade I*, *grade II* or *grade III* were used for *in vitro* maturation. After classification, the COCs were washed in PBS and 5% fetal bovine serum (FBS) solution and placed in cryovials containing maturation culture media TCM-199 (pyruvate 2.2 mg/ml) plus FSH (0.01 Ul/ml), LH (0.24 Ul/ml), and 10% FBS gentamicin (0.5 mg/ml) to be transported to an oocyte incubator located in the central laboratory. In the central laboratory, mineral oil (200 µl) was added to the cryovials, which were then
transferred to a 39° incubator at 5.5% CO₂ and saturated humidity, where they remained for 22-24 hours, in accordance with the beginning of the maturation process at the farm. After the period of in vitro maturation (IVM), the oocytes were transferred to individual Petri dishes and four-well plates, containing 400 µl of Tyrodes solution with albumin, lactate and pyruvate (TALP Fert culture; pyruvate 2.2 mg/ml and gentamicin 0.5 mg/ml) with heparin (0.2 mg/ml) and PHE (penicillamine 0.27 µg/100ul; hypotaurine 0.10 µg/100ul; epinephrine 0.033 µg/100ul) already stabilized and submerged in 4.0 ml of mineral oil inside the well. Straws of sexed and frozen semen from eight Jersey bulls prepared through a Percoll gradient were used for IVF. The straws were defrosted for 20 seconds in warm water at 36°C, and then emptied in 1.5 ml microtubes containing the stages of 45% and 90% of Percoll gradient, to first be centrifuged at 4932G for 5 minutes, followed by two centrifugation steps at 3086 G for 3 minutes. After each centrifugation, the supernatant was removed, leaving a deposit of sperm, and TALP Fert culture media was added. Spermatozoa were counted and an aliquot of sperm suspension was added each well, to obtain a final concentration of 2 x 10⁶ spermatozoa/ml. After insemination, the dishes were incubated at 39°C with 5% CO₂ and saturated humidity for 18 to 22 hours. Considering the fertilization day as day zero (D0), on day one (D1), the presumptive zygotes were denuded by multiple pipetting, washed in SOF ("Synthetic oviduct Fluid") and incubated in 400 µl drops of SOF medium with SFB 2% and pyruvate (2.2 mg/ml) covered with mineral oil and incubated at 38.5°C under 5% CO₂ and saturated humidity until day seven (D7) in the in vitro culture media (IVC). The cleavage rate was evaluated on D2 and embryo development was evaluated on D7, considering the embryos that were in a developmental stage consistent with the day of the IVC to be viable. The embryos were classified according to IETS criteria (Wright, 1998), and only embryos at grade 1 or 2 were used. For data analysis, the embryos were classified into total blastocysts (considering grade 1 and...
2) and grade 1 only blastocysts. The total blastocyst rate was performed considering the relation between number of viable oocytes by number of blastocysts at D7.

Statistical analyses

All statistical analyses were performed using NCSS 2005. The variables were analyzed as repeated measures, considering the effects of treatments (CAT vs. CONT), category (lactating and non-lactating), collections (14; 28; 42 days) and their interactions, as well as the female random effect was included in all models. Bull effect was initially considered in the model. However, there was no effect ($P > 0.10$) of sire, therefore it was removed from the model. The reproductive variables considered were: total number of follicles, and the number of follicles divided into five classes of size (3 mm; 4-6 mm; 7-10 mm; 11-13 mm; and > 13 mm), as well as, viable and recovered oocytes, and embryos produced. Chi-Square test ($X^2$) was used to evaluate differences in retrieved oocytes, cleaved oocytes and blastocysts rates. The results are presented as the mean ± standard error of the mean (SEM). The value of $P \leq 0.05$ was considered to be significant and $P \leq 0.10$ was considered as a trend.

RESULTS AND DISCUSSION

In this study, the CAT injections interval was based on the best results obtained in another experiment from our laboratory (Pereira et al. 2013a) using postpartum dairy cows. Besides that, we considered the average follicular wave development interval (5 to 7 days) (Adams et al. 1992). Therefore, this experiment was designed to evaluate CAT supplementation since the beginning of follicular wave emergence, oocytes development until FA. Butaphosphan and cyanocobalamin administration promoted a tendency ($P = 0.10$) of increase in the number of total follicles collected in CAT group (Table 1), which is possible explained by the increase ($P = 0.10$) in follicles collected at 42 days (Fig. 2A). Those tendencies of increase in number of follicles aspirated were possible driven by
positive metabolic environment that cianocobalamin and phosphorus can create (Pereira et al. 2013a), which promoted an increase in follicular development. Considering the other compound in the CAT group (vitamin B12), it plays a role in cellular metabolism and is essential to DNA, RNA and proteins sintesys, as well as to energy production. Also, its action on the energy status in dairy cows is verified by the increased availability of glucose and reduction concentration of BHBA (Graulet et al. 2007 and Preynat et al. 2009). Ovarian studies with intramuscular administration of folic acid and B12 vitamin observed increased expression of granulosa cell genes in postpartum dairy cows (Preynat et al. 2010) and faster follicular growth (Gagnon et al. 2015). Furthermore, B12 deficiency in female mammals during pre-conception and pregnancy periods can be a negative factor to fertility, folliculogenisys and embryo development (Laanpere et al. 2010).

Likewise, there was a tendency of interaction ($P = 0.07$) between treatment and lactation on the number of 3 mm follicles, as well as, on the number of follicles 4-6 mm collected (Table 1). These suggest a potential stimulus of butaphosphan and cyanocobalamin administration (CAT group) on initial growing follicles, particularly in high energy demanding cows. Furthermore, CAT group have an increase ($P = 0.02$) in follicles 4-6 mm throughout the three trials (Fig. 2B). Thus, it can be postulated a residual effect of multiple treatments of butaphosphan and cyanocobalamin on follicle development.In addition to the improved energy mechanisms mediated by cyanocobalamin, CAT group also provided phosphorus to animals. Phosphorus is essential to transfer and use of energy, adenosine triphosphate (ATP) and adenosine monophosphate (AMP) (Cunningham 2002). A study with 0.35% and 0.47% phosphorus supplementation in the diet of dairy cows showed an increase in the average number of follicles (6 to 9 mm) in supplemented group with 0.47% phosphorous (1.2 vs 1 9 follicles, respectively) (Tallam et al. 2005).There was no treatment influence ($P > 0.05$) on the other groups of follicles size (7-10; 11-13 and greater than 13 mm).
According Seneda et al. (2001), there is a strong positive correlation between the number of follicles aspirated and the number of oocytes recovered. However, in our study the increase in the number of follicles aspirated with diameters of 4-6 mm in CAT group and as a result, the tendency of increase in the number of total follicles, did not result in any improvement ($P>0.05$) in the total number of oocytes retrieved, recovery rate, oocytes viable, although it may be influenced the oocyte quality by the increase in oocyte grade II number, that had increase throughout the trials (Figure 2C). However, even with this potential improvement in quality, it did not affect the results of total blastocysts, and blastocyst grade 1 (Table I). Moreover, we could not observe any difference ($P>0.05$) in retrieved rate (59.0% CONT vs. 61.0% CAT), cleavage rate (30.9 CONT vs. 36.7 CAT) and blastocyst rate (19.8 CONT vs. 15.6 CAT), this results were similar to those produced by Holstein cows (Bridges et al. 2010).

Considering the quality of aspirated oocytes, there was a greatest number of oocytes grade 2 collected from the CAT group (Fig. 2C), which is important because this grade is related to further capacity to become blastocyst stage, possibly due to greater cytoplasmatic maturity (Blondin 1995). Therefore, we believe that the improvement of oocyte quality has also been mediated by a possible increase in glucose levels and a reduction in the BHBA concentration (Nielsen and Ingvartsen 2004). Consequently, endocrine and metabolic signals that regulates follicle growth (Gutierrez 1997) also have influence on oocyte development not only through changes in circulating growth hormones, but also by the presence of these growth factors in the follicular fluid, which promote interactions between the granulosa cells and the oocyte (Webb 1999). Scaramuzzi et al. (2011) suggest that the system glucose-insulin not only have specific health functions maintaining cell integrity, but may also have specific functions affecting cells of the granulosa and theca. The possible environment created by butaphosphan and
cyanocobalamin would be a positive signal preventing the toxic effects of NEFA and BHBA on maturation of oocytes (Leroy et al. 2006).

CONCLUSIONS

Successive butaphosphan and cyanocobalamin administration can potentially increase in vitro embryo production and quality in Jersey cows during ovum pick up protocol once it has positive effects on early follicle development, particularly pronounced in lactating cows. Nevertheless, further studies are required to completely understand the mechanisms of action of butaphosphan and cyanocobalamin on oocytes and in vitro embryo production.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1: Schematic representation of experiment timeline. Treatments (Treat.) either CAT (4500 mg butaphosphan and 2.25 mg cyanocobalamin, 45 mL) or Control (0.9% NaCl, 45 mL). FA = follicular aspiration. Follicles aspired at day 0 were not considered in the analyses.
Fig 2. Effect of butaphosphan 4500 mg and 2.25 mg of cyanocobalamin (CAT group, n = 17) or placebo (0.9% NaCl) (CONT group, n = 19) during experimental period Total on total follicles collected (A); Follicles 4-6 mm (B); and oocytes grade II (C).
Table 1. Reproductive evaluations of in vitro embryo production during ovum pick up protocol in Jersey cows treated with butaphosphan 4500 mg and 2.25 mg of cyanocobalamin (CAT group, n = 17) or placebo (0.9% NaCl) (CONT group, n = 19)

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<th>CONT</th>
<th>CAT</th>
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<td>non-lactating</td>
<td>lactating</td>
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<tr>
<td>Follicles aspirated total</td>
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<td>17.27</td>
<td>22.68</td>
</tr>
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<td>Follicles 3 mm</td>
<td>11.18</td>
<td>13.22</td>
<td>17.94</td>
</tr>
<tr>
<td>Follicles 4-6mm</td>
<td>3.23</td>
<td>2.25</td>
<td>3.94</td>
</tr>
<tr>
<td>Follicles 7-10mm</td>
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<td>0.67</td>
<td>0.69</td>
</tr>
<tr>
<td>Follicles 11-13mm</td>
<td>0.50</td>
<td>0.56</td>
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<tr>
<td>Follicles &gt;13 mm</td>
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<td>Grade I oocytes</td>
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<td>Viable oocytes</td>
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**Note:** Means within a column not sharing a lowercased italic letter differ significantly at the *P* < 0.05 level.

*a* Standard error of mean.

*b* Category was considered the effects of lactating or non-lactating cows.