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Follicular dynamics and production of oocytes in young Nellore heifers with energetic supplementation

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ABSTRACT - To verify the effects of energy supplementation and fat on follicular dynamics, metabolic profile and the production of oocytes, 15 young heifers (*Bos taurus indicus*) with an average age of 14 months and with an initial weight of 256.35 kg were assigned to two treatments according to weight and follicular population as evaluated by ultrasonography: in T1 (1.0 × M), animals received 100% of the energy requirements for maintenance; in T2 (1.7 × M), animals received 170% of the energy requirements for maintenance, achieved by the addition of 200 g of Megalac®. After a period of adaptation to the diet, the treatments, blood collection and follicular aspirations were started using a randomized design. The dry matter intake and weight gain were lower in the 1.0 × M group than in the 1.7 × M group. No differences were found in the plasma progesterone concentrations, albumin, glucose, urea or gonadotropin (FSH and LH) levels between the groups. The mean concentrations of cholesterol were higher in the 1.7 × M group. The total number of small (<4 mm) and medium follicles (4-8 mm) was not altered by the treatments, but the number of small follicles increased on days 1 and 2 of the estrous cycle, with higher values found in the 1.7 × M group. The average of the oocytes also increased (9.50±2.1 and 12.5±4.4 for the 1.0 × M and 1.7 × M groups, respectively). The rapid increase in the amount of energy offered in the diet changes the amount of follicles and oocytes available for follicular aspiration (OPU) in young heifers without changing their metabolic profile.

Key Words: metabolic profile, nutrition and reproduction, OPU

Introduction

Bos indicus cattle breeds have made significant contributions to beef and dairy cattle in many tropical regions of the planet. Reproductive performance data, such as the pregnancy, weaning and survival rates of calves, have shown variations in the performance of *Bos indicus* cattle. However, these animals appear to be superior in performance to the *Bos taurus* breeds when raised in tropical or subtropical environments, where stressors such as high temperatures, humidity, ectoparasites and low-quality forage are greater. Although *Bos indicus* cattle constitute a majority of the national herd, much of the data on the reproductive physiology, hormone levels, and certain aspects of reproduction of *Bos indicus* cattle are not clearly elucidated (Bo et al., 2003). Nutrition and its relation to the type and amount of supplement given produce major effects on reproductive processes in cattle. While the effects on follicular development resulting from changes in nutrition for prolonged periods, particularly dietary restriction, have been studied extensively in animals, the effects of feed supplementation for short periods are less understood. It is known that feeding 200% of the maintenance requirements for three weeks increases the population of smaller follicles (<4 mm) in *Bos taurus* (Gutierrez et al., 1997). Other studies have demonstrated that steroidogenesis by granulosa cells, the development of pre-ovulatory follicles and oocyte maturation are also positively changed as a result of the supplementation of heifers with 200% of the maintenance requirements in diets with high available energy (Armstrong et al., 2001; 2002). The effects of the supply of fat on the ovarian follicular population have been observed for dairy and beef cattle, in all stages of lactation. Some authors observed an increase in the follicular population as a result of fat supplementation in dairy cows (Butler et al., 1996). An increase in the number of viable follicles is currently required to obtain the best results with respect to assisted reproduction in cattle. Obtaining viable oocytes *in vivo* has been a valuable tool to enhance the production of animals of great genetic potential by the use of *in vitro* fertilization (IVF).

The objectives of this study were to evaluate the effects of increased energy supply on the follicular dynamics, metabolic profile and production of oocytes in young Nellore heifers.

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Material and Methods

Fifteen heifers, averaging 14 months of age and with an average weight of 256.35±18.95 kg and a body score of 4.2±0.3 (scale 1-9), were divided into two groups according to weight and follicular population as evaluated by ultrasonography: T1 - 1.0 × M (n = 8): young heifers receiving 100% of maintenance energy requirements, calculated according to recommendations of the NRC (1996), and T2 - 1.7 × M (n = 7): young heifers receiving 170% of maintenance energy requirements, with addition of 200 g of protected fat/animal/day (Megalac®).

The adaptation period for the diets was 14 days, and the animals were fed *Brachiaria decumbens* hay and a concentrate supplement with 180 g/kg crude protein (CP) and 650 g/kg total digestible nutrients (TDN) to meet 100% of the maintenance requirements for energy and protein. The supply of feed was provided twice per day, at 8:00 and 16:00, on the premises of EMBRAPA-CNPGC in Campo Grande, Mato Grosso do Sul, Brazil.

Before the delivery of the supplement, the leftovers from the previous meal were evaluated and weighed to measure individual consumption. After the adaptation period, the animals were supplemented with the experimental diets (Table 1) until the end of the experiment.

After fifteen days of feeding the experimental diets, the animals were timed to present ovulation through the use of an intravaginal device containing 1.0 g of progesterone (Cronipress® - Biogenesis, Arg) and the application of 2 mg of estradiol benzoate (Estrogin® - Farmavet, Br) at the time of the device insertion. The intravaginal device was maintained for eight days, and two days before its removal, the animals were administered a dose of 150 mg of D-cloprostenol (Croniben® - Biogenesis - Arg). One day after the removal of the device, the animals received 100 mg buserelin acetate (Conceptal® - Intervet, Br) to induce ovulation, and visual identification of the estrus and ovarian ultrasound were performed. After viewing the preovulatory follicles using an ultrasound device equipped with a 7.5 MHz transrectal transducer (Falcon 100, Pie Medical®, Netherlands), the daily follicular development was monitored until the eighth day of the estrous cycle for the identification and aspiration of the dominant follicle, as described by Figueiredo et al. (1997). The follicles were identified, measured and drawn in diagrams, and the number of follicles at wave emergence, the diameter of the dominant and pre-ovulatory follicles and the diameter of the corpus luteum were evaluated. To obtain blood plasma for metabolic and hormonal measurements, samples of blood were collected via the coccygeal vein into tubes containing heparin on days one and two after ovulation (two samples, collected at 7:00 and 18:00), and from the third day to the eighth day of estrus (collected at 7:00, before the supplementation). The tubes were kept on ice until centrifugation at 1500 *g* for 10 minutes. After centrifugation, the samples were placed in plastic tubes and stored at -20 °C until determination of the hormone concentrations.

Table 1 - Composition of experimental diets

Component	Treatment	
	1.0 × M	1.7 × M
Hay (kg/day)	3.40	2.20
Corn (kg/day)	0.60	3.00
Soybean meal (kg/day)	0.20	0.50
Mineral supplement (6% P)	0.02	0.02
Urea (kg/day)	0.09	0.01
Fat (kg/day)	-	0.2
Total digestible nutrients (g/kg)	532.00	735.0
Crude protein (g/kg)	116.00	116.00

The plasma glucose concentration was determined using the diagnostic Labtest® kit, utilizing the enzyme glucose oxidase method described by Tietz (1982) and reading the results by colorimetry (500 nm). The plasma total cholesterol was determined using the diagnostic kits Labtest®, utilizing the cholesterol oxidase enzyme method, and reading of the results by colorimetry (500 nm). The plasma concentration of urea was determined using the diagnostic kits Labtest®, utilizing the enzyme urease method, and reading of the results by colorimetry (600 nm). The plasma concentration of albumin was determined using the diagnostic kits Labtest®, utilizing the reaction with bromocresol green and buffered method, and reading of the results by colorimetry (630 nm).

The dosages of follicle stimulating hormone (FSH), luteinizing hormone (LH) and progesterone (P4) were performed at the Laboratory of Animal Endocrinology, Faculdade de Medicina Veterinária - UNESP Araçatuba. A commercial kit was used for the P4 assay (Coat-a-Count Diagnostic Products Corporation, CA, USA). The kit sensitivity was 0.01 ng/mL, and the intra-assay coefficient was 2.4%.

The dosage of FSH was determined using a validated radioimmunoassay for cattle, bovine FSH (USDA-bFSH) for iodination and standard reference and NIDDK-OFSH as anti-first antibody, previously described by Bolt & Rollins (1983). The sensitivity was 0.009 ng/mL, and the intra-assay coefficient was 4.93%. The assays for bovine LH were determined as described by Bolt & Rollins (1983) and Bolt et al. (1990), using radioimmunoassay.

After eight days of follicular growth, the dominant follicles and all follicles larger than 5 mm were aspirated for synchronization of a new follicular wave using a 18 g needle attached to a convex transvaginal probe with the aid of a suction pump vacuum (70 mm/hg), which produced a flow of 15 mL/minute; 150 g of D-cloprostenol (Croniben® - Arg Biogenesis) were applied. Three days after the aspiration of the dominant follicle, all the follicles in the ovaries were visualized and aspirated. Subsequently, the follicular fluid was inspected in a petri dish under a stereomicroscope to recover the cumulus-oocyte complexes (COCs). Statistical analysis was performed employing software SAS (Statistical Analysis System, version 9.2), through command PROC GLM. The data on weight, feed intake, hormone concentrations and follicular dynamics (number and size of follicles) were evaluated using analysis of variance, adopting $\alpha = 0.05$ in a completely randomized design.

Results and Discussion

In the beginning of the treatments, the weight of the heifers was similar between groups, with an average of 258.7±7.1 kg and 254.0±9.0 kg for 1.0 × M and 1.7 × M, respectively. At the end of the experiment, the total weight gain for the animals in group 1.7 × M (18.6±8.4 kg) was higher ($P<0.05$) than group 1.0 × M (2.1±5.2), which was expected due to the lower feed supply in the latter. The feed intake per day was lower ($P<0.05$) for the animals of group 1.0 × M (4.5±0.8/kg DM/day) compared with group 1.7 × M (5.4±0.3/kg DM/day).

Of the 15 synchronized heifers, three heifers from group 1.0 × M and two heifers from group 1.7 × M showed no ovulation after removal of the intravaginal devices, so the data on the follicular dynamics of these animals were removed from the statistical analysis.

The concentrations of FSH, similar to those observed in other studies (Adams et al., 1994; Gong et al., 1995), varied according to the day of the cycle, although no difference between days resulted from the nutritional treatment in the total concentration of this hormone ($P>0.05$). Similarly, LH showed no difference between the treatments ($P>0.05$) (Table 2).

The P4 values ranged from 0.2 ng/dL on the first day of the cycle after ovulation to 3.8 ng/dL on day 8. Differences were found in the daily concentration of this hormone on days 6 and 7 of the synchronized estrous cycle, with higher values for group 1.7 × M, but the total amounts of P4 did not differ between the groups ($P>0.05$) (Table 2), as observed by Guardiero et al. (2010).

Because circulating cholesterol is the primary substrate for the synthesis of progesterone in mammals, added fat may permit increased synthesis of hormones, causing a rise in plasma progesterone levels. This effect was observed for milk cows and beef cows. The concentration of progesterone in the follicular fluid and luteal tissue can increase, as shown in some (Ruas et al., 2000; Ryan et al., 1992), but not all studies (Lammoglia et al., 1997), when adding fat to the diet. Grummer & Carroll (1991) reviewed several studies in which lipids were added to the diet of dairy cows. These authors found that the concentrations of plasma cholesterol were consistently higher in the diets supplemented with fat compared with the control diet. Similarly, in the present study, higher values of plasma cholesterol in group 1.7 × M were found, compared with the control group ($P<0.05$), but no direct relationship with plasma P4 was found (Table 2).

No differences were found between the diets in the total concentration of glucose based on the day of the estrous cycle ($P>0.05$), a fact previously reported by some authors (Gutierrez et al., 1997; Ruas et al., 2000). Ruminants utilize volatile fatty acids (VFA) as a main source of energy, and changes between states of hunger or overfeeding are rapidly regulated by insulin, which promotes an almost- constant blood glucose concentration.

The urea concentrations are indicators of protein intake and, as expected, have a positive correlation with the diet. The maximum and minimum values of plasma urea were found to be equal to 8.06 and 12.02 mg/dL of plasma urea nitrogen (PUN), respectively; these levels are considered adequate (Elrod & Butler, 1993; Barton et al., 1996; Butler et al., 1996). These authors found PUN concentrations up to 21 mg/dL and suggested that the plasma urea nitrogen can impair fertility when concentrations exceed 18 mg/dL. Such values were not achieved in this study. Armstrong et al. (2001) showed

Table 2 - Average daily gain (ADG), feed intake, metabolite blood concentrations (albumin, cholesterol, glucose and urea) and hormones (FSH, LH, P4) according to the treatment

	Treatment		P value
	1.0 × M	1.7 × M	
ADG (kg/animal/day)	0.1±0.1	0.7±0.1	0.003
Feed intake DM(kg/day)	4.5±0.8	5.6±0.3	0.043
FSH (ng/mL)	0.8±0.1	0.6±0.1	0.466
LH (ng/mL)	1.4±0.3	1.3±0.5	0.836
Progesterone (ng/mL)	1.6±0.7	1.8±0.7	0.605
Cholesterol (mg/dL)	101.4±35.8	129.7±39.7	0.001
Glucose (mg/dL)	65.6±21.4	74.6±30.8	0.153
Urea (mg/dL)	18.1±6.8	21.9±9.2	0.169
Albumin (g/dL)	2.0±0.5	2.1±0.4	0.177

1.0×M - heifers receiving 100% of the energy requirements for maintenance.
1.7×M - heifers receiving 170% of the energy requirements for maintenance, with adition of 200 g of protected fat/animal/day (Megalac®).

that the quality of oocytes from small follicles was negatively correlated with plasma urea concentrations, which most certainly did not occur in this experiment. No differences were found in the total plasma concentration of albumin between the groups (P>0.05), suggesting a good adaptation of the diets compared with the protein. The evaluation of plasma albumin is a way of monitoring the ingestion of feed protein.

The treatments did not affect the total number of follicles smaller than 4 mm that were measured by ultrasound, as reported by Bastos et al. (2007). However, an increase (P<0.05) was observed in the number of follicles in the 1.7 × M group versus the 1.0 × M group from day 1 to day 2 of the estrous cycle (Figure 1). After 3 days, however, this effect was no longer observed (P<0.05). The increased number of small follicles in the first two days of the estrous cycle occurred in the treatment with increased energy and fat without being accompanied by increased concentrations of FSH in the 1.7 × M group. Other studies have shown that increasing the number of follicles with no change in FSH (Beam & Butler, 1997; Gutierrez et al., 1997) as a result of energy supplementation or fat can positively influence female reproduction by changing the ovarian follicle and CL function through improved energy status and an increase in the precursors of the synthesis of reproductive hormones such as steroids and prostaglandins (Mattos et al., 2000; 2002). Plant oils are rich in oleic acid C18:1 and linoleic acid C18:2, which increase gluconeogenesis by increasing the production of propionate in the rumen (Chalupa et al., 1986). Because of this effect, the concentration of circulating insulin can also increase (Sartori & Mollo, 2007). Additionally, IGF-1 can increase as a result of the increased supplementation and produce follicle-enhancing effects (Zulu et al., 2002), which was not evaluated in this study. The same effect is expected in the size of the dominant and pre-ovulatory follicles (Table 3), which increased (P<0.05) as a result of supplementation in group 1.7 × M.

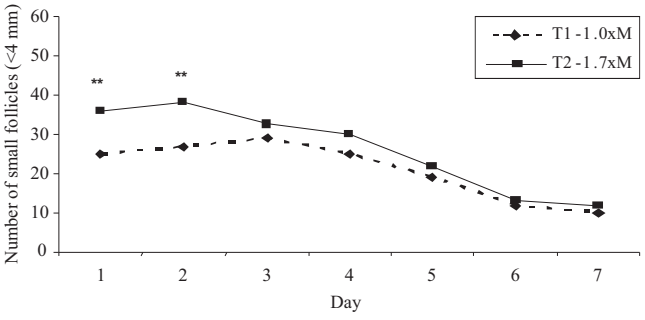

Figure 1 - Number of small follicles (<4 mm), according to the treatment type and day of the estrous cycle.

The average number of follicles with a diameter between 5 and 8 mm and large diameter, 8 mm, varied only with the day of the cycle and was not influenced by the treatments (P>0.05; Table 3). These data indicate that the increased intake of energy affects the recruitment of smaller follicles but does not alter the dominant follicle in *Bos indicus* animals, as previously observed by Gutierrez et al. (1997) and Armstrong et al. (2002) in *Bos taurus*. Some studies have demonstrated the increased number of smaller follicles in *Bos taurus* in response to increasing the amount of energy consumed or the application of GH (Gong et al., 1991), but few data are shown in *Bos indicus*. In a previous study, Maurasse et al. (1985) demonstrated that changes in the nutritional plan produced changes in the appearance of follicles of various sizes and a sharp increase of the follicles in cattle, as partially observed in this experiment. The increase in the number of smaller follicles may reflect a greater selection of structures available for further development. A larger number of follicles may indicate a change in the process of follicular selection and an increased selection of structures available for ovulation (OPU), which is of interest for application in assisted reproduction programs using *in vitro* production of embryos. This fact was confirmed by the increased production of oocytes in the group supplemented with increased energy (Table 3).

The diameter of the preovulatory follicle in group 1.7 × M was 1.21 cm, and the follicles were larger than those in group 1.0 × M (P<0.05). The size of the dominant follicle on the aspiration on day 8 of the cycle was affected by the treatment (P<0.05), showing a lower value in group 1.0 × M. The CL diameter on day 8 of the cycle did not differ between treatments (P>0.05), as observed by Guardieiro et al. (2010).

All the animals were aspirated again for a total of fifteen sessions of suction to the animals of groups 1.0 × M and 1.7 × M, resulting in 165 oocytes, which accounts for, on average, 11.0 oocytes per heifer per session of aspiration.

Table 3 - Effect of treatments on follicular and luteal characteristics and results of ovum pick-up

	Treatment		P value
	1.0 × M	1.7 × M	
Dominant follicle (cm)	0.9±0.1	1.1± 0.2	0.019
Pre-ovulatory follicle (cm)	0.9±0.2	1.2±0.2	0.015
Corpus luteum (cm)	1.2±0.4	1.5±0.5	0.221
Small follicles (<4 mm) (n)	23.4±12.4	27.5±16.1	0.318
Medium follicles (5-8 mm)	2.3±1.4	1.9±1.0	0.244
Aspirated follicles (n)	13.2±6.3	19.5±5.4	0.041
Recovered oocytes (n)	9.5±2.1	12.5±3.6	0.042
Oocytes (grade 1-3)	7.2±1.1	9.3±1.7	0.018
Oocytes (denuded/atretic)	2.3±0.5	3.2±1.8	0.133

SEM - standard error of the mean.

Regarding the effects of feed supplementation on oocyte quality, the literature data are controversial. Many of the studies indicate that increasing the amount of energy in diets for beef and dairy cows and heifers produces deleterious effects on oocyte competence and embryo quality (Armstrong et al., 2001; McCaffery et al., 2000; Nolan et al., 1998; Freret et al., 2006); other studies have shown beneficial effects of supplementation and fat (Fouladi-Nashta et al., 2007). However, most studies disregard the effects of the initial body condition of the animals or their overconditioning as for the response of the yield and quality of oocytes and embryos. In this regard, Adamiaki et al. (2005) reported that the increased energy is beneficial to oocyte quality only in animals of moderade body condition score (BCS) and is harmful for animals with a high BCS. In this study, the heifers were BCS-moderate (4.2 on a scale of 1-9) at the beginning of the treatment, which had no negative effect on oocyte quality because, despite the number of grade 1-3 oocytes being higher in group 1.7 × M ($P<0.05$), the proportion of the total number of oocytes did not differ between the groups ($P>0.05$).

Conclusions

A diet with greater amounts of energy and with the inclusion of fat (200 g/animal/day) in *Bos indicus* cattle with moderate body condition score increases the number of follicles at the beginning of the follicular wave and the number of oocytes available after follicular aspiration without altering the hormone concentrations or blood metabolites, with the exception of cholesterol.

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