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Oocyte aspiration and *in vitro* embryo production in Jersey cows with selenium-supplemented diet

[*Aspiração de oócitos e produção de embriões in vitro de vacas Jersey suplementadas com selênio na dieta*]

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ABSTRACT

The effects of selenium (Se) in Jersey cows' diet on the aspiration of oocytes and production of embryos in vitro were studied. Groups with five Jersey cows received 3.2mg or 9.6mg Se daily, provided in the feed concentrate. Six follicular aspirations were carried out every 15 days, using only the last 5. The oocytes were classified, and standard procedures were carried out for maturation, fertilization and cultivation. The total number of oocytes (35.11±2.65 *vs* 23.10±2.16) and degree 1 oocytes (11.61±2.65 *vs* 4.75±0.97) were higher in the group that received 9.6mg Se and the quantity of naked oocytes (3.23±0.87 *vs* 6.22±1.18) was lower in this group. The aspirated oocytes from the cows treated with 9.6mg Se/day resulted in higher (P<0.05) embryo production 21.98±2.37 *vs* 13.12±1.59). No difference was observed in serum Se concentration between the two groups. It is recommended that the daily diet be supplemented with 100g mineral salt containing 9.6mg Se, since this rate rendered a larger production of oocytes, higher quantity of degree 1 oocytes and greater production of embryos in the process of in vitro fertilization.

Keywords: cattle dairy, antioxidant, glutathione peroxidase, *in vitro* fertilization, oocyte quality

RESUMO

Avaliou-se o efeito do selênio (Se) adicional na dieta de vacas Jersey na aspiração de oócitos e produção de embriões in vitro. *Dez vacas Jerseys receberam 3,2mg de Se por dia ou 9,6mg, vinculado ao concentrado. Realizaram-se seis aspirações foliculares, com intervalo médio de 15 dias, aproveitando as cinco últimas. Os oócitos foram classificados e realizaram-se os procedimentos padrões de maturação, fertilização e cultivo* in vitro. *O total de oócitos, 35,11±2,65* vs *23,10±2,16, e oócitos de qualidade 1, 11,61±1,58* vs *4,75±0,97, foram mais elevados no grupo que recebeu 9,6mg de Se e a quantidade de oócitos desnudos mais baixa, 3,21±0,87* vs *6,22±1,18. A produção de embriões foi maior no grupo tratado com 9,6mg de Se/vaca/dia, 21,98±2,37* vs *13,12±1,59. Não se observou diferença na concentração de Se no soro entre os dois grupos. Conclui-se que é possível recomendar o fornecimento de 100g de sal mineral, contendo 9,6mg de Se, adicionado à dieta, pois resultou em maior produção de oócitos, maior quantidade de oócitos de grau 1 e maior produção de embriões no processo de fecundação in vitro.*

Palavras-chave: bovino de leite, antioxidante, fertilização in vitro, *glutatione peroxidase, qualidade de oócitos*

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INTRODUCTION

Selenium (Se) is one of the microminerals which are important for the different functions of animals, benefiting production (McDowell, 1992; Boland, 2003) and reproductive activity (Boland, 2003), and it should be in the diets of domestic animals at concentrations of 0.1 to 0.3ppm (National..., 1983). However, Underwood and Suttle (1999) pointed out that it was difficult to determine the tolerance of the animals to high levels of Se, because there are many factors that interfere with the chemical characteristics of the element to be ingested, the time of ingestion, the criteria to determine tolerance, the diet and the genotype of the animals. Se is a micromineral that is part of the enzyme glutathione peroxidase (GSH-Px), and it inactivates free radicals derived from oxygen as a consequence of metabolism (Ceballos *et al*.,1999; Luberda, 2005; Livingston *et al*., 2009). Wittwer *et al*. (2002) reported on the positive effects of Se in forage with GSH-Px in blood (0.74 to 0.97) and Ceballos *et al*. (1998; 1999) of the Se in blood with the level of GSH-Px.

GSH-Px protects cells from the damage caused by reactive oxygen species and by the reduction of hydrogen peroxides (Sandholm, 1980; Barbosa *et al*., 2005; Livingston *et al*., 2009). The authors also stated that GSH-Px favors the synthesis of hormones derived from arachidonic acid, the metabolism of foreign compounds in the animal and the transport of some amino acids in the kidneys.

Free radicals cause damage to the cell membrane, reducing the quality of the gametes (Luberda, 2005; Livingston *et al*., 2009). Ortiz *et al*. (2011), in an evaluation of human semen, noted that the antioxidant action of methionine protects the sperm cell membrane from free radicals, namely reactive oxygen species (ROS), and facilitates sperm penetration of the egg. Oocytes of good quality, with good morphologic and development characteristics are the first requisite for successful *in vitro* production of embryos (Seneda *et al*., 2001; Paschoal and Gradela, 2007).

There are 10 identified selenoproteins, including GSH-Px, which still hase unclear funcions (Arthur *et al*., 1990). Among the selonoproteins, diodinase converts thyroxine (T_4) into triidothyronine (T_3). In the case of Se deficiency, it can elevate TSH, and as a consequence, a deficiency of T_4 and T_3 can result (Arthur *et al*., 1993; OMS, 1998), which could harm animal development (Oblitas *et al*., 2000).

Hurley and Doane (1989) affirm that Se can be associated with the production of prostaglandins and that there is an accumulation of Se in placentomes, ovaries, and pituitary and adrenal glands, suggesting that there are specific requirements in these tissues. It is also pointed out that it can reduce the retention of the placenta and increase reproductive performance, since there are indications that GSH-Px protects the oocyte membrane against oxidative damage (Livingston *et al*., 2009), which in part also coincides with the observations of Luberda (2005). According to Eppig (1996), Krisher and Bavister (1998) and Livingston *et al*. (2009), GSH-Px has proved to be important in the maturation process of oocytes, involving the synthesis of biochemical components, phosphorylation of proteins and the activation of specific metabolic pathways.

Despite the advances in the *in vitro* production of embryos, it is still necessary to better understand the competence of the gametes (Paschoal and Gradela, 2007). Thus, the aim of this study was to determine the influence of dietary Se supplementation on the *in vitro* production of oocytes and embryos, to contribute to the production of competent gametes

MATERIAL AND METHODS

This experiment was carried out in agreement with the Ethical Principles in Animal Research approved by the Universidade Estadual do Maringá's Ethical Committee for Animal Research.

Ten primiparous Jersey cows, mean age of 3 years, with mean initial weight of 240.6kg, from the same genetic group, were randomly divided into control (GC) and treatment (GT) groups. The animals received 10kg natural matter (MN)/cow/day of whole corn plant silage (2.68kg dry matter-MS), 2.5kg (2.25kg DM)/cow/day of concentrate (90.0% dry matter; 16% crude protein; 13.8% rumen degradable protein; 9.8% crude fiber; 2% ether extract; 30% fiber in neutral detergent; 3.9% nonprotein

nitrogen; 70% total digestible nutrients; 10% mineral matter; 1% calcium and 0.45% phosphorus), 100g of mineral salt, especially prepared for this experiment (Potensal®)/cow/day and had access to stargrass pasture (*Cynodon spp*.) with low forage availability. The level of Se in the grass *Cynodon* ssp. was 0.51mg/kg MS, in the silage it was 0.25mg/kg MS and in the concentrate it was 0.35mg/kg MS.

The difference between the two experimental groups was in the concentration of selenium (sodium selenite) contained in the mineral salt, in which GC received the salt containing 32mg of Se/kg (3.2mg of Se/cow/day) and GT, 96mg of Se/kg (9.6mg Se/cow/day). Day zero (D0) was when the animals began to receive the selenium treatments, at which time the animals were wormed with 1% ivermectin (Supramec®, Schering-Plough).

Analyses of the diet with regard to dry matter, crude protein, ether extract, non-protein nitrogen and calcium were carried out in accordance with AOAC (Official…, 1990). Neutral detergent fiber and acid detergent fiber were determined according to Van SOEST *et al*. (1991) and phosphorus according to Fiske and Subbarow (1925).

The animals were weighed on day zero (D0; beginning) and on day 119 (D119; end), as well as four intermediate times, according to the farm's routine. Weighings were done in the morning before the animals were fed. After 20 days of treatment, the first transvaginal ultrasound-guided follicular aspiration (FA) was performed. This first FA was disregarded, and then five more aspirations were carried out, with a mean interval of 15 days. The follicular aspirations were performed utilizing an Aloka SSD-500 ultrasound apparatus equipped with 5/7.5 MHz intravaginal sectorial probe and needle guide for follicular aspiration, connected to a vacuum pump, with 40 to 50mmHg pressure. Follicles of 3 to 9mm were aspirated (Perry, 2007).

The oocytes collected were transported to BIOTEC, in a warm water bath at 36°C, in maturation medium (MIV-T Nutricell®). At BIOTEC the oocytes were classified according to Lonergan (1992) in degrees of I, II, III, expanded, naked and atresic, respectively, where I, II, III, naked and expanded oocytes were considered viable and degenerated or atretic as nonviable.

The viable oocytes were washed in maturation medium (MIV-T Nutricell®) and transferred to a 35x15mm Petri dish, containing seven 90-µL drops of maturation medium, in which each drop contained oocytes aspirated from one of the cows and were covered with mineral oil (Sigma®); the oocytes were tested for embryo culture and then incubated for 22 to 24h at 38ºC, with 5% CO_2 in air and saturated humidity. After maturation, the oocytes were washed and transferred to a fertilization plate, containing fertilization medium (Nutricell®), to which heparin (Sigma Chemical®) and PHE penicillin, hypotaurine and epinephrine (PHE) were added.

In in vitro fertilization (FIV), semen from a Jersey bull previously tested in FIV was utilized. The sperm were selected through PERCOLL gradient medium, in a 15-mL conical tube with two gradients, namely 45% and 90%. The semen was thawed in a warm water bath at 36ºC for one minute and placed in a tube containing the two Percoll gradients and centrifuged at 600g for 10min. After centrifugation, the pellet was removed and resuspended to a concentration of $2x10^6$sperm/mL. For FIV, 10µL of this suspension per drop were used.

After the addition of the semen, the FIV plate was incubated at 38ºC, in an atmosphere of 5% CO_2 in the air and saturated humidity for 18 to 22h. After completing the FIV, the structures were again washed and transferred to a culture plate containing culture medium (SOF Nutricell®) and incubated at 38ºC, in an atmosphere of 5% CO_2 in the air and saturated humidity.

The first evaluation was performed 48 h after fertilization (day 2) and the second on day 7, and the embryos were classified as morula, initial blastocyst, blastocyst and expanded blastocyst (Leibfried and First, 1979).

Blood was drawn with an 18G needle from the jugular vein (10mL), on D0 and again on day D119, to determine selenium in serum. The samples of pasture grass, silage and concentrate

were also obtained on the same days as blood samples. Blood was centrifuged at 3000rpm (1600g) for 15min, and the serum was frozen at -18°C until time of analyses by Laboratorio Green Lab, Porto Alegre, RS, using an atomic absorption spectrophotometric method – method SM 3500 Se (Eaton *et al*., 2005).

A completely randomized design was used, with analysis of variance and test of means for comparison of treatments, utilizing the Bayesian model for discrete variables. By preserving the classic characteristics and presumptions, the discrete random variables (quality and type of oocytes and embryos) of the control group (Y_{iC}) and treatment group (Y_{jT}) showed a Poisson distribution with parameter θ, where:

$Y_{iC} \sim$ Poisson (θ_C), i =1, 2, ..., n_C;
$Y_{jT} \sim$ Poisson (θ_T), j = 1, 2, ..., n_T.

For θ_C and θ_T, the non-informative gamma distribution and the *a priori* distribution, with parameters "a" and "b", where:

$\theta_C \equiv \theta_T \sim$ Gamma (a, b), with $a = b = 10^{+3}$.

Maintaining the classic characteristics and presumptions, continuous random variables (serum selenium levels) of the control group (Y_{iC}) and treatment group (Y_{jT}) were considered to have normal distribution with mean μ and variance σ^2, where:

$Y_{iC} \sim$ Normal (μ_C, σ^2_C), i = 1, 2, ..., n_C;
$Y_{jT} \sim$ Normal (μ_T, σ^2_T), j = 1, 2, ..., n_T.

For each μ_C and μ_T, the non-informative normal distribution with parameters "c" and "d" was considered the *a priori* distribution, while each σ^2_C and σ^2_T, the non-informative gamma distribution with parameters "e" and "f", was considered the *a priori* distribution, where:

$\mu_C \equiv \mu_T \sim$ Normal (c, d), with the hyperparameters c = 0 and $d = 10^{+6}$;
$\sigma^2_C \equiv \sigma^2_T \sim$ Gamma (e, f), with the hyperparameters $e = f = 10^{+3}$.

The *a posteriori* distributions for the parameters of interest were obtained with the WinBUGS (Spieglhalter *et al*., 1996) software, utilized to obtain the sample estimates.

A total of 10,000 samples were generated, of which the first 1000 were discarded. The last samples were taken at intervals of 10, producing samples of 900 for each parameter of interest.

The monitoring of the convergence of the chains generated by the Gibbs sampler was done through graphic analysis and the utilization of the diagnostic tests of Geweke (1992) and of Heidelberger and Welch (1983), available in *Convergence Diagnosis and Output Analysis* (CODA; (Spiegelhalter *et al*., 1994), implemented in the R program (R Development ..., 2007).

The *a posteriori* summary of the estimates (mean, standard deviation and *a posteriori* median) of the parameters of interest and for the difference between the parameters of the groups compared, with their respective confidence intervals (percentiles $P_{2.5\%}$-$P_{97.5\%}$) at the 95% level are presented in the tables. For intervals where "zero" does not belong to 95% CI = [$P_{2,5\%}$; $P_{97,5\%}$], there is a significant difference between the groups analyzed.

RESULTS AND DISCUSSION

During the 119 days of the experiment, the animals showed a mean weight gain of 49.9±13.47kg, ranging between 23 and 69kg, with a mean final weight of 298kg for the animals in GC and 283 for those in GT. The animals received the same daily amounts of silage, concentrate and mineral salt during the experiment.

The results on the production of total oocytes and classification according to the quality are shown in Table 1. The total number of oocytes collected and the number of degree 1 and 2 structures was higher in females that received 9.6mg of Se daily ($P<0.05$) and the number of naked oocytes was lower ($P<0.05$). However, the total number of nonviable oocytes and of atretic oocytes was higher in animals treated with 9.6mg of Se daily ($P<0.05$).

Table 1. Estimated means and standard deviations for oocyte production per aspiration in Jersey cows (n=5) supplemented with selenium (Se), 3.2mg/day/animal or 9.6mg/day/animal, for 119 days

Parameter	3.2mg Se/day/animal	9.6mg Se/day/animal
Oocytes completely viable	23.10±2.16	35.11±2.65*
Oocytes, degree 1	4.75±0.97	11.61±1.58*
Oocytes, degree 2	4.57±0.98	7.17±1.32
Oocytes, degree 3	8.90±1.32	16.40±2.10*
Oocytes, naked	6.22±1.18*	3.24±0.87
Oocytes, expanded	2.33±0.89	3.69±1.16
Oocytes, nonviable	5.48±1.16	11.23±1.65*
Oocytes, atretic	4.49±1.08	10.68±1.62*
Oocytes, degenerated	1.32±0.66	0.99±0.71

* P<0.05, for difference between Se doses.

Through the observations made, it was noted that the animals showed weight gain compatible with the activity of supplying oocytes by means of ultrasound, because there was weight gain, indicating that the performance of the animals should not have interfered with the production of oocytes, as noted by Boland (2003), in which cows that lose weight in post-partum do not show good ovulogenesis. Therefore, the effects observed can be attributed to Se (Table 1). The animals that received 9.6mg of Se/day showed a better performance with regard to oocyte production, when compared to animals that received 3.2mg of Se/day (P<0.05), where the latter amount is considered normal for animal production (National..., 1983). This allows us to propose that reproductive activity requires higher levels of Se, which is in agreement with the observations of Wittwer *et al*. (2002), who found a positive correlation of 0.74 to 0.97 between Se level and glutatione peroxidase (GSH-Px) or of Ceballos *et al*. (1998;1999) between blood Se level and GSH-Px. Also, Livingston *et al*. (2009) affirmed that free radicals such as reactive oxygen species (ROS) hamper the reproductive activity of sheep *in vivo*, and that antioxidants such as glutathione (GS) or glutathione peroxidase (GPx) or synthetic pathway enzymes are present in the follicular fluid, cumulus oophorus of the oocytes and in secretions of the reproductive tract of ewes.

Some hypotheses can be pondered in favor of the treatment with a greater level of Se, such as the possibly constant elevated blood levels of Se (Tables 2 and 3) could have contributed to the improvement of the growth, maturation and competence of the oocytes, a factor pointed out by Pavlok *et al*. (1992), Golsden *et al*. (1997) and Perry (2007), who observed better competence among oocytes from follicles with more than 2mm in diameter, because those obtained from smaller follicles were not capable of surviving after cleavage into eight cells. Thus, the formation of more GSH-Px (Wittwer *et al*., 2002) could have favored folliculogenesis and the growth of larger and more homogeneous follicles than in those animals treated with 3.2mg Se, although no statistical difference in blood Se level was observed between the treatments. Langlands *et al*. (1996) observed that ewes submitted to a high level of Se increased urinary or fecal losses or resulted in unavailable forms. Another hypothesis that can be considered is the antioxidant property of Se, combating free radicals resulting from atoms or molecules that have one or more unpaired electrons (Stryer, 1996), which are highly toxic and deleterious to cells and tissues in general (Rodriguez *et al*., 2004; Luberda, 2005). Livingston *et al*. (2009), in studying sheep, pointed out the importance of antioxidants in protecting oocytes and embryos, *in vivo*.

Table 2. Estimated means and standard deviations for the production of embryos *in vitro*, per aspiration of Jersey cows (n=5) supplemented with selenium (Se) at 3.2mg/day/animal or 9.6mg/day/animal for 119 days

Parameter	3.2mg Se/day/animal	9.6mg Se/day/animal
Total embryos (D-7)	13.12±1.59	21.98±2.37*
Initial blastocyst (D-7)	2.78±0.77	4.49±1.05
Blastocyst (D-7)	3.01±0.89	5.27±1.16
Expanded blastocyst (D-7)	6.64±1.15	10.95±1.64*

*P<0.05, for difference between Se doses.

Table 3. Serum selenium (Se) concentration in the beginning and end of the experiment in Jersey cows (n=5/treatment) supplemented with selenium at 3.2mg/day/animal or 9.6mg/day/animal for 119 days (mg/L)

Parameter	3.2mg Se/day /animal	9.6mg Se/day/animal
Serum selenium (D-0)	0.057±0.017	0.064±0.017
Serum selenium (D-119)	0.087±0.025	0.102±0.026

* P>0.05, no difference.

Thus, when there is an imbalance between free radicals (oxidants) and antioxidants (defense agents) that favors the oxidants, there is a surge of oxidative stress, and the oxidants attack the cells, producing cell damage or the death of the cells (Rodriguez *et al*., 2004). Therefore, as the treatment of the animals with 9.6mg Se per day maintains higher blood Se levels (Tables 2 and 3) and since Se is involved in the synthesis of peroxidases such as GSH-Px, localized in the cytosol and in the mitochondria, such animals should have higher levels of GSH-Px (Wittwer *et al*., 2002) than those that received 3.2mg Se. This could have resulted in greater protection of the oocytes against oxidants, particularly hydroxyl radicals (OH). Tinggi (2003) also emphasized the essentiality of Se for animals and humans, since Se is a component of glutathione peroxidase, an antioxidant that protects cell membrane lipids from peroxidation.

Degree 1 oocytes were predominant, a factor that confers greater competency to the oocytes, leading to a greater production rate of embryos *in vitro*; according to Golsden *et al*. (1997) and Seneda *et al*. (2001), the layers that surround the oocytes are important for the nutrition and regulation of the metabolic activities of the oocytes. Besides, Livingston *et al*. (2009) observed that 100% of cumulus oophori of the oocytes obtained from ewes contained glutathione peroxidase.

The number of nonviable oocytes, especially degenerated and atretic, was higher (P<0.05) in the treatment with 9.6mg Se in relation to treatment with 3.2mg Se, which was expected because there was a greater total production of oocytes and greater production of degree 1.

In the nutritional process of animals that are oocyte donors or reserved for reproduction, an important aspect in ruminants, microminerals such as Se are essential, which are responsible for the synthesis of enzymes, forming metalloproteins required for life, and where their deficiencies cause severe metabolic disturbance and severe pathological consequences (McDowell, 1992; Underwood and Suttle, 1999; Fisher Jr., 2000).

The level of Se considered normal in domestic animals varies from 0.1 to 0.3ppm (National..., 1983); Se is involved in the formation of glutathione enzymes, particularly GSH-Px, the most important (Rodriguez *et al*., 2004) with the function of catalyzing the reduction of peroxides to protect the cells from oxidative damages (Ceballos *et al*., 1998; Oblitas *et al*., 2000; Boland, 2003; Rodriguez *et al*., 2004; Luberda, 2005; Livingston *et al*., 2009).

Rodriguez *et al*. (2004) pointed out that the imbalance between free radicals (oxidants) and antioxidant defense agents favoring oxidants leads to oxidative stress, which results in cell damage and death. There can also be alterations in the activity of intracellular organelles such as mitochondria and lysosomes, membranes and other cellular structures, especially DNA (Combs *et al*., 1975; Fisher Jr., 2000).

The total production of embryos *in vitro* was higher in GT (P<0.05). The numbers of initial blastocysts and blastocysts did not differ between the two groups (P>0.05). The results are presented in Table 2.

The analyses revealed that in submitting the oocytes to the maturation process and *in vitro* fertilization, a mean total number of blastocysts of 21.98±2.37 was obtained in animals that were treated with 9.6mg of Se/day in comparison to 13.12±1.58 for animals that received 3.2mg Se/day, showing a significant difference (P<0.05). This is probably the result of the production of oocytes of better quality (degree 1) in the animals that received 9.6mg/day of Se, which can be explained by the observations of Uhm *et al*. (2007), who worked with pig embryos and found that the oocytes cultivated and fertilized in medium containing 25mg/mL sodium selenite showed better development and quality at seven days post-fertilization in relation

to those cultivated in medium without sodium selenite.

According to Uhm *et al*. (2007), Se guarantees adequate biosynthesis of selonoprotein, protecting the embryos' cells against oxidants and apoptosis. In relation to selenoprotein, we can cite the studies of Wittwer *et al*. (2002), who found a positive correlation of 0.74 between the amounts of Se ingested by the animal and GSH-Px, and of Livingston *et al*. (2009), who emphasized the importance of Se in sheep reproduction *in vivo*, when assessing the quality of oocytes matured *in vivo* and of the embryos.

The mean concentration of Se did not significantly differ between the two groups ($P<0.05$), as shown in Table 3. There were no statistical differences observed in serum Se concentration, comparing levels of the first and last day of the experiment in GT ($P>0.05$). These results possibly reflect the observations of Langlands *et al*. (1996), who reported that sheep with elevated Se levels showed increased excretion of Se via feces and urine; there could have also been variability of the animals with respect to Se handling. Nonetheless, based on the results obtained, it is possible to deduce that the ingestion of the greater amount of Se by the animals could have increased the level of GSH-Px in tissues.

In relation to serum Se levels, an increase of 34.5% was noted comparing the beginning and end of the experiment, in the animals treated with 3.2mg of Se/day, although there was no significant difference ($P>0.05$). However, in those that received 9.6mg of Se/day, there was a significant increase in serum Se of 37.2%, comparing the beginning and end of the experiment.

These values suggest the importance of Se in the process of oocyte production with better competency for generating embryos to be fertilized *in vitro*, among other factors, such as the increase in the synthesis of GSH-Px, although not evaluated. Two other important factors could be improvement in immunity and better growth of the animals (Ceballos and Wittwer, 1996).

CONCLUSIONS

It can be concluded that higher addition of Se to the diet resulted in a higher production of oocytes, larger number of degree 1 oocytes and higher production of embryos in the *in vitro* fertilization process.

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