EFFECT OF ESTRADIOL BENZOATE ON OOCYTES/EMBRYOS RECOVERY AND HEMATOLOGICAL PARAMETERS OF MISMATED BITCHES

(EFEITO DO BENZOATO DE ESTRADIOL NA RECUPERAÇÃO DE OÓCITOS , EMBRIÕES E NOS PARÂMETROS HEMATOLÓGICOS DE CADELAS RECÉM ACASALADAS)[1]

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SUMMARY

We investigated estradiol benzoate effects on oocytes/embryos recovery rate and the influence of this drug on the hematopoietic system. Twenty four bitches were divided in two groups, Group I, 12 females that received a single shot of estradiol benzoate, 0.2 mg/kg intramuscularly, between 2 and 7 days after the date of the last mismating or insemination and, Group II (control), 12 bitches that received 0.2 ml/kg of oily diluent, in corresponding dates. The bitches were ovary-hysterectomized and the uterus/oviduct were isolated and flushed with a PBS, heparin and polyvinyl alcohol solution. Oocytes and embryos were quantified and classified according to their stage of development. Blood counts were performed on M1 (before drug administration), M2 (15 days after drug administration) and M3 (40 days after drug administration). Pearson correlation coefficient was used to analyze the variable retrieval structures, while Fisher exact test was used for the analysis of embryonic viability. ANOVA was used to analyze repeated measurements and Tukey test for hematological parameters. All tests were performed at 5% significance level. The recovery rate of total structures in group I was lower (22.88%) than group II (65.85%). A lower embryo recovery (ratio 3: 52) rate and a greater number of degenerated structures (ratio 11: 1) were observed in group I. Hematological parameters showed significant difference in erythrocytes, hematocrit and hemoglobin concentrations 15 days after drug administration and difference in leukocytes concentration 40 days after using the medication in bitches of group I, however, at the end of the experiment all bitches had blood counts considered normal.

KEY-WORDS: Estradiol benzoate. Bitch. Contraception

RESUMO

Nosso objetivo foi avaliar os efeitos do benzoato de estradiol[7] sobre a taxa de recuperação oocitária e embrionária e sua influência sobre o sistema hematopoiético. Vinte e quatro fêmeas foram utilizadas: GRUPO I – 12 cadelas que receberam uma única aplicação de benzoato de estradiol, dose de 0,2 mg/Kg, via intramuscular, entre o 2º e 7º dia do último acasalamento ou inseminação. GRUPO II - 12 cadelas que receberam solução oleosa diluente, dose de 0,2 ml/Kg em datas correspondentes. As cadelas foram submetidas a ovariohisterectomia e as tubas uterinas e útero lavados, com solução de PBS, álcool polivinil e heparina. Os oócitos e embriões foram quantificados e classificados de acordo com seu estádio de desenvolvimento. O hemograma foi realizado em **M1** (antes da aplicação do fármaco), **M2** (15 dias após a aplicação do fármaco) e **M3** (40 dias após a aplicação do fármaco). Para as variáveis recuperação de estruturas foi utilizado o coeficiente de correlação de Pearson e teste exato de Fisher na análise de viabilidade embrionária, análise de variância – ANOVA para medidas repetidas e o teste de Tukey para os parâmetros hematológicos. Todos os testes foram realizados a 5% de significância. A taxa de recuperação de estruturas no grupo I foi menor (22,88%) quando comparada ao grupo II (65,85%). Foi observada uma menor recuperação embrionária (proporção 3: 52) e um maior número de estruturas degeneradas (proporção 11: 1), no grupo I. Os parâmetros hematológicos mostraram diferença significativa em relação a concentração de hemáceas, hemoglobina, e volume globular, 15 dias após a aplicação do fármaco e diferença na concentração de leucócitos 40 dias após o uso dessa medicação em cadelas do grupo I, no entanto, ao final do experimento todas as fêmeas apresentavam hemogramas considerados normais.

PALAVRAS-CHAVE: Benzoato de estradiol. Cadelas. Contracepção

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INTRODUCTION

Unwanted matings have always been a concern of dog and cat owners, however, when it comes to contraception, especially the use of contraceptives, there is conflict and disagreement about the used of these drugs. The action mechanism of drugs used to prevent, terminate and/or abort pregnancy, depends on the stage of the estrous cycle, pregnancy stage where they are used, the ability to generate fetal death, the absorption and/or abortion or even induction of labor (HOFFMAN, 1999).

The use of estradiol benzoate to prevent implantation on mismated bitches is a form of contraception, which can be used in programs that aim to control the population of dogs in a less expensive way. Studies on the dose, side effects, right timing regarding the animal estrous cycle, could intensify the use of this hormone and increase its effectiveness.

Esters, especially estradiol benzoate, have been used to stop unwanted pregnancy in bitches for many years. Estrogens alter the transit time of the zygote in the uterine tubes, modify uterine biochemistry and cause embryo degeneration due to its embryotoxic effect. Estrogens can stimulate uterine contractions and cervical opening (BRUNCKHORST et al, 2000; FONTBONNE, 2010).

More recent publications have shown that the use of estrogen in low doses, in mismated bitches, is safe and effective (TSUTSUI, 2006). These studies are being conducted in an attempt to rescue the popularity of exogenous estrogen as a method of abortion. This drug, considered inefficient for many years, was causing serious side effects in bitches such as bone marrow suppression and subsequent aplastic anemia.

Therefore, the objective of this study is to evaluate the effects of estradiol benzoate on the oocyte recovery rate and/or embryonic vitality of recovered structures and the influence of this drug on the blood counts of mismated bitches.

MATERIAL AND METHODS

Twenty-four crossbred bitches, medium size, aged between 1 and 8 years old, that had been newly mismated or in early follicular phase, were selected for full general clinical examination, vaginal cytology and serum progesterone levels. A score of 80% of superficial cells in the smear was the criteria to confirm the stage of the estrous cycle. Progesterone levels were measured by RIA (SRIKANDAKUMAR et al, 1986) and all bitches included in the study had progesterone levels between 0.52 and 33 ng/ml, as well as 80 to 100 % of superficial cells. These parameters were used, respectively, as criteria to determine the pre-ovulatory surge of luteinizing hormone (LH) and to determine the stage of the estrous cycle .

We used both, newly mismated bitches (between 2 and 7 days) and bitches that were verified to be in the follicular phase of the cycle, which were artificially inseminated (A. I.) with fresh semen of a donor dog trained for manual collection and routinely used in other experiments. The artificial insemination protocol used was: two A. I. with 48-hour interval, on days D4 and D6 of preovulatory LH surge, determined according to Feldman and Nelson (1996). The insemination dose was approximately 330×10^6 motile sperm.

These females were randomly assigned to two groups, Group I, formed by 12 bitches that received a single dose of estradiol benzoate, 0.2 mg/Kg intramuscularly, between the 2nd and 7th day of the last mating or insemination (TSUTSUI et al. 2006) and Group II, the control group, formed by 12 bitches that received a dose of diluent oil solution, 0.2 ml/Kg on corresponding dates.

A conventional ovary-hysterectomy (OHE) was performed in all animals, between 15 and 18 days of the preovulatory LH surge, which was determined by serum progesterone. The fallopian tubes and uterus were isolated and the uterine horns were divided into left and right and sectioned longitudinally in Petri dishes. The fallopian tubes and the uterus were carefully washed in order to recover the *cúmulus oophorus* complex *(COCs)* and/or embryos in a solution of PBS, with polyvinyl alcohol and heparin at 30°C.

The COCs and the recovered eggs were collected, transferred to Petri dishes and observed under stereomicrocospe (Leica MZ 12.5) for evaluation and counting of the morphological structures. The retrieved structures were classified into oocyte, morula, early blastocyst, expanded or hatched blastocyst, following the classification recommended by Stringfellow & Seidel (1998).

Embryonic viability was performed by evaluating the integrity of the nuclear chromatin by staining with propidium iodide and Hoechst 33342. Subsequently, it was examined with an inverted microscope equipped with fluorescent light (blue filter 535 and 617 nm). The embryonic structures that became bright blue with intact plasma membrane were considered viable, while the ones that became red or pink were considered non-viable. Embryos with less than 50% red cells were considered viable, following criteria proposed by Watt et al. (2009).

Blood samples were collected to perform blood counts. Complete blood counts were performed using the automated method to study morphology in stained smears. The reference values were the same adopted by Meinkoth & Clinkenberard (2000).

The occurrence of hematological abnormalities was evaluated over time, time 1 (T1) before drug administration, time 2 (T2) 15 days after drug administration and time 3 (T3) 40 days after drug administration.

Statistical analysis of oocyte and embryo retrieval variables was performed using the Pearson correlation coefficient. The analysis of embryonic viability was performed using Fisher exact test. Blood count results were submitted to ANOVA and Tukey test. All tests were performed at 5% significance level.

The procedures were approved by the Ethics and Biosafety Committee of the Institution on September 21, 2006.

RESULTS AND DISCUSSION

The values for oocyte and embryo retrieval are shown in Tables 1 and 2. The number of structures recovered in dogs of Group I was lower (22.88%) compared to Group II (65.85%). There was a 13.6% embryo retrieval and 9.4% oocyte recovery in Group I and embryo and oocyte retrieval rates of 96.2% and 1.2%, respectively, in Group II, indicating the effect of estradiol benzoate on dogs of Group I. Only 3 embryos and 11 degenerated structures were recovered in Group I, the group that received estradiol benzoate, while 52 embryos were recovered in control group II. Estradiol benzoate causes embryonic degeneration due to its embryotoxic effect (BRUNCKHORST et al, 2000).

Oocytes and embryos were absent in 5 bitches of Group I despite the presence of corpora lutea. These females mated between day 0 of the estrous cycle (D0, day of the onset of pre-ovulatory LH surge) and 5 days later. The estradiol benzoate was probably responsible for the degeneration of oocytes and embryos, since 95% of reproductively normal bitches that mate during their fertile period become pregnant (REYNAUD et al. 2006).

The change of gametogenic and embryo motility, inducing oocyte and embryo degeneration may also have occurred due to the use of estradiol benzoate. The action mechanism of exogenous estrogen is related to changes of the tubal micro-environment, which slows the gametogenic and embryo transport promoting the closing of the uterus-tubal junction, with subsequent retention of embryos and oocytes in the fallopian tubes, followed by the degeneration process (BRUNCKHORST et al, 2000; FONTBONNE, 2010).

The low recovery rate seen in Group I can also be a consequence of increasing uterine and tubal contractility stimulated by estradiol benzoate. Treatment with estradiol in women increases the circulating levels of oxytocin (AMICO et al. 1981; MITCHELL et al. 1998). This also occurs with dogs, which could explain the increase of uterine and tubal contractility and lead to tubal absence of oocytes and embryos in these females (FONTBONNE, 2010).

Table 1 - Number of corpora lutea and structures (oocytes, embryos and degenerated structures) recovered in the fallopian tubes and uterus of dogs in group I (GI), after a single intramuscular shot of estradiol benzoate[8], 0.2 mg/kg dose, between 2 and 7 days from the last mating.

Group I	**Corpora lutea**	**Number of structures retrieved**		
		oocyte	**embryos**	**degenerated**
E 01	-	0	0	0
E02	4	0	0	0
E03	8	8	0	0
E04	5	0	0	0
E05	9	0	0	2
E06	14	0	1 morula	0
E07	6	0	0	0
E08	6	0	0	2
E09	9	0	1 hatched blastocyst	0
E10	5	0	1 morula	0
E011	13	0	0	7
E13	6	0	0	0
Total	**85**	**8**	**3**	**11**
Total	**85**		**22**	

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Table 2 - Number of corpora lutea and recovered structures (oocytes, embryos and degenerated structures) in the fallopian tubes and uterus of dogs in group II (GII).
* corpora lutea count was not performed in this bitch.

Group II	Corpora lutea	Number of retrieved structures		
		Oocyte	Embryo	Degenerated
C01	-	0	3 hatched blastocyst	**0**
C02	9	1	0	**0**
C03	9	0	3 hatched blastocyst	**0**
C04	6	0	6 hatched blastocyst	**0**
C05	9	0	2 hatched blastocyst	**0**
C06	11	0	8 hatched blastocyst	**0**
C07	8	0	6 expanded blastocyst	**0**
C08*	-	0	8 expanded blastocyst	**0**
C09	9	0	7 hatched blastocyst	**0**
C11	9	0	6 initial blastocyst	**0**
C12	3	0	1 morula	**0**
C13	9	0	2 expanded blastocyst	**1**
Total	**82**	**1**	**52**	**1**
Total	**82**		**54**	

Table 3 – Analysis of embryo viability observed in uterine flushings of dogs in group I and group II.

	Embryo viability			
	Collected embryos	**Analyzed structures**	**Viable**	**Non viable**
Group I	3	3	2 (75%)	1 (25%)
Group II	52	23	16 (69.56)	7 (30.43)

Despite the low recovery of oocytes and embryos in Group I, these structures when retrieved were viable (group I – 75% and group II- 69.56%), as shown in Table 3. The percentage of viable structures were similar in both groups.

Estradiol benzoate is a short shelf-life drug, 3 days. This characteristic is beneficial because the connection time of the drug with its receptor is relatively short, which causes less severe side effects (COWEN et al. 1985), compared to other estrogen compounds.

The analysis of the blood counts (Table 4) shows significant differences ($p<0.05$) for the variables erythrocytes ($p=0.015$), hemoglobin ($p=0.001$) and globular volume ($p=0.012$), 15 days after drug administration (T2) without, however, exceeding the limits considered standard for the species. This fact was considered an effect of estradiol benzoate, without excluding the possibility of the occurrence of coagulopathy.

The changes observed for these parameters can also be attributed to blood loss caused by surgical procedure, since at T2 four bitches had already undergone OHE.

Table 4 - Mean and standard deviation values of red blood cells (HE), hemoglobin (HG), packed cell volume (VG), platelets (PL) and leukocytes (LC) in group I over the three time interval studied T1 (before drug administration), T2 (15 days after drug administration) and T3 (40 days after drug administration) and reference values adopted by Meinkoth and Clinkenberard (2000).

T	HE (5.5 a 8.5 (X 10^6 µL)	HG (12 a 18 (g/dl)	VG (%) (37 a 55%)	PL (180,000 µL)	LC (6- 17 (x $10^{3/}$ µL)
1	6627500 ± 172614.15[a]	15.54 ± 0.56[a]	45.08 ± 1.46[a]	272614.58 ± 48600.58	13879.17 ± 804.52[a]
2	6010833.33 ± 257806.77[b]	13.59 ± 0.43[b]	40.33 ± 1.65[b]	226843.75 ± 55396.78	13658.33 ± 1467.55[ab]
3	6111666.67 ± 184935.83[b]	14.26 ± 0.41[b]	43.17 ± 1.11[ab]	236325 ± 33229.73	10394.91 ± 740.33[b]
p	0.015	0.001	0.012	0.324	0.031

Different letters (a, b) in the column mean significant differences ($p<0.05$).

At T3, leukocyte values were statistically different from the values T1 ($p = 0.031$), however, the state of leukopenia was not characterized. In general, all bitches had blood counts considered normal for the species, which confirms the findings of Weiss & Klausner (1990) who identified a fast and transitory effect of estrogen on blood.

Another important consideration about the use of estradiol benzoate is the individual sensitivity and severity of side effects strong relationship with the used dose. No other symptom related to the reproductive tract was identified in the animals studied, although the evaluation period was relatively short to diagnose uterine or breast changes.

CONCLUSION

The oocyte and embryo retrieval was lower in females who received estradiol benzoate (0.2 mg/Kg intramuscularly), which confirms the contraceptive effect of the drug when used between 2 and 7 days after mating and no changes were observed in the blood counts of the studied bitches until 40 days after drug administration.

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