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POSSIBILITIES OF IMPLEMENTING PROGESTERONE EIA TEST IN THE CONTROL OF REPRODUCTION IN DAIRY COWS

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ABSTRACT

The aim of this study was to implement the progesterone EIA test, developed in our laboratory by using an anti-progesterone antibody (Yamaguchi University, Japan), in order to determine the optimal moment for artificial insemination (AI) and to detect pregnancy in Holstein-Friesian cows according to the progesterone concentration in the whole milk. Also, the influence of β-carotene, applied at the day of insemination and human chorionic gonadotrophin applied on day 7 after AI on the progesterone level and the pregnancy rate were evaluated.

For the accuracy of oestrus detection, the milk samples from 70 cows were collected on the day of insemination. Milk samples from 148 cows were collected 19-22 days following insemination for pregnancy check.

After detection of naturally occurring oestrus (day 0) and AI, cows were divided into the following groups: group A (n = 19) was treated with 200 mg β-carotene (20 ml Carofertin® i.m. Alvetra u. Werfft Gmbh, Austria), group B (n = 17) was treated with 1500 IU hCG i.m. (Schering-Plough, the Netherlands) and control (non treated) group C (n = 18). The milk samples for EIA progesterone concentration analysis were collected on the day of AI, the 14th and the 20th day of the oestrus cycle.

Oestrus detection errors and inappropriate moments of insemination according to the progesterone concentration were detected in 22.86% animals (16/70). The test accuracy for non-pregnant cows was 90.48% (76/84). The accuracy of the progesterone test in pregnant cows was 75% (48/64). False positive results (high progesterone level, but the cows were not pregnant) was detected in 25% of cows (16/64) as a result of a prolonged oestrus cycles, embrional mortality and endomethritis (10/16 cases).

The treatment of cows with 1500 IU of hCG, on the day 7 of the oestrus cycle, resulted in statistically significant increase of progesterone concentration in the dioestrus ($p < 0.01$). The most successful insemination was in the group of cows that was treated with hCG (47.05%; 8/17), then in the control group (38.88%; 7/18) and the least in the Carofertin group - 36.84% (7/19). These differences were only numerical ($p>0.05$).

The EIA test developed in our laboratory could be used for accurate progesterone determination in the whole milk during implementation of different methods for control of bovine reproduction.

Key words: β-carotene, cows, EIA test, hCG, milk, progesterone

INTRODUCTION

Diagnosis of pregnancy in cattle is of special interest because succesful reproduction is often crucial to cows life time milk production.

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Progesterone is a steroid hormone secreted by ovarian *corpus luteum* following ovulation in the luteal phase of the sexual cycle in cattle. Concentration of progesterone in the blood plasma of cows indicates secretory activity of the luteal tissue, since there is no other significant source of progesterone (1).

Progesterone is necessary to preserve the gravidity and luteal deficiency is considered as one of the causes of infertility (2). Progesterone concentration decline in the early period of

pregnancy, leads to embryonic death or abortion. As the *corpus luteum* is a main source of progesterone with the task of maintaining pregnancy at the beginning of gestation, stimulating its activity has been the subject of several studies in the last decade. The aim of this research was to find positive effects in the procedures designed to stimulate the *corpus luteum* function in order to increase the conception rate. Numerous authors considered that the application of hCG between 7 to 15 days after oestrus may improve the fertility of the cows (3, 4, 5).

Corpus luteum in cows contains two to five times more β-carotene compared to other tissues (liver, adipose tissue, plasma), even in deficient conditions. In animals with reduced β-carotene in forage, there is only small decrease of progesterone concentration in the *corpus luteum* tissue compared to animals that had enough β-carotene (6). Several studies on the impact of β-carotene in improving reproductive parameters have shown incoherent results (7, 8, 9, 10).

Determining progesterone concentrations in the blood and milk is carried out, not only in terms of diagnosis and exclusion of pregnancy, but also in discovering cyclic dysfunction and disorders of ovarian function. Although the differences in the concentration of progesterone is clearly visible on the 17th day after mating or insemination, the most reliable results in the diagnosis of pregnancy rates are achieved from the 19th to the 24th day of the cycle (11, 12, 13).

Despite significant advances in technology, the measurement of progesterone remains still one of the earliest, indirect ways of detecting nonpregnant cows and an excellent marker for ovarian function.

High reliability progesterone test requires continuous records, which plays a key role in the control of reproduction in dairy farms. The success of reproduction depends on many factors, but oestrus detection and determination of the optimal time of insemination is one of the main problems in management of high-yielding dairy cows.

The aim of this work was to implement the progesterone EIA test developed in our laboratory by using an anti-progesterone antibody (Yamaguchi University, Japan) in order to determine:

1) the accuracy of oestrus detection on the day of insemination;

2) the reliability of the test in detection of pregnant and nonpregnant cows from days 19 to 22 after AI;

3) the effect of application of β-carotene at the moment of insemination on the increase of the progesterone level and the pregnancy rate, and

4) the effect of application of hCG on day 7 after AI on the progesterone level and the pregnancy rate.

MATERIALS AND METHODS

The experiment was conducted on a commercial farm with 450 milking cows. The cows were between 60 and 180 days of lactation and were milked twice a day. The average milk production on the farm was 6,500 l calculated at 305 days of lactation. Oestrus was detected on the basis of external signs of oestrus, rectal examination and vaginal inspection (by speculum).

The pregnancy check was carried out by ultrasound scanner (285 SIUI CTS-V, Shantou, China) using a rectal probe of 7.5 MHz, 27-35th day after insemination. The final confirmation of pregnancy rates was performed by rectal exploration with ≥ 2 months after insemination to detect cases with embryonic mortality.

Approximately 8 ml of milk from clinically healthy quarters (n=232) were sampled in plastic 10 ml tubes (Spectar, Cacak, Serbia). Potassium dichromate tablets of 3.3 mg (Merck, Darmstadt, Germany) were used as a preservative during transportation and storage of milk samples. Samples were stored in a refrigerator at +4 °C until analysed.

Levels of progesterone in whole milk through the use of EIA progesterone tests were determined in the Scientific Veterinary Institute "Novi Sad", Novi Sad. Anti-progesterone antibodies, protocol for assay and protocol of production of HRP-P4 were obtained from the Laboratory of Theriogenology, Department of Veterinary Medicine, Faculty of Agriculture, Yamaguchi University, Japan. Substrates for EIA were produced in INEP, Zemun (Serbia). Optical density (extinction) was recorded by using a plate reader (Rayto Life and Analytical Science Co., Ltd, P. R. China), with filter wavelength of 450 nm.

The "Dairy Quest" (Profit Source, Wi, USA) software program was inplemented on farm for data tracking of production, reproduction, health and results of EIA test. Milk sampling was based on the generated list of cows in the "Dairy Quest" software.

Milk samples from 70 cows were collected on the day of insemination for the accuracy of oestrus detection. One hundred and foury eight milk samples were collected on days 19, 20, 21 or 22 following insemination for indirect early pregnancy check/ detecting the resumption of new oestrus. The values of progesterone concentration in whole milk ≥4 ng/ml, obtained in days 19 to 21 of the cycle were used as a possible pregnancy indicator. Values under this levels indicated that the cows were not pregnant.

The influence of β-carotene, applied at the day of insemination and human horionic gonadotrophin (hCG) applied on day 7 after AI, on the progesterone level in milk on day 14 and 20 after insemination on pregnancy rate of Holstein-Friesian cows were also evaluated. After naturally occurring oestrus (day 0) and AI, the cows were randomly divided into the following groups:

- group A ("Carofertin" group, n = 17) was treated with 200 mg β-carotene (20 ml Carofertin® i.m., Alvetra u. Werfft Gmbh, Austria);
- group B ("hCG" group, n = 17) was treated with 1500 IU hCG i.m. (Schering-Plough, the Netherlands) and
- group C ("control group", non treated, n = 18).

Milk samples for EIA progesterone concentration analysis were collected on the day of AI, at the 14th and at the 20th day of the estral cycle. Cows with high levels of progesterone on the day of insemination were not included in the experiment.

RESULTS

1. Accuracy of oestrus detection at insemination based on the progesterone level

The results for the oestrus detection at insemination based on the progesterone level are showed in Table 1. Out of 70 samples, 16 (22.86%) had a progesterone concentration higher than 2 ng/ml on the day of insemination, which indicates poor oestrus detection technique and insemination at the incorrect time.

Table 1. Progesterone concentration in milk samples collected on the day of insemination

Categories	Number of cows	(%)
low progesterone (0 to 1.9 ng / ml)	54	77,14
suprabasal level (2.0 to 3.9 ng / ml)	12	17,14
high level (≥ 4 ng / ml)	4	5,72
Total:	70	100,00

2. Pregnancy/oestrus detection based on progesterone level at 19th, 20th, 21st or 22nd day after AI

For early detection of pregnancy 148 milk samples between the 19th – 22nd day after insemination were analysed. The results are showed in Table 2. Low levels of progesterone indicated the occurrence of oestrus and high levels of progesterone indirectly pointed to conception (pregnancy).

Table 2. Results of the analysis of milk samples analysed 19-22nd days after AI

Groups according to the level of progesterone	Number of samples	Pregnancy rates			
		Pregnant (No.)	(%)	Non-pregnant (No.)	(%)
High (≥ 4 ng / ml)	64	48	75,00	16	25,00
Low (<4 ng / ml)	84	8	9,52	76	90,48
Total:	148	56	37,83	92	62,17

3. Influence of β-carotene and hCG aplication on the milk progesterone level and the conception rate

3a. Effect at the 14th day of the cycle:

The effect of human chorionic gonadotropin (hCG), applied on the 7th day after AI and β-carotene, applied on the day of insemination (day 0) on the level of progesterone resulted in a statistically significant increase of progesterone concentration in dioestrus-day 14th between hCG and the Control grup ($p < 0.01$). Carofertin treatment resulted in a slight progesterone increase, but this value was only numerical.

Table 3. Descriptive statistics values for the concentration of progesterone on the 14th day of the cycle after AI. The analysis included all cows, regardless of the outcome of insemination (pregnant or nonpregnant)

Groups in the experiment	Concentration of progesterone (ng/ml), 14th day					
	n	$\overline{x}$	SD	Sx	CV%	I.V.
A - Carofertin	19	10,53	9,70	2,23	92,15	0,36-30,5
B – hCG	17	16,97	9,93	2,41	58,52	0,30-34,50
C - Control	18	7,28	5,63	1,33	77,30	0,05-19,50

3b. Results of the determination of progesterone concentration on the 14th day of the cycle between pregnant and nonpregnant cows within Carofertin, hCG and control groups

Mean values of concentrations of progesterone on the 14th day of the cycle, when analyzed on the basis of future outcomes insemination, showed a tendency to increase in the hCG and Carofertin groups of pregnant cows compared to the nonpregnant group. However, a large standard deviation and coefficient of variation could be seen in all groups. A statistically significant increase of progesterone concentration was noted between the hCG pregnant and the Control pregnant group at day 14th ($p < 0.001$).

Table 4. Descriptive statistical parameters for progesterone concentration on the 14th day of the cycle (pregnant and nonpregnant, within the group)

Groups in the experiment	Concentration of progesterone (ng/ml), 14th day					
	n	$\overline{x}$	SD	Sx	CV%	I.V.
Carofertin pregnant	7	14,40	11,43	4,32	79,38	1,50-30,50
Carofertin nonpregnant	12	8,28	8,23	2,38	99,40	0,35-30,00
hCG pregnant	8	21,91	7,03	2,49	32,09	10,75-34,50
hCG nonpregnant	9	12,59	10,39	3,46	82,51	0,30-30,00
Control pregnant	7	5,77	2,99	1,13	51,77	2,15-11,00
Control nonpregnant	11	8,24	6,77	2,04	82,19	0,05-19,50

Table 5. Statistical significance of differences in the concentration of progesterone on the 14th day of the cycle (pregnant and nonpregnant, within groups)

Groups in the experiment	hCG pregnant	Control pregnant	Carofertin nonpregnant	hCG nonpregnant	Control nonpregnant
Carofertin pregnant	p > 0,05 (p=0,281)	p > 0,05 (p=0,2593)	p > 0,05 (p=0,3402)	–	–
hCG pregnant	–	p < 0,001 (p=0,0006)	–	p > 0,05 (p=0,0592)	–
Control pregnant	–	–	–	–	p > 0,05 (p=0,6916)

Mann-Whitney-Wilcoxon rank-sum test

3c. Effect on the 21st day of the cycle

Mean values of concentrations of progesterone on the 20th day after AI were highest in the A-Carofertin and the B-hCG group, but no statistically significant differences were detected between pregnant cows in the groups based on the progesterone values (Mann-Whitney-Wilcoxon rank-sum test). A large standard and coefficient deviation could also be noticed in all the groups.

Table 6. Descriptive statistical parameters for progesterone concentration on the 20th day of the cycle in pregnant cows

Groups in the experiment, 20 days after insemination	Progesterone concentration (ng/ml), 20th day after AI					
	n	$\overline{x}$	SD	Sx	CV%	I.V.
A-Carofertin pregnant	7	22,12	11,78	2,23	53,26	8,00-40,00
B-hCG pregnant	8	19,32	6,62	2,41	34,29	5,53-26,50
C-Control pregnant	7	15,22	10,31	3,90	67,72	3,90-29,67

Pregnancy rates in this experiment were diagnosed by rectal examination in the period of 45 to 60 days after insemination. The results of artificial insemination are shown in Table 7.

Table 7. The results o f artificial insemination of cows in groups according to different treatments

Groups in the experiment	n total	n pregnant	n nonpregnant	% pregnant
A-Carofertin	19	7	12	36,8
B-hCG	17	8	9	47,1
C-Control	18	7	11	38,9

DISCUSSION

In this study we have exeminated the possibilities of implementing progesterone EIA test in the control of reproduction in dairy cows. The absolute incorect time for insemination was determined by the progesterone concentration ≥ 4 ng/ml and the suprabasal progesterone levels were defined as 2-4 ng/ml. The suprabasal level was considered as insemination in proestrus or at the beginning of metoestrus. Out of 70 samples, 16 (22.86%) had a progesterone concentration higher than 2 ng/ml on the day of insemination, which indicates poor oestrus detection technique and insemination at the incorrect time.

The accuracy of oestrus detection in the experiment was 77.14% (54/70). None of the cows that had a high concentration of progesterone on the day of insemination became pregnant (≥ 4 ng/ml). Out of the 12 cows with a suprabasal progesterone concentrations (2.0 to 3.9 ng/ml), two were pregnant, but they were inseminated again after 24 hours. Oestrus detection errors and incorrect moment of insemination according to the progesterone concentration (> 2 ng/ml of progesterone) were detected in 22.86% of the animals (16/70). According to the results reported by other authors (14, 15, 16), 5.1%, 19% and 22.1% of cows, respectively, were inseminated in the luteal phase of the cycle. Comparing with the literature data (15, 21) and based on our clinical experience, it can be concluded that one of the major problems of large farming systems (capacity of 200 or more dairy cows) is poor oestrus detection.

Regarding the oestrus detection based on progesterone level at 19th, 20th, 2nd or 22nd day after AI, we found that out of 64 cows with high progesterone concentrations, pregnancy was confirmed by clinical examination in 48 cows (75%), while 16 cows (25%) were not pregnant. Out of these 16 false positive samples (cows with high-progesterone, but not pregnant), 62.50% (10/16) had embryonic death (finding empty embryonic vesicles with turbid liquor, ondulating membranes and no heart beat at 32-38 post AI - 3 cows) or uterus inflammation process (abnormal vaginal discharge and clinical endometritis, - 5 cows) or hormonal disturbance (luteal cysts - 2 cows). In the 6 remaining cows (6/16; 37.50%) there were no clear indications for the absence of pregnancy, despite high levels of progesterone.

The test accuracy for nonpregnant cows was 90.48% (76/84). All cows with zero progesterone levels from 19-22nd days after AI had characteristic clinical signs on the reproductive organs (estral mucus, postoestrus blood discharge, failed fertilization).

A false negative result (low levels of progesterone, but the cows were pregnant) was detected in 9.52% of the cows (8/84). The average level of progesterone was 1.88 ± 0.68 ng/ml (CV = 36.39%). This is the most critical group for the accuracy of the test and deserves special attention. For more precise control of milk samples with progesterone values close to the "cut off" level, the recommendation is to re-sample milk ater 2-3 days (at the 24th day of cycle) (22).

The effect of human chorionic gonadotropin (hCG), applied on the 7th day after AI and β-carotene, applied on the day of insemination (day 0) on the level of progesterone resulted in a statistically significant increase of progesterone concentration in dioestrus-day 14th between hCG and the Control gruop ($p < 0.01$). Carofertin treatment resulted in a slight progesterone increase, but this value was only numerical.

On other side, the results of the determination of progesterone concentration on the 14th day of the cycle between pregnant and nonpregnant cows within Carofertin, hCG and control groups showed that the mean values of concentrations of progesterone on the 14th day of the cycle, when analyzed on the basis of future outcomes insemination has a tendency to increase in the hCG and Carofertin groups of pregnant cows compared to the nonpregnant group. However, a large standard deviation and coefficient of variation could be seen in all groups. A statistically significant increase of progesterone concentration was noted between the hCG pregnant and the Control pregnant group at day 14th ($p < 0.001$).

Additionaly, in this study we have examineted the influence of β-carotene and hCG aplication on the milk progesterone level and the conception rate. The results are in accordance with the results of Mann et al. (17) showing that the concentration of progesterone in early pregnancy has an impact on the outcome of insemination. The concentration of progesterone in dioestrus is lower in open cows than in pregnant cows. Also, Lamming et al. (18) stated that early progesterone increase (from 12

to 17 days after AI) can influence the outcome of insemination, but stressed that there is not strictly a high level of progesterone, which certainly ensures the preservation of embryos.

The concentrations of progesterone on the 20th day after AI were highest in the A-Carofertin and the B-hCG group, but no statistically significant differences were detected between pregnant cows in the groups based on the progesterone values. A large standard and coefficient deviation could also be noticed in all the groups.

Pregnancy rates in this experiment were diagnosed by rectal examination in the period of 45 to 60 days after insemination.

The most successful insemination results were in the group of cows that were treated with hCG (47.05%; 8/17), then in the control group (38.88%; 7/18) and least of all in the Carofertin group (36.84%; 7/19). These differences were only numerical ($p>0.05$).

The difference in the conception rate between the hCG and the C-control group (+8.17%) and between the hCG and the Carofertin group (+10.21%) may indicate that embryo loss is caused, to a certain extent, by the lack of endocrine communication between mother and embryo.

Delayed embryo development or a poorly developed embryo is not able to produce enough interferone τ, failing to prevent luteolyse and to ensure further development. Fertility reduction of 25-30% occurs because of early embryonal loss and lack of communication between mother and fetus. Further knowledge of the control of embryonal development and production of interferone τ is of great importance in the development of strategies to reduce the high incidence of clinically invisible embryo mortality in dairy cows (19, 20).

In order to have more convincing results of this experiment, a greater number of samples and/or analysis of blood serum, which is expected to have less fluctuations in the progesterone concentration than in whole milk is needed to confirm the hypothesis of Lamming et al. (18) that the amount of progesterone may determine the fate of the embryo from day 7 of the cycle.

The results of our study indicate that treatment of cows with hCG leads to increased levels of progesterone in dioestrus (the middle of the sexual cycle). Application of β-carotene had no effect on milk progesterone concentration and pregnancy rate.

CONCLUSIONS

The accuracy of oestrus detection and determining the optimal time of insemination based on the progesterone concentration in milk was 77.14%. Oestrus detection errors were detected in 22.86% of the animals. The accuracy of the progesterone test in pregnant cows was 75%. False positive results (high progesterone level, but the cows were not pregnant) was detected in 25% of cows and some are recognised as a consequence of a prolonged oestrus cycles, embrional mortality and endometritis.

The treatment of cows with 1500 IU of hCG, on day 7 of the oestrus cycle, resulted in a statistically significant increase of progesterone concentration in dioestrus ($p<0.01$). The most successful insemination was in the group of cows that were treated with hCG, then in the control group and least of all in the Carofertin group, but these differences were only numerical ($p>0.05$).

The progesterone EIA test developed in our laboratory by using the anti-progesterone antibody (Yamaguchi University, Japan) could be used for reliable quantitavive detection of progesterone concentration in the whole milk.

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