

Monitoring of early pregnancy and early embryonic mortality by ultrasound and determination of pregnancy-associated glycoproteins and progesterone in cows

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ABSTRACT

The aim of study was to investigate the role of pregnancy-associated glycoproteins (PAG) in early pregnancy, as well as the possibility of using PAG and ultrasound as diagnostic tools in the diagnosis of embryonic mortality. Our research included 73 Simmental cows, 3-7 years old, which calved every year. According to ultrasound findings (on the 17th, 24th, 35th and 45th day following AI), the cows were divided into 3 groups: pregnant cows (n = 34), non-pregnant cows (n = 18) and cows which had suffered embryonic mortality (n = 21). Blood samples were collected every 72 hours between the 12th and 45th day following AI and levels of progesterone and pregnancy-associated glycoproteins (PAG) were determined. Statistical analysis of variance for progesterone at days 12, 21 and 35 following AI showed significant differences between pregnant cows and both the non-pregnant and embryonic mortality groups (P>0.05). PAG variance analysis at days 24, 30 and 34 following AI showed highly significant differences (P>0.01) between the non-pregnant and both the embryonic mortality and pregnant groups. On the other hand, variance analysis showed that mean values for PAG at days 40 and 45 following AI were highly significantly different (P>0.01) between the pregnant and both the non-pregnant and embryonic mortality groups. The conclusion emerged that it is impossible to determine embryonic mortality merely on the basis of progesterone profile, but it is easy to distinguish pregnant from non-pregnant cows, supposing cows to be more than 21 days pregnant. It is very easy and accurate to distinguish non-pregnant cows from cows that have suffered early embryonic mortality. Furthermore, 98% of the cows in our research which had experienced embryonic mortality, lost their embryos 17-24 days after AI, visible in a drastic decrease in PAG seven and half to nine days later. Using PAG for pregnancy diagnosis enables us to prove the existence of live, vital embryos in utero 24 days after conception.

Key words: cow, pregnancy-associated glycoproteins, progesterone, early pregnancy, embryo mortality

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Introduction

Realtime B-mode grey-scale ultrasound scanning is the method of choice for the early diagnosis of pregnancy in cows. Using a 7.5 MHz transducer, BOYD et al. (1988) were able to visualize cow embryos as early as 9 days, whilst PIERSON and GINTHER (1984), using a 5 MHz transducer were able to do so at 12 and 14 days in heifers. Accurate proof of the existence of live, vital embryos, according to ultrasound findings, could be made on day 24, when the heart beat became visible (GODDARD, 1994). There are also some laboratory methods widely used for early pregnancy diagnosis. The most common is the determination of progesterone in blood and milk, 21 days after oestrus, and the determination of different pregnancy specific proteins, used in the pregnancy diagnosis of ruminants until the early nineties (ZOLI et al., 1992; KAREN et al., 2003). The main advantage of pregnancy specific proteins (PSP) is their ability to prove the existence of placentation and the presence of live, vital embryos, while progesterone only proves the existence of corpus luteum.

The most commonly used pregnancy specific protein for pregnancy diagnosis in cows is PAG (pregnancy-associated glycoprotein). Pregnancy-associated glycoprotein has been found in the serum of pregnant cattle and used as a pregnancy marker (PERENYI, 2002). As pregnancy failure occurs, PAG concentrations drop and disappear from maternal blood. Sequential assays of similar trophoblast proteins from the same group (PSP60), between day 24 and term, are useful for studying the course of pregnancy, although this does not allow discrimination between early embryonic mortality and non-fertilisation (MIALON et al., 1993). The same author noticed that the biological half-life for PSP60 was seven and a half to nine days. As PAG molecules are also the products of the trophoblastic cells, it was suggested that their determination in maternal blood can be useful for predicting foetal well-being and help detect early placental abnormalities, embryonic mortality or abortion (ECTORS et al., 1996).

After *in vitro* production of embryos or cloning, the PAG follow-up in plasma samples collected weekly was able to monitor embryonic or foetal mortality (ECTORS et al., 1996 and 1997). In these studies, it was concluded that significantly increased PSP60 levels could indicate abnormal placental development.

In veterinary practice, PAG and PSPB RIAs in biological fluids are helpful in confirming clinical diagnoses established by ultrasonography (SZENCI et al., 1998 and 2000). The aim of both studies was to investigate the role of different pregnancy-associated glycoproteins (PAG) in early pregnancy, as well as the possibility of using PAG and ultrasound as diagnostic tools in diagnosing embryonic mortality. The goal of our study was to determine the possibility of distinguishing early embryonic mortality from non-pregnancy in cows. Due to the great significance that subfertility and infertility

in cows play in the dairy industry, we expect that our results may have not only a scientific but also a positive economic impact.

Materials and methods

According to anamnestic data (absence of pregnancy failures and reproductive problems) we formed research herd of 73 simmental cows. They all have already calved, they were 3-7 years old and kept in the four stables in Čakovec region. According to ultrasound findings (17, 24, 35 and 45 day after artificial insemination; AI) the cows were divided in 3 groups: pregnant cows (n = 34), nonpregnant cows (n = 18) and cows suffered embryonic mortality (n = 21).

Cows which were suspect to be pregnant by all four examinations were alligned into pregnant group. Cows considered to be pregnant according to the first or the first two ultrasound findings and nonpregnant according to the last or last two findings were alligned into embryonic mortality group. Cows considered to be nonpregnant by all four ultrasound findings were alligned into nonpregnant group. We collected blood from all 73 cows using the same pattern, according to diagnostic significance (for progesterone at days 14, 21 and 35 after AI and for PAG at days 24, 30, 34, 40 and 45 after AI) We determined level of PAG and progesterone using RIA method.

Cows were examined four times (on the 17th, 24th, 35th and 45th day following artificial insemination (AI), using the same technique. After clinical and rectal examination, transrectal ultrasound examination was performed using a Sonovet 2000 portable ultrasound device. A linear ultrasound probe was used on frequencies of 5 and 7.5 MHz. Thorough visualisation of both uterine horns was followed by saving all important images and short films for each animal in a local computer database.

Blood samples were collected every 72 hours between the 12th and 45th day following AI for PAG and every 48 hours for progesterone assays. We used only samples which could be diagnostically valuable. For progesterone, this was at days 14, 21 and 35 after AI and for PAG at days 24, 30, 34, 40 and 45 after AI.

The PAG measurements were performed according to the method of ZOLI et al. (1992), with some modifications. In brief, the previously weighed standards and plasma samples (0.1 mL) were diluted in 0.2 mL of Tris buffer at pH 7.5 {0.025 M Tris, 0.01 M MgCl₂, 0.01% (wt/vol) sodium-azide, 0,05% (wt/vol) bovine serum albumin (BSA)}. The standard curve ranged from 0.2 to 25 ng/mL and in order to minimise non-specific interference, 0.1 mL virgin heifer plasma was added to each tube of the standard curve. After the addition of appropriate dilutions of antisera, the plasma samples and the standards were incubated overnight at room temperature. Antisera titres were determined to obtain a tracer binding-ratio in the zero standards of approximately 20-30%. The following day, tracer (0.1 mL or 25 000 cpm) was added to all the tubes and the tubes were incubated for

4 hours at room temperature. The total assay volume was 0.5 mL. The separation of the free and bound fractions was carried out by centrifugation (20 min at 1500×g, 10 °C) after the addition of 1 mL second antibody polyethylene glycol (PEG) solution {0.17% (v/v) normal rabbit serum, 0.83% (v/v) sheep anti-rabbit IgG, 0.3% (wt/vol) BSA, 4% (wt/vol) polyethylene glycol 6000 in Tris buffer}. After the tubes had been incubated for 1 hour with the second antibody PEG solution, 2 mL of Tris buffer was added and the tubes were centrifuged (20 min at 1500×g). The supernatant was aspirated and 4 mL of Tris buffer was added to each tube. The tubes were centrifuged (20 min at 1500×g) and the supernatant aspirated. The ¹²⁵I in the pellet was quantified using a gamma counter (LKB Wallac 1261 Multigamma counter, LKB, Turku, Finland) with a counting efficiency of 75%. The level of progesterone in serum samples was determined using the standard RIA method with conjugated steroids (Coat-A-Count TKPG, Diagnostic product Corporation) without pre-incubation. The ¹²⁵I in the pellet was quantified using a gamma counter (LKB Wallac 1261 Multigamma counter, LKB, Turku, Finland) with a counting efficiency of 75%. All samples in both methods (PAG and progesterone) were analysed in duplicate, taking the mean value as result and all doubtful results were repeated. The results were processed by the ANOVA statistical method, Tukey HSD tests of post-hoc analysis and also correlations between laboratory and ultrasound findings were determined.

Results

Comparison of pregnancy diagnosis between PAG, progesterone and ultrasound findings have shown 96% match. Comparison between two positive pregnancy diagnosis (ultrasound finding and one or both laboratory finding-high progesterone and high PAG) and data about calvings for our cows had shown 100% match.

Variance analysis has shown that there was significant difference ($P<0.05$) for mean values of PAG levels measured on the 24th day after AI, 30th day after AI and 34th day between all three groups. Variance analysis for PAG levels determined on the 40th and 45th day after AI has shown that there is significant difference ($P<0.01$) between pregnant and both nonpregnant and embryonic mortality groups.

Variance analysis for progesterone levels on 21st day after AI has shown significant difference ($P<0.05$) between pregnant cows and both nonpregnant and embryonic mortality group. Variance analysis for progesterone levels determined 14 and 35 days after AI didn't show any significant difference between the groups.

Analysis of linear correlations between the group didn't show any significant linear correlation between the parameters or groups.

Table 1. Level of pregnancy associated glycoproteins (PAG) and progesterone given in ng/mL for sera samples of pregnant cows

Days after AI	N° of cows	Mean	Minimum	Maximum	Variance	SD	SE
24 (PAG)	34	0.895	0.3	1.784	0.081	0.286	0.050
30 (PAG)	34	1.454	0.702	2.436	0.181	0.425	0.075
34 (PAG)	34	2.104	1.327	3.234	0.352	0.593	0.104
40 (PAG)	34	2.701	1.56	5.223	0.606	0.778	0.137
45 (PAG)	34	3.520	1.767	5.883	0.907	0.952	0.168
14 (PROG)	34	6.379	2.761	9.671	4.018	2.004	0.354
21 (PROG)	34	7.587	3.178	12.364	5.467	2.338	0.413
35 (PROG)	34	8.869	4.605	17.445	10.415	3.227	0.570

Table 2. Level of pregnancy associated glycoproteins (PAG) and progesterone given in ng/mL for sera samples of cows which suffered embryonic mortality

Days after AI	N° of cows	Mean	Minimum	Maximum	Variance	SD	SE
24 (PAG)	21	1.115	0.574	1.872	0.101	0.318	0.069
30 (PAG)	21	0.905	0.3	2.436	0.271	0.519	0.113
34 (PAG)	21	0.577	0	2.865	0.857	0.926	0.202
40 (PAG)	21	0.419	0	3.639	1.158	1.076	0.235
45 (PAG)	21	0.405	0	3.924	1.372	1.172	0.256
14 (PROG)	21	4.876	2.364	8.56	3.533	1.879	0.411
21 (PROG)	21	1.01	0.165	1.837	0.179	0.423	0.092
35 (PROG)	21	4.592	2.016	9.355	3.446	1.856	0.405

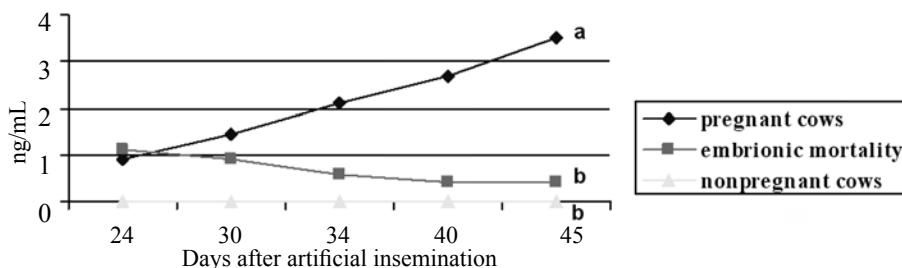


Fig. 1. Average PAG level in blood of pregnant, nonpregnant and embryonic mortality group in ng/mL (Values with different superscripts differ significantly $P < 0.05$, ANOVA)

Table 3. Level of pregnancy associated glycoproteins (PAG) and progesterone given in ng/mL for sera samples of nonpregnant cows

Days after AI	N° of cows	Mean	Minimum		Variance	SD	SE
24 (PAG)	18	0.011	0	0.124	0.001	0.033	0.008
30 (PAG)	18	0.020	0	0.358	0.007	0.084	0.0198
34 (PAG)	18	0.006	0	0.112	0.001	0.026	0.006
40 (PAG)	18	0	0	0	0	0	0
45 (PAG)	18	0	0	0	0	0	0
14 (PROG)	18	3.761	0.792	5.85	1.446	1.203	0.283
21 (PROG)	18	1.019	0.111	4.193	0.969	0.984	0.232
35 (PROG)	18	4.108	0.265	7.794	2.175	1.475	0.322

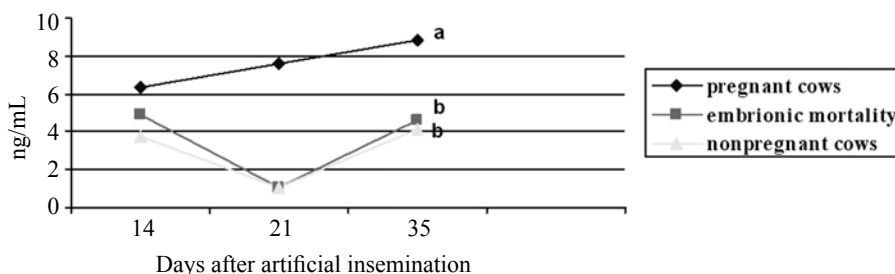


Fig. 2. Average progesterone level in blood of pregnant, nonpregnant and embryonic mortality group in ng/mL (Values with different superscripts differ significantly $P < 0.05$; ANOVA)

Discussion

The aim of study was to investigate the role of pregnancy-associated glycoproteins (PAG) in early pregnancy, as well as possibility of using PAG and ultrasound as diagnostic tools in the diagnosis of embryonic mortality. Due to the great significance that subfertility and infertility in cows play in the dairy industry, we expect that our results may have not only a scientific but also a positive economic impact. Namely, the intensive selection of cows for milk production in the dairy industry has had negative effects on the fertility rate, which decreases year by year, and poses to date the most significant problem in dairy cow reproduction. Furthermore, daily ultrasound monitoring of early pregnancy in dairy cows shows that most are capable of conceiving, but somehow lose their embryos before day 24 and positive pregnancy checking simply does not take place. However, it is not

suitable in everyday practice to check dairy cows so often, because it is time consuming, expensive and could cause the cows discomfort and iatrogenic pregnancy failure. There is an obvious need to find another method of monitoring the continuation or failure of early pregnancy in dairy cows. According to numerous studies (MIALON et al., 1993; SZENCI et al., 1998; PERENYI, 2002; ZOLI et al., 1992; BECKERS et al. 1994; KAREN et al., 2003; PRVANOVIĆ et al., 2003), constant attempts are being made to research the physiology of early pregnancy in cows, in order to develop an accurate and reliable method for distinguishing late embryonic mortality from non-pregnancy. In our study, we combined three of the most popular methods of early pregnancy detection (ultrasound, PAG and progesterone) and tried to find an ideal pattern of sampling and examination that would enable us to find and identify cows that had suffered from early embryonic mortality, and which would be both practical and accurate at the same time. A comparison of positive pregnancy diagnosis between PAG, progesterone and ultrasound findings showed a 96% match. A comparison between two positive pregnancy diagnoses (ultrasound findings and one or both laboratory findings of high progesterone and high PAG) and data on calving for our cows showed a 100% match.

A statistical analysis of variance for progesterone at days 12, 21 and 35 after AI showed a significant difference between pregnant cows and both the non-pregnant and embryonic mortality groups ($P > 0.05$). PAG variance analysis at days 24, 30 and 34 after AI showed a highly significant difference ($P > 0.01$) between the non-pregnant and both the embryonic mortality and pregnant groups. On the other hand, variance analysis showed that the middle values for PAG at days 40 and 45 after AI were highly significantly different ($P > 0.01$) between the pregnant and both the non-pregnant and embryonic mortality groups. Our results are similar to results obtained by SZENCI et al. (1998 and 2000) who performed similar research on 138 Holstein Friesian cows. He found significantly lower levels of embryonic mortality in a significantly smaller number of cows (7 cows monitored for PSPB and four cows monitored for PAG). The same author also confirmed that embryonic mortality could be ascertained using PAG or PBSP profiles. He also noticed that levels of progesterone could not be used for diagnosing embryonic mortality, because the corpus luteum remained on the ovary in three out of four cows which had experienced embryonic mortality. Previous research performed by HUMBLOT et al. (1988) using PSPB concentrations showed that although researchers could determine embryonic mortality, it was impossible to determine its exact timing and they could only confirm it if embryonic death occurred after day 24. In contrast, ECTORS et al. (1997). determined the exact timing of embryonic or foetal mortality on the basis of PAG levels, collected weekly during pregnancy, after cloning and embryo transfer. Our results measuring PAG in blood during early pregnancy in cows confirmed all these data, but we monitored a higher number of animals with early pregnancy failures ($n = 21$). In our research, we could distinguish very easily and accurately non-pregnant cows

from cows that had suffered early embryonic mortality. Furthermore, 98% of the cows in our research which had experienced embryonic mortality, lost their embryos 17 to 24 days after AI, evident in a drastic decrease in PAG seven and half to nine days later. The biological half-life for PAG during the first month of pregnancy is seven and half to nine days, as already published by MIALON et al. (1993). Using PAG for pregnancy diagnosis enabled us to prove the existence of live, vital embryos in utero 24 days after conception.

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SAŽETAK

Svrha ovog rada bila je istražiti ulogu trofoblastičnoga proteina (PAG) u fiziologiji rane gravidnosti u goveda kao i mogućnost njegove primjene te ultrazvuka u dijagnostici rane embrionalne smrtnosti. Promatrane su bile 73 krave simentalne pasmine, u dobi od tri do sedam godina, u kojih nisu ustanovljene poteškoće s plodnošću. Sve su držane na pet obiteljskih gospodarstava u Međimurskoj županiji, u uzgojima koji su brojili od osam do 20 krava. Krave su bile podijeljene u tri skupine: gravidne krave (n = 34), krave sumnjive na ranu embrionalnu smrtnost (n = 21) i negravidne krave (n = 18). Na osnovi ultrazvučnih pretraga i laboratorijskoga praćenja razine progesterona i trofoblastičnoga proteina u serumu od 12. do 45. dana nakon UO donijeti su sljedeći zaključci. Na osnovi razine progesterona u serumu moguće je razlučiti gravidne od negravidnih krava, ali nije moguće dijagnosticirati embrionalnu smrtnost. Na temelju razine trofoblastičnoga proteina moguće je sa sigurnošću razlikovati krave kod kojih je nastupila embrionalna smrtnost nakon 16. dana gravidnosti od krava koje nisu postale steone. Embrionalna smrtnost vidljiva je na osnovi znatnoga pada razine trofoblastičnoga proteina u serumu majke, i to 7,5-9 dana nakon uginuća embrija, koliko iznosi i biološki poluzivot trofoblastičnoga proteina u prvom mjesecu gravidnosti. Praćenjem razine trofoblastičnoga proteina u serumu moguće je pouzdano dokazati postojanje živoga vitalnoga embrija u maternici steone krave već nakon 24. dana steonosti. U istraživanju je 98% krava u skupini u kojoj je nastupila embrionalna smrtnost izgubilo embrij kad su bile gravidne 17-24 dana što predstavlja kritično razdoblje za preživljavanje embrija.

Ključne riječi: krava, steonost, embrionalna smrtnost, trofoblastični protein, progesteron, embrij
