

Relationship Between IFN γ Production, Antibody and Hormone Levels in Naturally *Neospora caninum*-infected Pregnant Dairy Cows

Jeong-Hoon Son, Bae-Keun Park, Hwa-Young Son, Ju-Young Jung, Sang-Joon Park*,
Tae-Hwan Kim*, Sung-Whan Cho¹ and Si-Yun Ryu¹

College of Veterinary Medicine, Research Institute of Veterinary Science, Chungnam National University, Daejeon, 305-764, Korea

*College of Veterinary Medicine, Kyungpook National University, Daegu, 702-701, Korea

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Abstract : Neosporosis is a widespread parasitic disease caused by *Neospora caninum*, an intracellular protozoan parasite. It causes economic losses due to reproductive failure. The potential relationship between pregnancy outcomes and levels of IFN γ , hormones, and antibodies in naturally *N. caninum*-infected cows was examined in the blood samples collected every 2 or 4 weeks in 26 pregnant cows from 4 different farms. The mean S/P value of seropositive non-aborting animals (n = 14) reached peak levels 15 weeks prior to parturition, and declined thereafter to parturition. The S/P value 13 weeks prior to abortion in seropositive aborting cows (n=3) remained at high levels, and abortions occurred at 20 (142 days), 26 (185 days), and 28 weeks (199 days) after artificial insemination. IFN γ levels in the seropositive non-aborting group varied by individuals and gestational periods; IFN γ levels stayed at elevated levels or increased abruptly close to abortion in seropositive aborting cows. IFN γ level patterns in the seronegative group (n = 9) were similar to the seropositive non-aborting group, although IFN γ amounts were lower than the seropositive group. The mean progesterone levels in the seropositive non-aborting and seronegative groups decreased markedly 7 weeks prior to parturition. The mean progesterone levels 5 and 7 weeks prior to abortion were lower than the other groups 5 and 7 weeks prior to parturition. The mean 17 β -estradiol levels in the seropositive aborting cows increased close to abortion; the produced amounts were lower than those of seropositive non-aborting and seronegative groups close to parturition. These results suggested that lower levels of progesterone and β -estradiol in *Neospora*-infected cows may lead to increases in IFN γ production and in turn may result in abortion.

Key words : *Neospora caninum*, dairy cow, IFN γ , progesterone, β -estradiol.

Introduction

Bovine neosporosis (*Neospora caninum*) has been reported in many countries (2,8,11,26,35), and may result in economic losses, including still births, neonatal mortality, early fetal death which may present clinically as a return to cycle and/or increased calving intervals, increased culling, reduced milk production, and reduced value of breeding stock (11,37). The relationship between *Neospora* infection and abortion has been established based on a significant association between seropositive animals and abortion (10). Abortion in the field primarily occurs between 4 and 7 months of gestation (5,9,19). Abortions in herds can result from point source infection or parasite reactivation in chronically infected cows (24). ELISA assay using antibodies to *N. caninum* is a useful tool in epidemiological diagnosis, and is used in herd management (3,10,15).

A Th-1/Th-2 response during the gestational period is essential for maintenance of pregnancy due to appropriate immune regulation by cytokines (5,15,21). Certain cytokines are bene-

ficial (CSF-1, TGF β , GM-CSF and IL-10) for pregnancy while others are detrimental (TNF α , IFN γ and IL-12) (12). However, some cytokines may have both beneficial and detrimental effects, depending on cytokine concentration or stage of pregnancy (12). For example, the generation of a Th-1 type response involving pro-inflammatory cytokines including IFN γ may inhibit parasite multiplication by activation of cytotoxic mechanisms (14,21). Conversely, excessive Th-1 responses may result in destruction of normal maternal cells in the placenta and lead to abortion (5,12,15,19,21,31).

Progesterone and 17 β -estradiol play central roles in pregnancy and can influence the cytokine profiles of antigen-presenting cells and T-cells (12). Progesterone is associated with the development of IL-4- and IL-5-producing Th-2 type cells (12,28,29,31). Progesterone supplementation in cows with high *N. caninum* antibody titers increases the risk of abortion by affecting cell-mediated immune responses (5). In addition, 17 β -estradiol promotes both IL-10 and IFN γ secretion by antigen-stimulated T-cell clones. High concentrations of estradiol favor IL-10 production, while lower concentrations favor IFN γ production (12). The relationship between IFN γ production, hormone and antibody levels, and pregnancy outcomes in naturally

¹Corresponding author.
E-mail : syryu@cnu.ac.kr

Neospora-infected dairy cows was examined in the present study.

Materials and Methods

Animals

Animals came from farms (n = 4) located near Gongju city and Yeongi gun, Chungnam Povince, Korea. The farm had been previously exposed to *N. caninum* as determined by ELISA.

All experimental animals were tested for confirmation of tuberculosis and brucellosis prior to artificial insemination (AI). Cows received a killed mixed vaccine (Bar Vac Elite 4-HS, Boehringer Ingelheim, St Joseph, MI) which included bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, Myxovirus parainfluenza3, bovine respiratory syncytial virus, and *Haemophilus sommus*. All cows were bred by AI. Pregnancy was confirmed 5 to 7 weeks after AI by transrectal ultrasonography and was re-confirmed 10 to 12 weeks post-AI by rectal examination. Dams were rechecked for tuberculosis by the PPD test and brucellosis by the Rose Bengal test in case of abortion.

Blood sampling

Blood was collected every 2 or 4 weeks by coccygeal venipuncture after the rectal confirmation of pregnancy until either parturition or abortion. Blood samples were centrifuged and sera were stored at -70°C until analysis.

Serological assay

Serum samples were diluted 1:10 in sample diluents and tested for *N. caninum* antibodies with an enzyme linked immunosorbent assay (ELISA) kit (Chekit *Neospora*, IDEXX Laboratories, Liebefeld-Bern, Switzerland) according to the manufacturer's instructions. Duplicate determinations were performed on each sample and the optical density (OD) was measured. For each sample, the ratio of the OD of evaluated sample to the mean OD of the positive control was calculated as the S/P value (%): $\text{S/P value (\%)} = (\text{sample mean OD} - \text{negative control mean OD}) / (\text{positive control mean OD} - \text{negative control mean OD}) \times 100$. Samples with an S/P value ≥ 40 were classified as the positive infected group, S/P value ≥ 30 to < 40 were classified into the suspect group, and S/P value < 30 were classified into the negative group according to manufacturer's recommendations.

IFN γ assay

Duplicate serum samples were tested for IFN γ using ELISA. Briefly, a purified bovine polyclonal capture antibody (Pierce, Rockford, IL) was adsorbed overnight to a 96-well assay plate (Costar, Corning, NY) at a concentration of 5 $\mu\text{g/ml}$ in 0.1M Na_2HPO_4 (pH 9.0) at 4°C , washed, and blocked with 20% FBS (Hyclone, Logan, UT) in PBS. Serum samples and 2-fold serial dilutions of recombinant bovine IFN γ standards (Thermo Scientific, Pierce, Rockford, IL) were dispensed and incubated overnight at 4°C . Samples were washed with 0.5 ml/L Tween-20 (Sigma, St. Louis, MO) in PBS and biotinylated detection polyclonal antibody (Pierce) was added to each well. The ELISA was developed using Avidin-horseradish peroxidase

(Vector Laboratories, Burlingame, CA) and ABTS (2, 2'-Azino-bis(3-Ethylbenzthiazolin-6-sulfonic acid) substrate in 0.1M citric acid (Sigma). Spectrophotometric readings were recorded at 405 nm, and mean cytokine concentrations for duplicate assays were calculated. Our limit of detection for IFN γ was 15.625 pg/ml.

Hormone assay

Hormone levels were assessed with solid-phase Coat-A-Count ^{125}I radioimmunoassays provided by the Diagnostic Products Corporation (Los Angeles, CA). Water-based dilutions of all standards and controls were used to determine hormone concentrations. Samples, standards, and controls (400 μl) were pipetted into antibody-coated tubes, and radio-labeled tracer (1 ml) was added to each tube. All tubes were incubated overnight. Tubes were aspirated and counted for 3 min with a Gamma counter (EG & G, Wallce, Finland). Assay reliability was evaluated with inclusion of control samples with known hormone concentrations in each assay. Intra- and inter-assay coefficients of variation (CV) of the progesterone assay were 5.3% and 8.6%, respectively. Intra- and inter-assay CV of the 17β -estradiol assay were 8.7% and 12.3%, respectively.

Results

Serum antibody

Serum samples were assayed with an ELISA kit at the time of AI to assess the prevalence of neosporosis. As shown in Table 1, the percentage of seropositive cows was relatively high (65.4%), and abortion occurred in seropositive cows at a single farm (17.6%). Abortion times from 3 cows were 20 (142 days), 26 (185 days) and 28 weeks (199 days) after AI, respectively.

The mean S/P value of non-aborting seropositive animals reached peak levels ($123.14 \pm 21.65\%$) on 15 weeks prior to parturition and subsequently decreased over time. Further, the relative percentage to the peak levels during 7 weeks prior to parturition sharply decreased (80.1%, 77.46%, 65.1%, 48.77%, 46.15%) as seen in Fig 1. The S/P value during 13 weeks prior to abortion did not demonstrate significant changes in seropositive aborting cows and remained at high levels (Fig 2). There were no significant changes in the mean S/P value (Fig 1) throughout the examination period in seronegative control group, and all 9 cows remained seronegative throughout this period.

Table 1. *N. caninum* seropositivity and abortion rates

Farm	n	Seropositive cows (%)	Seropositive aborting cows (%)
A	11	7(63.6) ^a	-
B	10	7(70) ^a	3(42.9) ^b
C	2	2(100) ^a	-
D	3	1(33.3) ^a	-
	26	17(65.4) ^a	3(17.6) ^b

^awith respect to the total number of cows.

^bwith respect to the total number of seropositive cows.

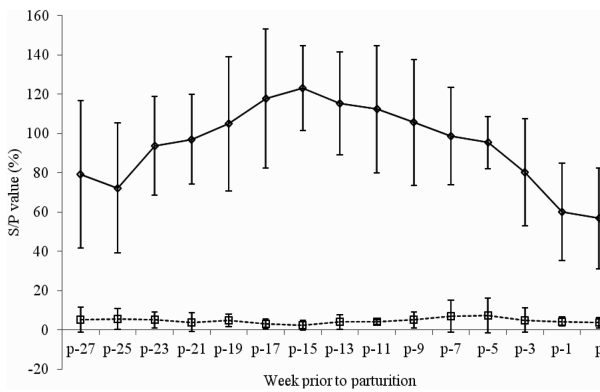


Fig 1. Antibody titers during pregnancy in seropositive non-aborting (◇) and seronegative cows (□). Results are represented as the mean ± SD. p; parturition.

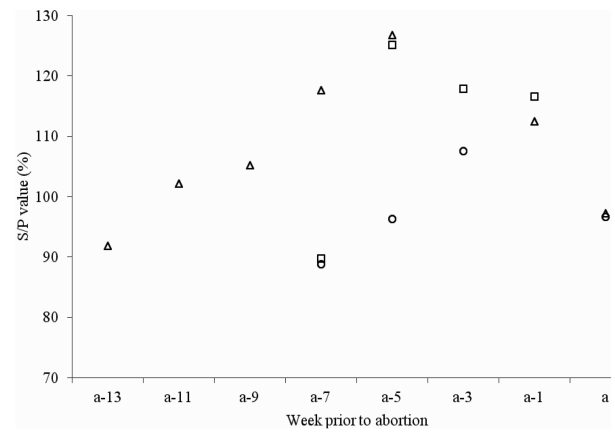


Fig 2. Antibody titers prior to abortion in 3 seropositive aborting cows. ○; B-22 cow, □; B-031 cow, △; B-342 cow. a; abortion.

IFN γ responses

IFN γ levels in seropositive non-aborting cows varied from undetectable levels to 1640.8 pg/ml according to an individual in the same gestational period; the levels varied with the gesta-

tional period in a single individual (Table 2).

Seropositive aborting cows maintained very high levels of IFN γ production or markedly increased IFN γ production close to abortion (Table 3). The general patterns of IFN γ levels in

Table 2. Serum IFN γ concentrations (pg/ml) in seropositive non-aborting and seronegative cows

Cow	Week prior to parturition														
	p-27	p-25	p-23	p-21	p-19	p-17	p-15	p-13	p-11	p-9	p-7	p-5	p-3	p-1	p
A-2 ^a										1187.2	873.5	181.5	-	-	-
A-14 ^a	-	-	-	-	-	-	-	-	19.5	19.1	17.1	-	-	-	-
A-17 ^a															
A-18 ^a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A-19 ^a										508.2	-	41.0	21.5	16.2	16.6
A-22 ^a						217.0		393.9	818.0	421.0	206.1	41.8	15.7	-	19.6
A-28 ^a						992.1	988.6	1195.7	1173.1	1130.2		1114.8	967.1	717.1	643.3
B-005 ^a		342.1	552.8	606.4											1305.2
B-030 ^a		95.6		142.15	26.5	49.2	40.9	135.1	-	15.7	-	-	-	-	-
B-83 ^a	1326.7		1308.8	1398.1	1445.7	1145.7	1088.6								1201.7
B-253 ^a						68.2	683.8	505.2							17.8
C-62 ^a										42.0		43.2		39.7	73.0
C-66 ^a													1640.8	1373.9	1375.1
D-66 ^a		-	-	-	-	-	29.3	31.0	15.7	17.5	21.8	-	-	-	18.8
A-5 ^b												18.1	101.8	-	-
A-11 ^b	-	-													
A-13 ^b											182.5	257.3	249.0	109.1	46.3
A-29 ^b								19.0	16.0	18.6	18.2	-	-	20.1	15.9
B-16 ^b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-353 ^b	16.4	23.0													
B-355 ^b	73.5	29.1		18.4											
D-63 ^b	152.4	222.9	190.8	147.7	167.3	83.5	50.5	54.5		97.6	163.4	172.0	179.2	188.8	
D-68 ^b		327.8	249.1	428.0	576.7	479.3	338.0	367.7	235.0		264.0		271.4	230.3	170.8

^aseropositive non-aborting cow, ^bseronegative cow, p; parturition, -; un-detection.

Table 3. Serum IFN γ concentrations (pg/ml) in seropositive aborting cows

Cow	Week prior to abortion							
	a-13	a-11	a-9	a-7	a-5	a-3	a-1	a
B-22				2997.5	3108.5	3061.2		3204.2
B-031				1065.1	1858.2	2099.9	2296.4	
B-342	344.6	561.2	779.6	837.8	1040.0		1337.5	1507.2

a; abortion

seronegative cows were lower than seropositive non-aborting cows (Table 2). A single animal in this group demonstrated high production responses in spite of S/P values is less than 5.4 during gestation.

Hormone responses

Based on our findings that the S/P value in the seropositive non-aborting group decreased 7 weeks prior to parturition, while changes in the S/P value in the seropositive aborting group were less distinct, time points from 7 weeks prior to parturition or abortion (p-7 to p, or a-7 to a) were chosen to ascertain whether or not hormonal responses were involved in these patterns.

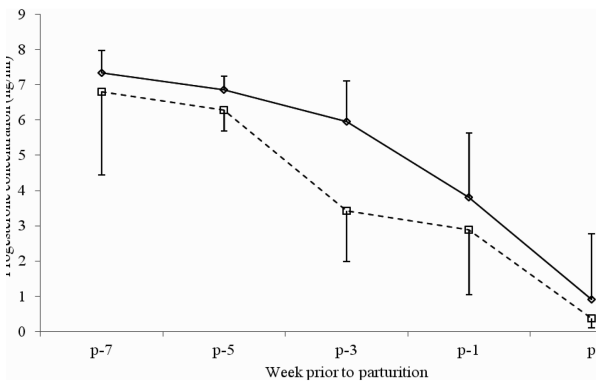


Fig 3. Mean (\pm SD) progesterone concentrations prior to parturition in seropositive non-aborting (◇) and seronegative cows (□). p; parturition

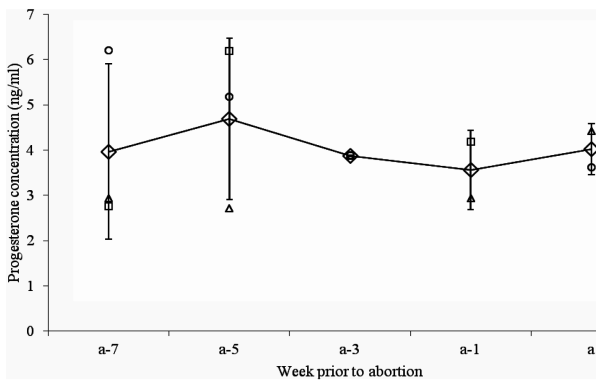


Fig 4. Progesterone concentrations prior to abortion in 3 seropositive aborting cows. ○; B-22 cow, □; B-031cow, △; B-342 cow, ◇; mean \pm SD. a; abortion.

The mean progesterone levels decreased markedly from 7.34 (p-7) to 0.92 ng/ml (p) in seropositive non-aborting cows (Fig 3). The seronegative control group demonstrated similar patterns to the seropositive non-aborting group (6.81 to 0.37 ng/ml), as seen in Fig 3. One cow demonstrated a slightly decreased pattern close to abortion (6.21 to 3.62 ng/ml) in the seropositive aborting group. Two cows did not demonstrate significant changes ranging from 2.72 to 6.19 ng/ml (Fig 4). However, the mean concentrations at a-7 (3.97 ng/ml) and a-5 (4.69 ng/ml) weeks were lower than those of seropositive non-aborting at p-7 (7.34 ng/ml) and p-5 (6.87 ng/ml) and seronegative groups at p-7 (6.81 ng/ml) and p-5 (6.29 ng/ml) weeks.

Changes in 17 β -estradiol levels in seropositive non-aborting and seronegative groups demonstrated similar patterns, and levels were abruptly increased just prior to parturition (Fig 5). However, the mean levels at each time point 7 weeks prior to abortion in seropositive aborting cows were lower (0.23, 0.47, 0.97, 1.53, 2.93 pg/ml) than seropositive non-aborting (1.01, 1.96, 3.03, 18.63, 19.03 pg/ml) and seronegative groups (2.24, 3.45, 2.59, 7.17, 10.44 pg/ml) 7 weeks prior to parturition (Fig 5, 6).

Discussion

Seropositive non-aborting cows remained seropositive at 2-to-4 week screening intervals during pregnancy in the present study. Mean *N. caninum* antibody titers during the mid-gestational period were higher compared to other periods and declined to parturition. This was supported by antibody levels

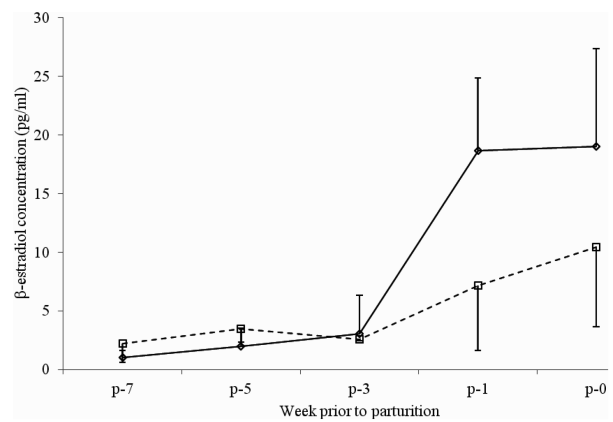


Fig 5. Mean (\pm SD) β -estradiol concentrations prior to parturition in seropositive non-aborting (◇) and seronegative cows (□). p; parturition.

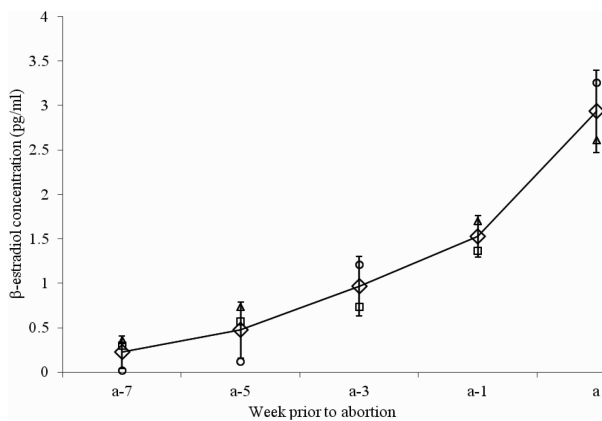


Fig 6. β -estradiol concentrations prior to abortion in 3 seropositive aborting cows. \circ ; B-22 cow, \square ; B-031 cow, \triangle ; B-342 cow, \diamond ; mean \pm SD. a; abortion.

which peaked at 6 to 7.5 months of gestation (6) or 2 to 4 months prior to parturition and followed by a decrease (35). Several similar reports have demonstrated that antibody levels rose during mid- and late-gestation (15,23). The rises in antibody levels could reflect the enhanced humoral responses by parasite activation and multiplication in the host (15,23,35).

In our study, seronegative cows demonstrated consistently seronegative responses at 2-to-4 week interval screenings during pregnancy. Several reports also demonstrated that changes in serological status during pregnancy are very low in endemically infected herd (24). These facts suggested that horizontally transmitted infection in the present study did not occur.

Abortion occurred between 20 weeks and 28 weeks of gestation in a total of 3 of 17 seropositive cows in the present study; previous reports suggested that most abortions occurred between 4 and 7 months of gestation (5,9,19). Antibody titers in seropositive aborting cows in our study continued to demonstrate high levels during the gestational period prior to abortion. Several reports demonstrated that the rises in antibody levels occurred prior to abortion and elevated antibody production or transiently increased antibody responses could serve as important indicators for abortion risk (23). Abortion rates in our study were 17.65% in seropositive cows, and could be secondary to *N. caninum* infection (10,20,24).

Cell-mediated immune responses are important for protection against *N. caninum*, and the character of the immunological response at the materno-fetal interface is important in determining whether *N. caninum* infection will lead to fetal death, fetal survival, and congenital transmission of infection (14,31). The generation of Th-1 type responses inhibits parasite multiplication by activation of cytotoxic mechanisms and IFN γ production (14,21,38). However, this pro-inflammatory cytokine result in the destruction of normal maternal cells in the placenta, causing abortion (2,12,16,21). Cell-mediated immune responses associated with IFN γ production were demonstrated in *N. caninum*-infected cattle (14,16,19,21). These results suggested that a certain IFN γ level during gestation in *N. caninum*-infected cows displayed beneficial effects on preg-

nancy maintenance, and when the IFN γ amounts within definite period of pregnancy result in high levels or increases acutely, abortions may occur as a detrimental effect. Our data suggested that maintenance of high IFN γ levels or marked increases in IFN γ production close to abortion in seropositive groups resulted in abortions due to IFN γ detrimental effect. In addition, it is likely that naturally-infected seropositive non-aborting cows have protective immunity against *N. caninum* as beneficial effects (39). Based on the previous reports on IFN γ effects in *Neospora*-infected cows, it is difficult to explain why 4 cows in the seropositive non-aborting group which had relatively high levels of IFN γ compared to the remaining 10 cows did not have abortions. Because the IFN γ level varied according to the individual and the gestation period of the individual, these results suggest that it is difficult to estimate accurately how much IFN γ produced in naturally-infected pregnant cows can promote abortion or protect against abortion.

High progesterone levels were maintained during pregnancy with steadily increase from early- to mid-gestation, and significantly fall at calving (4,15,30). These enhanced progesterone levels promote the production of Th-2 type cytokines, including IL-4 and IL-5 (12,17,28,29,31). Gestational progesterone diminishes the Th-1 response favoring pregnancy maintenance, with a bias towards Th-2 dominance. Conversely, a shift towards Th-1 dominance has been associated with abortion (4,7,13,15). The seropositive non-aborting group demonstrated higher progesterone levels prior to parturition compared to the seronegative group in the present study. This suggested that *Neospora* seropositivity induced higher progesterone production, resulting in reduced Th-1 activity (13), which could eventually lead to weakening of *N. caninum* protective immunity (15,38). Since cell-mediated immune mechanisms play an important role in reducing parasitaemia by diminishing parasite multiplication (5,14,38), we reasoned that these results explain why *Neospora*-infected cows have persistent seropositive during the experimental period.

Previous studies demonstrated that serum progesterone levels in abortion were significantly lower than those in normal pregnancies (1,34). A similar result was observed in the present study in which the mean progesterone levels of aborted 3 cows at 20-28 weeks after artificial insemination were lower than that of seronegative groups. These results suggest that the maintenance of the progesterone level within the range of control group is important to successful pregnancy.

The studies on the effects of β -estradiol on cytokine profiles demonstrated contradictory results. Several studies demonstrated that β -estradiol secreted in large amounts during pregnancy had no effects on Th-1 and Th-2 type cytokine production by T-cell lines and clones (28), and physiological β -estradiol levels did not reactivate *N. caninum* in mice (18). In contrast, results of several studies varied from suppression of IFN γ expression (22,32,33) to enhancement of IFN γ secretion by antigen-stimulated T cell clones (12). The mean 17 β -estradiol levels in seropositive aborting cows were lower than those of seropositive non-aborting groups in the present study. This

suggested that the maintenance of lower levels is related to high IFN γ level in aborting group.

The 17 β -estradiol levels were elevated from the end of first trimester and peaked at parturition (25,27). Estradiol levels in abortion were significantly lower than those in normal pregnancies (1,36). The mean estradiol levels in seropositive aborting cows prior to abortion were lower than those of seronegative groups prior to parturition in the present study.

To conclude, our data suggested that IFN γ levels in seropositive aborting cows were higher than those of seropositive non-aborting cows, while the levels of progesterone and β -estradiol were lower than those of seropositive non-aborting cows.

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*Neospora caninum*에 자연 감염된 임신우에서 IFN γ , 항체 및 호르몬 수준의 상관관계

손정훈* · 박배근* · 손화영* · 정주영* · 박상준** · 김태환** · 조성환*¹ · 류시윤*¹

*충남대학교 수의과대학 및 동물의과학연구소, **경북대학교 수의과대학

요 약 : 네오스포라 감염증은 세포내성 원충성 기생충인 *Neospora caninum*에 의해 발생하는 질병으로 전세계적으로 발생하고 있으며, 소에서는 번식장애로 인한 막대한 경제적 손실을 유발한다. 본 연구는 4 곳의 각기 다른 목장으로 부터 *N. caninum*에 자연 감염된 양성우와 음성우를 포함한 26두의 임신우를 선정하여 2주 내지 4주 간격으로 혈액을 채취하여 호르몬, 항체가, IFN γ 수준과 유산 혹은 분만과의 상관관계를 조사하였다. 유산하지 않은 *Neospora* 양성우 14두의 S/P 평균값은 분만 전 15주에 최고치를 보였고, 이후 분만 때까지 감소하였다. 유산한 양성우 3두의 유산 시기는 각각 인공 수정 후 20주 (142일), 26주 (185일), 28주 (199일)였으며, 이들의 S/P값은 유산 전 13주부터 유산 때까지 높은 수준을 유지하였다. 유산하지 않은 양성우에서 IFN γ 수준은 개체에 따라 그리고 동일한 개체라도 임신기간이 경과함에 따라 다양하게 나타났으며, 유산한 양성우에서 IFN γ 수준은 계속 높은 상태를 유지하거나 또는 유산시점에 근접하여 급상승하였다. *Neospora* 음성우 9두의 IFN γ 수준은 양성우보다 낮았지만, 그 양상은 유산하지 않은 양성우와 유사하였다. 유산하지 않은 양성우와 음성우에서 평균적인 progesterone수준은 분만 전 7주부터 뚜렷하게 감소하였다. 유산한 양성우에서는 유산 전 5주와 7주의 평균적인 progesterone 수준은 정상 분만한 음성우나 양성우의 분만 전 5주와 7주에 측정된 평균 progesterone 수준보다 낮았다. 유산한 양성우에서 17 β -estradiol의 평균 수준은 유산 시점에 근접할수록 증가하였지만, 혈청내 총양은 유산하지 않은 양성우와 음성우에서 분만시점에 근접하여 측정된 평균 17 β -estradiol 수준보다 더 낮았다. 이상의 결과로 미루어 보아 *Neospora*에 감염된 소에서 17 β -estradiol과 progesterone 수준의 저하는 IFN γ 산생을 증가시켜, 결국에는 유산을 초래할 수 있다고 생각된다.

주요어 : *Neospora caninum*, 젖소, IFN γ , progesterone, β -estradiol