

A STUDY OF SPONTANEOUS ROSETTE FORMATION  
IN THE BOVINE FEMALE DURING THE IMMEDIATE  
POSTESTRAL PERIOD

by

HISHAM AHMAD FAHMI

B.V.M.S., University of Baghdad, 1972

---

A THESIS

Submitted in partial fulfillment of the  
requirements for the degree

MASTER OF SCIENCE

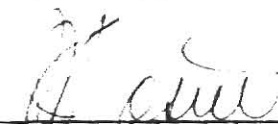
Department of Surgery and Medicine

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1981

Approved by:

  
\_\_\_\_\_  
Major Professor

**THE FOLLOWING  
PAGES ARE BADLY  
SPECKLED DUE TO  
BEING POOR  
QUALITY  
PHOTOCOPIES.**

**THIS IS AS  
RECEIVED FROM  
CUSTOMER.**

SPEC  
COLL  
LD  
2668  
J4  
1981  
F33  
c.2

11200 068180

## CONTENTS

	Page
ACKNOWLEDGEMENTS . . . . .	3
INTRODUCTION . . . . .	5
REVIEW OF LITERATURE . . . . .	8
MATERIALS AND METHODS . . . . .	16
Trial 1 . . . . .	18
Trial 2 . . . . .	18
Trial 3 . . . . .	18
RESULTS . . . . .	20
Trial 1 . . . . .	20
Trial 2 . . . . .	20
Trial 3 . . . . .	21
Pregnancy Results . . . . .	21
DISCUSSION . . . . .	23
Tables . . . . .	27
Figures . . . . .	34
REFERENCES . . . . .	37
APPENDICES . . . . .	40

## ACKNOWLEDGEMENTS

#### ACKNOWLEDGEMENTS

I would like to share the pleasure of this work with the people who supported me and made this work possible. I would like to convey special gratitude to Dr. Jacob E. Mosier for his unlimited support and encouragement, Dr. Stanley M. Dennis for his cooperation and participation in my examining committee, together with Dr. Russell A. Frey. Special thanks to Dr. David Carnahan for his tremendous support, advice and assistance. I would like to thank Dr. Neil V. Anderson for providing laboratory materials and facilities together with his technician, Aggie Moll. I am also very grateful to Mary Schmidt at the Kansas State University Dairy Teaching and Research Center for her great help to me.

A STUDY OF SPONTANEOUS ROSETTE FORMATION IN THE  
BOVINE FEMALE DURING THE IMMEDIATE  
POSTESTRAL PERIOD

## INTRODUCTION

Early detection of pregnancy is an appealing objective for reproductive biologists and of important practical application in animal husbandry. Early pregnancy detection has been successfully demonstrated in many species by using immunological material and conceptus relationships.

Rosette inhibition testing has been used to demonstrate early pregnancy in sheep, mice, and women<sup>3,10,12,13,18,19</sup>. In sheep, pregnancy was detected as early as 72 hours after mating<sup>10</sup> and in mice within six hours after mating<sup>18</sup>. The immunological concept for detecting early pregnancy is based on spermatozoa, zygote, embryo and fetus antigens being foreign to the mother<sup>2,17,18</sup>. Therefore, immunological rejection of the new conceptus could be expected, but that doesn't occur in nature<sup>18</sup>.

Different antibodies against spermatozoa and the products of conception have been detected<sup>6</sup>. Serum antispermatozoa antibodies have been detected in women with an unexplained infertility<sup>5,6</sup>. Antibodies against the zona pellucida were also detected in women with an unexplained infertility<sup>16</sup>. In cattle, lymphocytotoxic antibodies against fetal antigens were reported in heifers during their first gestation and in cows after the first gestation<sup>15</sup>.

An early pregnancy factor (EPF), believed to be an immunosuppressive factor, prevents immunological rejection of the conceptus during early stages of the pregnancy<sup>10,13,17,18</sup>. The rosette inhibition test is used to detect the suppressive effect of EPF on the rosette formation by material T lymphocytes<sup>4,17,18</sup>.

T lymphocytes have the ability to form rosettes when incubated with heterologous red blood cells<sup>18,19</sup>. T lymphocytes from pregnant females demonstrated more inhibition during the rosette inhibition test<sup>3,10,12,13,14</sup>. Rosette inhibition is a slow tedious procedure at the present time. It involves use of anti-T lymphocyte serum in a bifoldded series dilution and specific calculation for the inhibition titer<sup>13,18</sup>.

Early diagnosis is helpful for early confirmation of conception, embryo mortality, pregnancy diagnosis in dornor and recipient animals during embryo transfer and in seasonal breeders like horses and sheep, in addition to its importance in studying infertility; all have useful application in animal production and economics and are of scientific interest<sup>3,10,14,18</sup>.



## REVIEW OF LITERATURE

T lymphocytes are reported in thymus, paracortical zone of lymph nodes, periarteriolar sheath of spleen and in peripheral blood. They are found in less proportions in bone marrow, lymph node cortex and splenic follicles<sup>19</sup>. T lymphocytes have been demonstrated to have certain characters that differentiate them from B lymphocytes<sup>18,19</sup>.

Tizard<sup>19</sup> cited important characters of T lymphocytes. He indicated that they largely mediated cell-mediated immune responses and also influenced antibody production. T lymphocytes do not carry immunoglobulin molecules on their surface. One important character of T lymphocytes is that they have erythrocyte receptors and are capable of forming rosettes when heterologous erythrocytes are allowed to attach. This discovery was important in T lymphocyte assays and in rosette inhibition<sup>9,18,19</sup>.

To collect T lymphocytes, lymphocytes are first isolated from blood. Most methods for isolating lymphocytes involve Ficoll-hypaque solution<sup>1,9,13,15</sup>. Although, there are many methods for distinguishing T cells from B cells, few methods have been described for isolating T cells<sup>19</sup>.

Madsen et al<sup>9</sup> isolated T cells from other lymphocytes by allowing T cells to form rosettes with washed sheep erythrocytes, followed by use of Ficoll-hypaque and gradient centrifugation. Non-rosette forming cells were isolated from the interface while rosette forming cells were harvested from the pellet. This technique was called E-rosette gradient centrifugation<sup>9</sup>.

Julius et al<sup>7</sup> described a rapid technique for isolating T cells by using a nylon wool column. The nylon wool was soaked in normal saline and then washed three to four times in distilled water to remove toxic materials. The nylon wool was transferred to 5 ml plastic syringes to make the column and the lymphocyte solution was added by gentle dripping. The T lymphocytes

were isolated by eluting the column with warm medium after 45 minutes incubation at 37° C; the eluted fluid was centrifuged and 84 to 91% of the cells isolated were T lymphocytes with a possible contamination of 2 - 5% of B lymphocytes.

E-rosette formation was one method for distinguishing T cells from B cells in a lymphocyte population<sup>9,19</sup> by simply allowing washed erythrocytes to adhere to T lymphocytes that have erythrocyte receptors<sup>19</sup>. Adherence of erythrocytes to lymphocytes was described as rosette formation<sup>9,13,18</sup>. This affinity for rosettes formation was utilized for isolating T cells<sup>9</sup> and for the rosette inhibition test<sup>4,11,13,17,18</sup>. The rosette inhibition test was originally used for assessing immunosuppressive activity of anti-lymphocyte serum (ALS) and for preparing antilymphocytic globulin<sup>3,11,17</sup>. The test is based on measuring the inhibitory titer of ALS on rosette formation after incubating lymphocytes in different ALS dilutions<sup>13</sup>. The lymphocytes are allowed to form rosettes by incubating them with washed heterologous erythrocytes after one and a half hours incubation with ALS<sup>13</sup>.

Pregnancy occurs after ovum (a) are fertilized by spermatozoa producing a zygote. The male gamete (spermatozoan) are considered foreign to the female as are all the products of pregnancy<sup>18</sup>.

The zygote, embryo and fetus bear antigens that are foreign to the maternal immune system, therefore, they are expected to provoke immune reactions that might result in an immunological rejection of the conceptus; however, this is rare in nature<sup>3,19</sup>.

The spermatozoa sometimes provoke antibody stimulation capable of agglutinating human spermatozoa. Such antibodies may be demonstrated in women with unexplained infertility<sup>5,6</sup>. The antibodies are capable of

causing tail to tail and head to head agglutination of spermatozoa<sup>5</sup>. Such antibody-antigen reaction may follow natural copulation, therefore, it is concluded that the female immune system is able to recognize spermatozoa within the genital tract<sup>5,6</sup>.

Nishimoto et al<sup>16</sup> reported autoantibodies to zona pellucida in infertile and aged women. These antibodies were associated with women with unexplained infertility. This finding supported the need for appropriate immune suppression to protect the ovum during the early stages of pregnancy. Immunological failure may lead to recognition of the zona pellucida in the developing ova by the body immune system with formation of antibodies capable of reacting with the zona pellucida of the ovum<sup>6,16</sup>. Such antibodies were believed to coat the zona pellucida that might prevent sperm penetration and failure of fertilization or by inhibiting implantation of the growing zygote<sup>6,16</sup>.

Bracket et al<sup>2</sup> indicated in their study on fertilization and early development of cow ova, that remnants of spermatozoa stayed for a long time within the zona pellucida after fertilization. An additional factor is morphological changes of the ovum after fertilization. Immunological studies on this aspect have not been reported in cattle.

Newman and Hines<sup>15</sup> reported maternal antilymphocyte antibodies in first gestation bovine fetuses. They suggested maternal recognition of fetal lymphocytes might result from migration of fetal lymphocytes into the maternal system.

The presence of different antibodies in the maternal circulation against spermatozoa and products of conception suggested the possibility of maternal immunological reaction to pregnancy. the presence of antibodies also suggested absence of a totally impermeable barrier between the mother and the conceptus<sup>15,18</sup>.

During early pregnancy, the conceptus takes a similar course in most mammals but as pregnancy progresses, different relations are established between the dam and its conceptus. In farm animals, the conceptus attaches itself to the endometrium by the fetal and maternal placenta. In the cow, the fetal placenta (cotyledone) attaches to the endometrium in confined areas called caruncles (collectively placentomes) that establishes the placental barrier between the dam and conceptus, while in man, primates and some other animals, the embryo embeds itself within the endometrium thus providing greater opportunity of exposure to the maternal immune system<sup>15,17,18</sup>. Normally, the placenta is a barrier between the maternal immune system and conceptus antigens. Different types of placentation in different species may result in different levels of permeability of the placental barrier. However, the period before implantation is characterized by highly similar direct contact between the conceptus and genital tract in domestic animals and man<sup>18</sup>.

Several authors have indicated that the conceptus is protected by different methods against maternal immunological attack during the different periods of gestation. In all periods, however, at least partial suppression of the maternal immune response might take place<sup>13,18,19</sup>. Suppression of the maternal response is thought to occur from an alteration of cell-mediated immunity during pregnancy from altered T lymphocytes response. This has been determined in sheep and women<sup>12,13,14,17,18</sup>.

Shaw and Morton<sup>18</sup>, suggested that immunosuppression during pregnancy is likely to occur through qualitative changes in lymphocytes. At different stages of pregnancy, a variety of proteins have been reported to have immunosuppressive properties. Among these are a-feto-protein, pregnancy associated macroglobulin and human chorionic gonadotropin (HCG)<sup>13,17</sup>.

A specific protein detected during early stages of pregnancy with immunosuppressive properties has been designated as the early pregnancy factor (EPF). The EPF has been consistently detectable within six hours in mice and within 72 hours in sheep following fertile matings<sup>13,17,18</sup>. This factor is reported to be continuously present during the first two trimesters of pregnancy<sup>10,17</sup>. Other proteins of immunosuppressive properties have not been reported during the early stages of pregnancy<sup>12,13,17</sup>.

The immunosuppressive properties of pregnancy proteins have been measured by their effect on T lymphocytes activity. One method for measuring lymphocyte activity is rosette inhibition testing for determining qualitative changes in lymphocytes<sup>3,10,12,13,14,17,18</sup>. Detection of EPF by the rosette inhibition test is a useful method for detecting pregnancy primarily because EPF is thought to be present during the early stages of pregnancy<sup>3,10,13,14</sup>.

Evison et al<sup>3</sup> were able to detect pregnancy in sheep within 24-48 hours after mating by the rosette inhibition test using serum samples from pregnant and non-pregnant ewes. A detection of early embryonic mortality in ewes was suggested to be possible within 24 hours of death by the same test.

Early detection of pregnancy in domestic animals is important for economic and management reasons and also for infertility studies<sup>3,12,13,14</sup>. It was not possible to detect pregnancy in domestic animals before return of estrus or expected return of estrus. Progesterone levels increase with pregnancy and decrease with return to estrus<sup>8,14,17,18</sup>. Pregnancy detection was possible with progesterone assays in cows when three samples were taken at eight-day intervals; a 98% accuracy was reported<sup>8,18</sup>.

Early pregnancy detection by the rosette inhibition test was suggested to be an efficient biological test in many species<sup>3,10,12,13,14,18</sup>.

Morton et al<sup>10</sup> suggested using the rosette inhibition test for detecting EPF within 72 hours after mating. They reported EPF during the first month of gestation, and in some cases to four months of gestation. Morton et al<sup>12</sup> detected early pregnancy in seven ewes after mating by the rosette inhibition test on serum samples taken from the jugular vein and suggested the possibility of detecting early embryonic mortality.

Nancarrow et al<sup>14</sup> demonstrated the efficiency of the rosette inhibition test for detecting induced embryonic death in sheep. They monitored the decrease in the rosette inhibition titer that indicated decrease in EPF after surgical removal of the embryos from pregnant ewes and by inducing artificial abortion by an analogue of prostaglandin F-2a\*. They demonstrated the possibility of detecting ovine embryonic mortality within 48 hours of occurrence.

Morton et al<sup>13</sup>, using the rosette inhibition test, demonstrated the presence of an early pregnancy factor in serum of pregnant women. They tested lymphocytes from normal donors after incubation with serum from pregnant women. They excluded the effects of human chorionic gonadotropin by similar testing of the effect of this hormone. They concluded that serum of pregnant women contained a factor with immunosuppressive properties, likely to be an early pregnancy factor (EPF) in women that may be necessary for continued viability of the embryo during early pregnancy<sup>13</sup>.

The rosette inhibition test has been used to determine the inhibition titer of antilymphocyte serum (ALS) on the rosette formation of lymphocytes

\*Cloprostenol (estrumate<sup>R</sup>), I.C.I., Australia.

of pregnant animals and to test the effect of serum of pregnant animals on the inhibition titer of ALS on rosette formation<sup>3,4,10,11,12,13,14,17</sup>. When used for this purpose, the test was slightly modified to test the immunosuppressive properties of pregnant serum. Pregnant serum was inactivated for incubating at 56° C for 30 minutes to exclude other factors that might affect the rosette formation. Heterologous erythrocytes were washed and absorbed by pregnant serum as were normal lymphocytes from non-pregnant donors. Different dilutions of lymphocytes were incubated in antilymphocyte serum. Pregnant sheep and women had 25% inhibition in rosette formation at ALS dilution titer. These results corresponded to the dilution titer with non-pregnant sheep and women<sup>3,10,12,13,14</sup>.

While the test was considered to be highly accurate for detecting early pregnancy, it is not 100% accurate. It is a matter of unreality to expect absolute accuracy with any biological test<sup>17,18</sup>.



## MATERIALS AND METHODS

This study was conducted from January 16 to May 25, 1981, on 44 Holstein dairy cows, aged 2 to 7 years, housed within the Kansas State University Dairy Teaching and Research Center (KSUDTRC).

The cows were kept in open pens and observed daily for signs of estrus. The cows in estrus were bred artificially with frozen semen obtained from the Kansas Artificial Breeding Service Unit (KABSU).

Blood samples were drawn from the tail vein into test tubes containing 100 units of heparin (10 units per 1 ml of blood). Samples were drawn from 22 cows within the first four days after breeding. Other samples were drawn from 22 cows within the first four days after estrus. The study was divided into three trials and two groups of blood samples were tested in each trial. Spontaneous rosette formation (E-rosette test) was carried on the T lymphocytes in each sample of all groups within two hours after collection.

Ten ml heparinized blood from each sample was diluted with an equal volume of phosphate buffer solution (PBS), divided equally and gently layered on 8 ml Ficoll-paque solution<sup>A</sup> (density = 1.077 at 25° C) and centrifuged at 575 g for 30 minutes<sup>B</sup>. The lymphocytes were then harvested from the interface band into 5 ml PBS by a Pasteur pipette. The lymphocytes were centrifuged and the pellet washed in 10 ml PBS. Residual erythrocytes were removed by osmotic shock. The lymphocyte pellet was resuspended in 10 ml cold Rosville Park Memorial Institute (RPMI) medium

---

A Ficoll-paque, Pharmacia Fine chemicals, Piscataway, New Jersey.

B International Equipment Company, Model HN-S Centrifuge, Needham Heights, Mass.

1640<sup>C</sup>, centrifuged and resuspended into 2 ml RPMI with 10% inactivated fetal calf serum (FCS)<sup>D</sup>.

The T lymphocytes were isolated by a nylon wool column<sup>E</sup>. The column was prepared and washed by 20 ml RPMI with 10% FCS. The lymphocytes in 2 ml RPMI 10% FCS were gently dropped into the column and incubated for 45 minutes at 37° C in a CO<sub>2</sub> incubator<sup>F</sup>. The column was gently eluted by dripping 20 ml RPMI 10% FCS. The eluted fluid was centrifuged to concentrate the T lymphocytes. The pellet of T lymphocytes was resuspended in Hanks basic salt solution (HANKS BSS)<sup>G</sup> to a concentration of  $1 \times 10^4$  per ml.

Sheep erythrocytes were collected weekly and washed thrice in Hanks BSS and concentrated to  $1 \times 10^5$  per ml. Equal quantities of 0.1 ml of sheep erythrocytes and T lymphocytes were mixed, centrifuged immediately at 200 g for 5 minutes, and resuspended by centrifugation at 10-15 RPM for 5 minutes. The suspension was transferred to a hemocytometer chamber<sup>H</sup> and the rosettes counted in 400 lymphocytes for each sample. At least three erythrocytes adhered to a lymphocyte and was considered as a rosette.

---

C GIBCO, RPMI 1640, Grand Island, New York.

D KC Biological Inc., Inactivated FCS, Lenexa, Kansas.

E Fenwal Laboratories, Scrubbed nylon fiber (3 denier, 3.81 cm type 200).

F National Appliance Company, Model 3221 - 14 Incubator, Portland, Oregon.

G Appendix VII.

H American Optical Corporation, Improve Neubauer, Bright Line Hemacytometer, Buffalo, New York.

### Trial 1

The E-rosette test was carried on 12 cows within the first four days after breeding and 12 cows within the first four days after estrus served as controls.

In preparation for trials 2 and 3, pregnant and non-pregnant cow serums were obtained to test their effect on E-rosette formation in bred and non-bred cows. Twenty ml of blood was collected from the jugular vein into a test tube from 41 day pregnant cow to prepare pregnant cow serum. Another 20 ml of blood was collected from the jugular vein of non-pregnant cow to prepare non-pregnant cow serum. The blood from both cows was allowed to clot and the serum separated, inactivated at 56° C for 30 minutes and stored at -30° C.

### Trial 2

T lymphocytes isolated from 10 cows within four days after breeding were divided into two groups and processed. The first group incubated with the pregnant cow serum and the second group incubated with non-pregnant cow serum as controls. Lymphocytes from 1 ml containing  $1 \times 10^4$  per ml were incubated with 0.25 ml serum for  $1\frac{1}{2}$  hours. The cells then tested for rosette formation.

### Trial 3

The pregnant and non-pregnant cow serums were incubated with T lymphocytes isolated from 10 cows within four days after estrus. Incubation, and treatment and testing were the same as for the samples in trial 2.

## RESULTS

### Trial 1

The results of the individual cow lymphocyte rosette formation following breeding in 400 lymphocyte samples are given in Table 1. The average lymphocyte rosette formation of the 12 cows tested was 96.75 rosettes in 400 lymphocyte samples. The statistical mean of these rosette formations was between 73.87 and 119.63. The means of the rosette formation at different time intervals were presented in Fig. 1. The statistical data and the statistical figures are presented in Appendix I.

The average lymphocyte rosette formation in 400 lymphocyte samples of the 12 cows tested at different intervals after estrus was 152.83. The individual cow lymphocyte rosette formations were presented in Table 2 and the means of the rosette formation at different intervals in Fig. 1. The statistical mean of this group for the total rosette formations was between 136.46 and 169.20 rosettes in 400 lymphocyte samples (Appendix I).

### Trial 2

The lymphocyte rosette formation in 400 lymphocyte samples of the 10 individual cows after incubation with pregnant cow serum are listed in Table 3. The average lymphocyte rosette formation was 222.8 rosettes in 400 lymphocyte samples. The statistical mean averaged between 193.31 and 252.29 rosettes per 400 lymphocytes for the 10 samples tested (Appendix II).

The average rosette formation in 400 lymphocyte samples was 253.1 rosettes in the second 10 samples incubated with non-pregnant cow serum with a statistical mean lying between 227.58 and 278.52 rosettes in 400 lymphocyte samples (Fig. 2; Table 4).

### Trial 3

The results for individual cow lymphocyte rosette formation in 400 lymphocyte samples of the 10 cows tested after estrus and incubated with pregnant cow serum were given in Table 5. The average rosette formation was 267.8 rosettes in 400 lymphocyte samples. The statistical mean was between 232.62 and 299.98 rosettes in 400 lymphocyte samples (Appendix II) and the rosette means at different time intervals are depicted in Fig. 3.

The second group of samples had an average rosette formation of 300.3 rosettes in 400 lymphocyte samples. The individual rosette formation in 400 lymphocyte samples after incubation with non-pregnant cow serum after estrus are given in Table 6. The statistical mean in this group was between 278.69 and 321.91. The means of the rosette formation at different time intervals are given in Fig. 3.

### Pregnancy results

Eight of the 12 cows tested in the first group of Trial 1 were confirmed to be pregnant 40 days after insemination by rectal palpation (Table 7). One cow returned to estrus after 65 days, another returned to estrus after 46 days, another one had a short cycle of 14 days, and one had a normal cycle of 21 days.

Five of the ten cows in Trial 2 were confirmed to be pregnant 40 days after insemination by rectal palpation. One cow returned to estrus 36 days after insemination and the remaining cows returned to estrus between 19 and 24 days after insemination. These cows were identified by numbers 25 to 34 (Table 7).

## DISCUSSION



The results from the first trial revealed differences in lymphocyte rosette formation in the 12 cows tested following breeding and in the 12 cows tested after estrus. The average of the rosette formation was 56.08 higher in cows tested after estrus compared to cows tested following breeding. The difference remained higher (48-45 rosettes) after excluding the readings at 96 hours after estrus for the purpose of comparing the results in both groups at the same time intervals. The statistical mean of the group tested after estrus was 34.21 compared by two samples (independent t-test). The statistical mean difference after the reduction of the 96 hours readings on the second group was higher by 26.34. Comparing the means of the rosette formation at the certain time intervals were consistently higher in the group after estrus. The difference in the two groups was statistically significant and the rosette formation was constantly lower in the group following breeding.

Alteration of the rosette formation occurred with significantly lower readings in the group tested after breeding. Morton *et al*<sup>13</sup> demonstrated similar findings in lymphocytes of pregnant and non-pregnant women tested by spontaneous rosette formation. The spontaneous rosette formation occurred in Hanks BSS alone similar to the study reported here that indicates a factor had affected the lymphocytes after breeding. This factor has the affinity to inhibit rosette formation in Morton *et al* experiment<sup>13</sup> and similarly in our trial. Noonan *et al*<sup>17</sup> reported the EPF inhibited the immune response of T cells and could be detected by adoptive transfer of contact sensitivity as an efficient immunological technique to measure immunological lymphocytic suppression. Furthermore, the immunological

suppression of lymphocytes has been detected by rosette formation by the rosette inhibition test<sup>17</sup>.

The EPF has been successfully demonstrated in sheep<sup>3,10,12,14,17</sup> and in women<sup>13</sup> by the rosette inhibition test, spontaneous rosette formation and adoptive transfer of contact sensitivity<sup>17</sup>. Inhibition of rosette formation was found in lymphocytes after breeding in cows in the first trial. This might be due to the same factor that was suggested earlier in all mammals<sup>17,18</sup>. The mechanism of action of this factor on lymphocytes is not known; whether it occurs by coating the lymphocytes or by a more active method has yet to be clarified. Furthermore, 8 of the 12 cows tested after breeding in the first trial were confirmed pregnant by rectal palpation 40 days after insemination. Pregnancy confirmation in this group supported immunosuppressive activity during pregnancy in this experiment during the first three days after breeding. These findings agreed with earlier findings of a similar inhibitory factor (EPF) in sheep and women<sup>3,10,12,13,14,17</sup>.

In the second trial, lymphocyte rosette formation was significantly lower in samples incubated with pregnant cow serum when compared with samples incubated with non-pregnant cow serum. The average was higher by 30.3 rosettes with the non-pregnant cow serum treatment. The statistical calculations revealed significant differences between the two groups with lower rosette formations in the pregnant cow serum treatment. The pregnant serum was found to have an inhibitory effect on rosette formation; it should be noted that the lymphocytes were taken from the same cow at the same time and treated with pregnant and non-pregnant cow serums. Five of the ten cows were confirmed pregnant by rectal palpation 40 days after insemination. In this trial the serum from pregnant cows had a significant

inhibition on rosette formation of bovine lymphocytes that agrees with other findings with pregnant sheep<sup>3,10,12,14,17</sup> and women<sup>13</sup>.

Morton et al<sup>12</sup> and Noonan et al<sup>17</sup> reported the rosette inhibition effect in pregnant women and sheep serums after incubating serum at 56° C. They suggested that the immunosuppressive factor cannot be inactivated at this temperature but other serum factors that might have an effect on rosette formation are inactivated. The pregnant cow serum during this trial was treated similarly and it appeared that this treatment did not prevent the inhibitory serum factor. The presence of this factor was suggested to be presented during the first two trimesters of pregnancy<sup>3,10,12,13,14,17,18</sup>. The factor may be necessary to protect pregnancy during the early stages by its immunosuppression on T lymphocytes. No other similar factors during this period in the cow have been demonstrated. The mechanism of action of the serum factor is not clear. It could be an active modification of lymphocyte function or altered antigenic response following lymphocytic coating.

Results of the third trial revealed that serum of pregnant cows had an inhibitory effect on rosette formation of lymphocytes from cows after estrus compared with the same lymphocytes incubated with non-pregnant serum. The average difference was 32.5 rosettes higher in non-pregnant cow serum treatment and 17.56 rosettes statistical mean difference which is higher also in the non-pregnant serum treatment when compared by paired t-test.

The findings in trial 3 supported the inhibitory effect of the pregnant cow serum on rosette formation in trial 2. The pregnant cow serum was used on lymphocytes from non-pregnant cows that further confirmed the presence of an inhibitory factor in the cow serum during pregnancy.

Similar results were obtained when the data from group one of trial 2 when incubated with the pregnant cow serum and are shown in Table 3, and were compared to data from group one of trial 3 which were also treated with pregnant cow serum and presented in Table 5. The same results were obtained from comparing the data from group 2 which were incubated with non-pregnant cow serum in trial 2 presented in Table 4 with those data from group 2 of trial 3 which were incubated with non-pregnant cow serum presented in Table 6. Similar results might be obtained by comparing the results in trial 2 and 3.

It is concluded that significant inhibitory effect on E-rosette formation occurred due to the bovine pregnancy factor. The results do not provide a standard test for early pregnancy in cattle. However, useful information was obtained that encourages the use of the rosette inhibition test during early pregnancy in cattle. The rosette inhibition test might be a more accurate procedure for pregnancy diagnosis due to its large margin of inhibition of rosette formation between pregnant and non-pregnant animals. This study also encourages broader studies on this aspect of immunological events during early pregnancy in cattle.

TABLE 1. NUMBER OF LYMPHOCYTES IN A 400 LYMPHOCYTE SAMPLE SHOWING  
ROSETTE FORMATION IN INDIVIDUAL COWS AT SPECIFIC INTERVALS  
FOLLOWING BREEDING

Cow No.	Time*	Lymphocytes With Rosette Formation
1	24	72
2	24	112
3	24	86
4	48	120
5	48	81
6	48	84
7	48	101
8	48	88
9	48	140
10	72	102
11	72	146
12	72	109

\*Hours following breeding

TABLE 2. NUMBER OF LYMPHOCYTES IN A 400 LYMPHOCYTE SAMPLE SHOWING  
ROSETTE FORMATION IN INDIVIDUAL COWS AT SPECIFIC INTERVALS  
AFTER ESTRUS

Cow No.	Time*	Lymphocytes With Rosette Formation
13	24	160
14	24	124
15	24	120
16	48	164
17	48	150
18	48	140
19	48	122
20	48	172
21	72	132
22	72	168
23	96	200
24	96	182

\*Hours after estrus

TABLE 3. NUMBER OF LYMPHOCYTES IN A 400 LYMPHOCYTE SAMPLE SHOWING  
ROSETTE FORMATION AFTER INCUBATION WITH PREGNANT COW  
SERUM IN INDIVIDUAL COWS AT SPECIFIC INTERVALS FOLLOWING  
BREEDING.

Cow No.	Time*	Lymphocytes With Rosette Formation
25	24	238
26	24	245
27	48	131
28	48	235
29	48	238
30	48	209
31	48	268
32	72	240
33	72	251
34	96	176

\*Hours following breeding

TABLE 4. NUMBER OF LYMPHOCYTES IN A 400 LYMPHOCYTE SAMPLE SHOWING ROSETTE FORMATION AFTER INCUBATION WITH NON-PREGNANT COW SERUM IN INDIVIDUAL COWS AT SPECIFIC INTERVALS FOLLOWING BREEDING.

Cow No.	Time*	Lymphocytes With Rosette Formation
25	24	245
26	24	256
27	48	177
28	48	265
29	48	260
30	48	222
31	48	279
32	72	290
33	72	299
34	96	238

\*Hours following breeding



TABLE 5. NUMBER OF LYMPHOCYTES IN A 400 LYMPHOCYTE SAMPLE SHOWING  
 ROSETTE FORMATION AFTER INCUBATION WITH PREGNANT COW  
 SERUM IN INDIVIDUAL COWS AT SPECIFIC INTERVALS AFTER ESTRUS.

Cow No.	Time*	Lymphocytes With Rosette Formation
35	24	238
36	48	285
37	48	270
38	48	262
39	48	322
40	48	244
41	48	309
42	72	165
43	72	300
44	72	283

\*Hours after estrus

TABLE 6. NUMBER OF LYMPHOCYTES IN A 400 LYMPHOCYTE SAMPLE SHOWING  
ROSETTE FORMATION AFTER INCUBATION WITH NON-PREGNANT COW  
SERUM IN INDIVIDUAL COWS AT SPECIFIC INTERVALS AFTER ESTRUS.

Cow No.	Time*	Lymphocytes With Rosette Formation
35	24	321
36	48	310
37	48	291
38	48	300
39	48	347
40	48	290
41	48	316
42	72	229
43	72	300
44	72	299

\*Hours after estrus

TABLE 7. RESULTS OF PREGNANCY DIAGNOSIS BY RECTAL PALPATION AFTER  
FORTY DAYS FOLLOWING INSEMINATION.

Cow No.	Pregnancy
1	+
2	+
3	-
4	+
5	+
6	-
7	-
8	+
9	-
10	-
11	+
12	+
25	+
26	+
27	-
28	-
29	+
30	-
31	+
32	-
33	-
34	+

Fig. 1. Mean number of lymphocytes in 400 lymphocytes sample with rosette formation at specific intervals following breeding and after estrus.

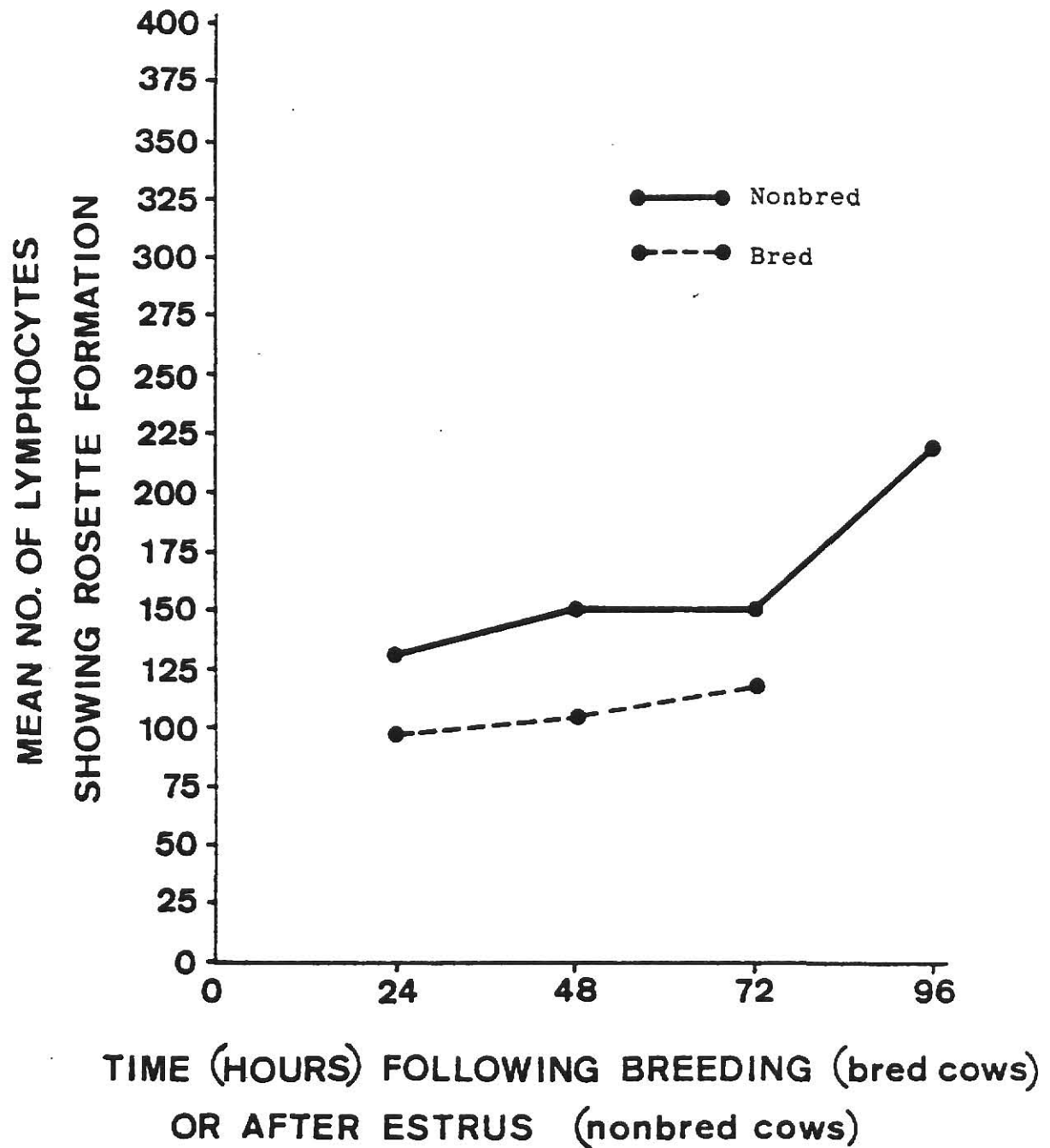


Fig. 2. Mean number of lymphocytes in 400 lymphocytes sample showing rosette formation after incubation with pregnant and non-pregnant cow serum at specific intervals following breeding.

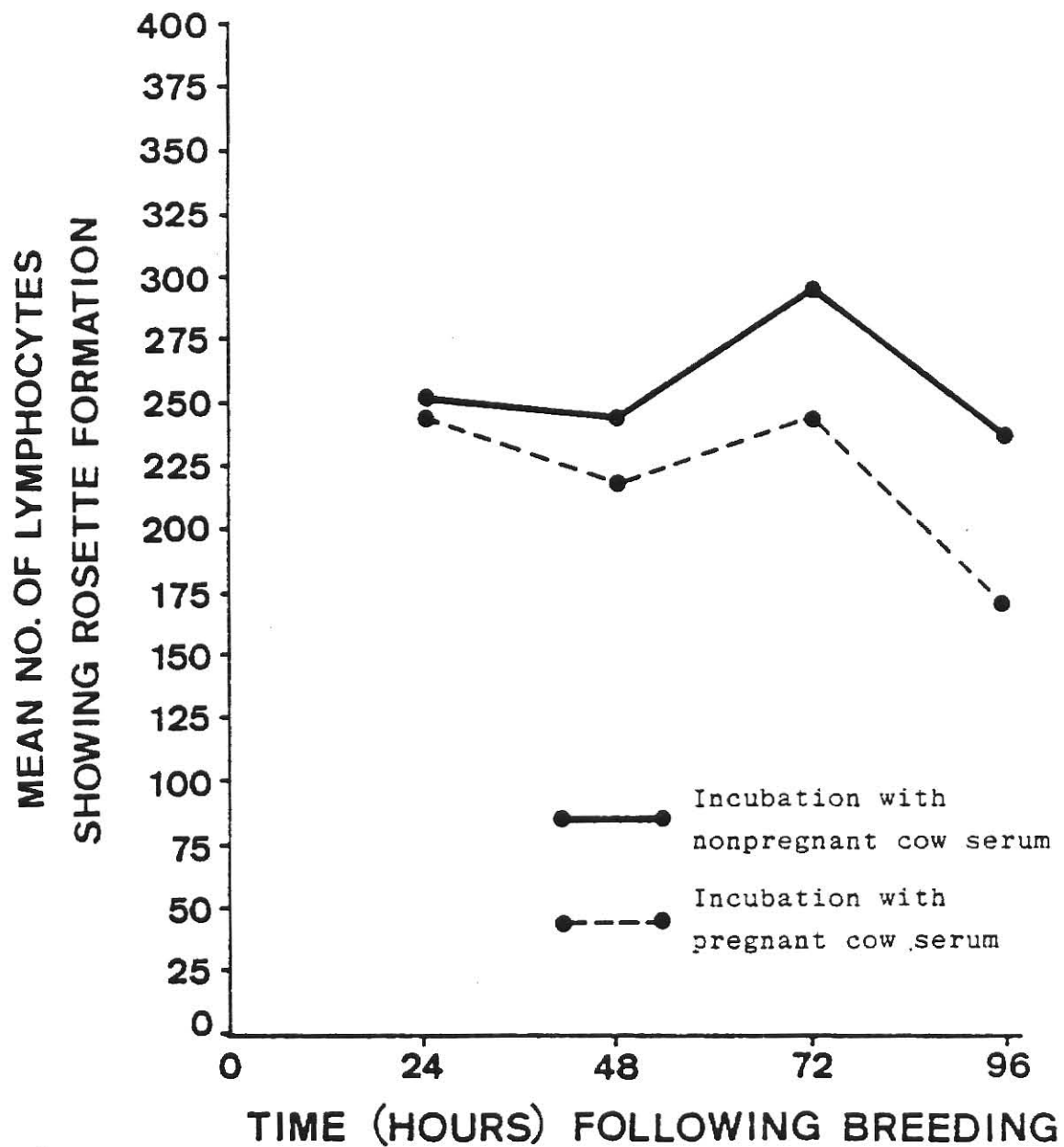
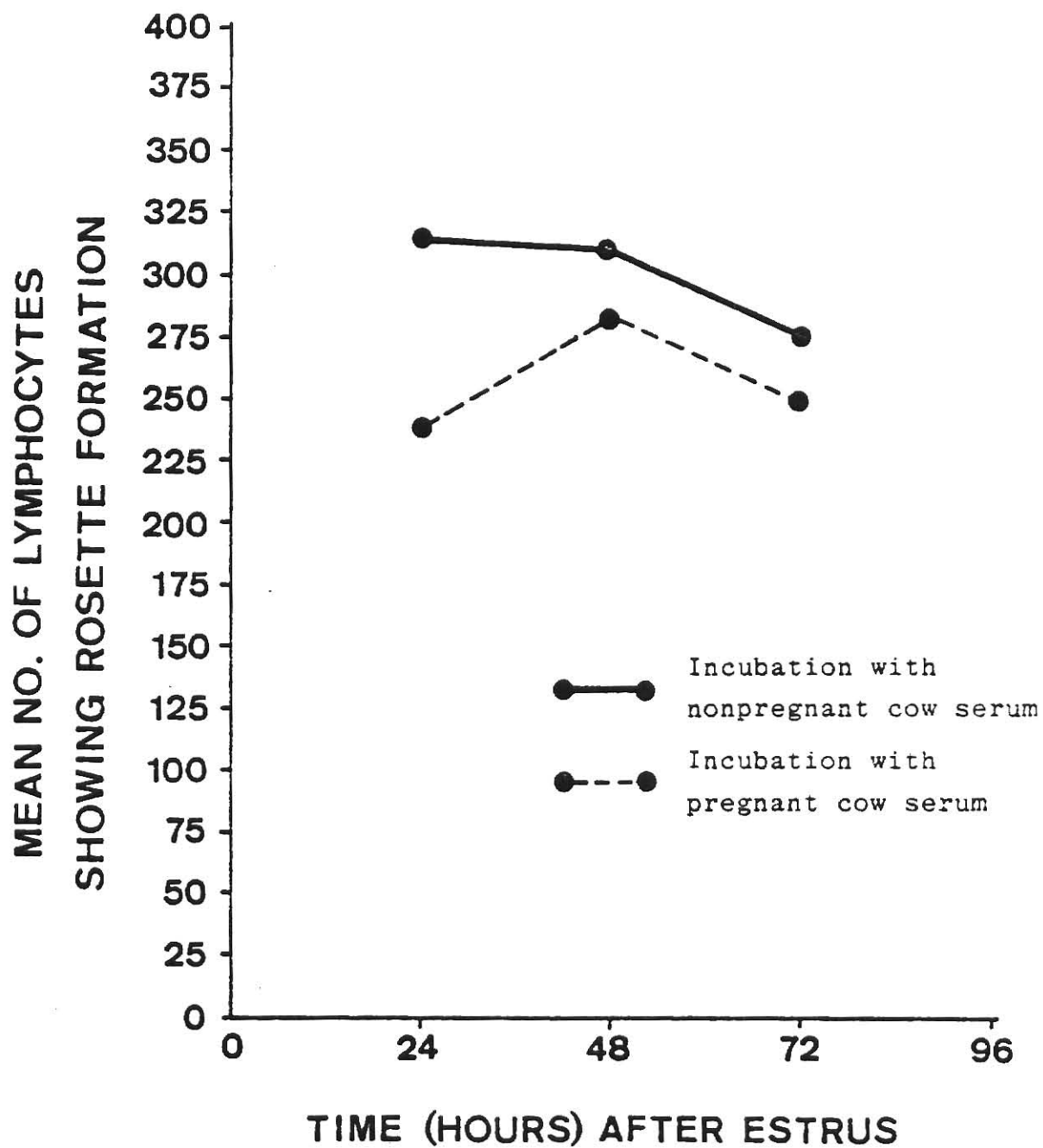


Fig. 3. Mean number of lymphocytes in 400 lymphocytes sample showing rosette formation after incubation with pregnant and non-pregnant cow serum at specific intervals after estrus.



## REFERENCES

1. Atikig B: Immune response to nanidt P-40 extracted, infectious, B Rh. virus antigen in rabbits. Thesis, KSU, Manhattan, Kansas, 66506, p 32, 1980.
2. Brackett BG, Oh Y, Evans JF, Donawick WJ: Fertilization and early development of cow ova. Biol Rep 23: 189-205, 1980.
3. Evison B, Nancarrow C, Morton H, Scaramuzzi R, Clunie JA: Detection of early pregnancy and embryo mortality in sheep by the rosette inhibition test. Theriogenol 8: 157, 1977.
4. Hogg DF, Clunie GJA, Morton H, Hegh V: The place of the rosette inhibition test in the preparation and assay of Antilymphocytic Globulin. Aust NZJ Surg 43: 188-192, 1973.
5. Ingerslev HJ, Ingerslev M: Clinical findings in infertile women with circulating antibodies against spermatozoa. Fert Sterl 33: 514: 520, 1980.
6. Jones WR: Immunologic infertility-fact or fiction? Fert Sterl 33: 577-586, 1980.
7. Julius MH, Simpson E, Herzenberg LA: A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3: 645-649, 1973.
8. Laing JA, Gibbs HA, Eastman SAK: A herd test for pregnancy in cattle based on progesterone levels in milk. Br. Vet. J. 136: 413-415, 1980.
9. Madsen M, Johnsen HE, Hansen PW, Christiansen SE: Isolation of human T and B lymphocytes by E-rosette gradient centrifugation. Characterization of the isolated subpopulations. J. Immunol Meth. 33: 323-336, 1980.

10. Morton H, Clunie GJA, Shaw FD: A test for early pregnancy in sheep. *Res Vet Sci* 26: 261-262, 1979.
11. Morton H, Hegh V, Clunie GJA: Antilymphocyte serum: studies of the specificity of the rosette-inhibiting antibody in rabbit anti-mouse ALS. *Australian Journal of Exp. Biol. and Med. Sci.* 53: 373-380, 1975.
12. Morton H, Nancarrow CD, Scaramuzzi RJ, Evison BM, Clunie GJA: Detection of early pregnancy in sheep by the rosette inhibition test. *J. Reprd Fert* 56: 75-80, 1979.
13. Morton H, Rolfe B, Clunie GJA, Anderson MJ, Morrison J: An early pregnancy factor detected in human serum by the rosette inhibition test. *Lancet* 19: 394-397, 1977.
14. Nancarrow CD, Evison BM, Scaramuzzi RJ, Turnbull KE: Detection of induced death of embryos in sheep by the rosette inhibition test. *J Reprod Fert* 57: 385-389, 1979.
15. Newman MJ, Hines HC: Stimulation of maternal antilymphocyte antibodies by first gestation bovine fetuses. *J Reprod Fert* 60: 237-241, 1980.
16. Nishimoto T, Mori T, Yamada I, Nishimura T: Auto-antibodies to zona pellucida in infertile and aged women. *Fert Sterl* 34: 552-556, 1980.
17. Noonan FP, Halliday WJ, Morton H, Clunie GJA: Early pregnancy factor is immunosuppressive. *Nature* 278: 649-650, 1979.
18. Shaw FD, Morton H: The immunological approach to pregnancy diagnosis: A review. *Vet Rec* 22: 268-270, 1980.
19. Tizard IR: Cellular basis of the immune responses, in *An Introduction to Veterinary Immunology*, W.B. Saunders Company, Philadelphia. pp 69-74, 1977.



## APPENDICES

## APPENDIX I

Data Set 1

$$n_1 = 12$$

$$\bar{x}_1 = 96.75$$

$$s_{x_1} = 36.03$$

$$s_{\bar{x}_1} = 10.40$$

95% C.I. for : 73.87      119.63

Data Set 2

$$n_2 = 12$$

$$\bar{x}_2 = 152.83$$

$$s_{x_2} = 25.79$$

$$s_{\bar{x}_2} = 7.44$$

95% C.I. : 136.46      169.20

Data Set 2 (Reduced - omitted cow No. 23 and 24)

$$n_2 = 10$$

$$\bar{x}_2 = 145.2$$

$$s_{x_2} = 20.16$$

$$s_{\bar{x}_2} = 6.38$$

95% C.I. : 130.78      159.62

## APPENDIX II

Data Set 3

$$n_3 = 10$$

$$\bar{x}_3 = 222.8$$

$$s_{x_3} = 41.28$$

$$s_{\bar{x}_3} = 13.05$$

$$95\% \text{ C.I.} : 193.31 \quad 252.29$$

Data Set 4

$$n_4 = 10$$

$$\bar{x}_4 = 253.1$$

$$s_{x_4} = 35.57$$

$$s_{\bar{x}_4} = 11.25$$

$$95\% \text{ C.I.} : 227.58 \quad 278.52$$

Data Set 5

$$n_5 = 10$$

$$\bar{x}_5 = 267.8$$

$$s_{x_5} = 45.04$$

$$s_{\bar{x}_5} = 14.24$$

$$95\% \text{ C.I.} : 232.62 \quad 299.98$$

Data Set 6

$$n_6 = 10$$

$$\bar{x}_6 = 300.3$$

$$s_{x_6} = 30.24$$

$$s_{\bar{x}_6} = 9.57$$

$$95\% \text{ C.I.} : 278.69 \quad 321.91$$

## APPENDIX III

## A. Compare 1 vs. 2

two-sample (independent) t-test

## (a) first check assumption

$$H_0: \quad \text{vs. } H_1:$$

$$= .10$$

$$\text{Test Statistic: } F = 1.95$$

$$\text{Critical Value: } F_{.95, 11, 11} = 2.8$$

Conclude: Accept  $H_0$  assumption of so use pooled variance

## (b) Pooled Variance

$$s_p^2 = 981.63$$

$$s_{\bar{x}_2 - \bar{x}_1} = 12.79$$

$$(c) \bar{x}_2 - \bar{x}_1 = 56.08$$

$$(d) H_0: \quad - \quad = 0$$

$$H_1: \quad - \quad = 0 \quad (\text{suspected a positive difference so one-sided test})$$

$$= .05$$

$$\text{degree of freedom} = 22$$

$$t_{.95, 22} = 1.717 \quad (\text{critical value})$$

$$\text{Test statistic: } t = 4.38$$

Conclude: Reject  $H_0$

Conclude

## (3) 95% one-sided CI

$$34.21$$

## (f) 95% two-sided CI

$$29.55 \quad 82.61$$

## APPENDIX III

(Continued)

(g) Same analysis on reduced data set 2

$$H_0 : \sigma_1^2 = \sigma_2^2 \quad H_1 : \sigma_1^2 \neq \sigma_2^2 \quad F = 3.19 \quad F_{.95,11,9} = 3.15$$

Reject (barely)  $H_0$ 

I used pooled variance anyway

$$t = 3.78 \quad t_{.95,20} = 1.725$$

Reject  $H_0$  conclude 2 = 1

$$95\% \text{ CI} \quad \sigma_2 - \sigma_1 \quad 26.34$$

$$\text{two-sided } 21.71 \quad \sigma_2 - \sigma_1 \quad 75.19$$

## APPENDIX IV

## B. Compare 3 and 4

## Paired t-test

$$(a) H_0 : \mu_4 - \mu_3 = 0 \text{ vs. } H_a : \mu_4 - \mu_3 \neq 0$$

$$\alpha = .05$$

$$D = x_4 - x_3$$

$$\bar{D} = \bar{x}_4 - \bar{x}_3 = 30.3$$

$$s_D = 20.57$$

$$s_{\bar{D}} = 6.50$$

$$\text{Test statistic } t = 4.66$$

$$\text{Critical value } t_{.95,9} = 1.833$$

Reject  $H_0$

Conclude  $\mu_4 \neq \mu_3$

(b) 95% one-sided C.I.

$$\mu_4 - \mu_3 \geq 18.39$$

(c) 95% two-sided C.I.

$$15.61 \leq \mu_4 - \mu_3 \leq 44.99$$

## APPENDIX V

## C. Compare 5 and 6

## Paired t-test

$$(a) H_0 : \mu_6 - \mu_5 = 0$$

$$H_1 : \mu_6 - \mu_5 > 0 \quad (\text{this positive difference was suspected so used one-sided test})$$

$$\alpha = .05$$

$$D = x_6 - x_5$$

$$\bar{D} = \bar{x}_6 - \bar{x}_5 = 32.5$$

$$s_D = 25.77$$

$$s_{\bar{D}} = 8.15$$

$$\text{Test Statistic } t = 3.99$$

$$\text{Critical Value } t_{.95,9} = 1.833$$

Reject  $H_0$

Conclude  $\mu_6 - \mu_5$

(b) 95% one-sided C.I.

$$\mu_6 - \mu_5 > 17.56$$

(c) 95% two-sided C.I.

$$14.08 < \mu_6 - \mu_5 < 50.92$$

## APPENDIX VI

## D. Compare 3 and 5

Independent two-sample t-test

Assumption  $\sigma_3^2 = \sigma_5^2$  is ok.

(a) pooled variance

$$s_p^2 = 1866.4$$

$$\bar{x}_5 - \bar{x}_3 = 19.32$$

 $v$  = degrees of freedom = 18(b)  $H_0 : \mu_5 - \mu_3 = 0$      $H_1 : \mu_5 - \mu_3 \neq 0$  $\alpha = .05$ Test Statistic  $t = 2.33$ Critical Value  $t_{.975, 18} = 2.101$ Reject  $H_0$ Conclude  $\mu_5 - \mu_3 \neq 0$ 

(c) 95% C.I.

$$4.41 \leq \mu_5 - \mu_3 \leq 85.59$$



## APPENDIX VII

## E. Compare 4 and 6

Independent two-sample t-test

Assumption  $s_4^2 = s_6^2$  is ok

(a) pooled variance

$$s_p^2 = 1089.83$$

$$s_{\bar{x}_6 - \bar{x}_4} = 14.76$$

v - degrees of freedom = 18

(b)  $H_0 : \mu_6 - \mu_4 = 0$  vs.  $H_1 : \mu_6 - \mu_4 \neq 0$  $\alpha = .05$ Test Statistic  $t = 3.20$ Critical value  $t_{.975,18} = 2.101$ Reject  $H_0$ Conclude  $\mu_6 \neq \mu_4$ 

(c) 95% C.I.

$$16.19 \leq \mu_6 - \mu_4 \leq 78.21$$

## APPENDIX VIII

I. Concentrated Hanks - 10 X:

Make the following solutions separately:

## Solution #1

1) NaCl	100 gms	Dissolve in 500 ml
2) KCl	5 gms	deionized water
3) $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	2.5 gms	

## Solution #2

1) $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	1.4 gms	Dissolve in 500 ml
or $12 \text{H}_2\text{O}$	1.9 gms	deionized water
2) $\text{KH}_2\text{PO}_4$	0.8 gms	

## Solution #3

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2.3 gms	Dissolve in 125 ml
or $\text{CaCl}_2$ (anhydrous)	1.74 gms	deionized water

Each salt should be dissolved before adding the next. Combine solutions #1 and #2, add 250 ml deionized water, shake well and add solution #3. Add .25 gms of phenol red. Stopped tightly and refrigerate. (Doesn't have to be sterilized.)

II. Complete Hanks - 1 X:

		<u>Amount per liter</u>
10 X Hanks		100 ml
Antibiotics		1 ml
Fungizone		0.2 ml
Dextrose (10%)		10 ml
Deionized water	add until	1,000 ml mark
$\text{NaHCO}_3$ (7.5%)	to pH 7.4 or 7.6	

Filter to sterilize (Seems to precipitate in the autoclave). Used for washing cells in autoradiography, etc.

## APPENDIX IX

I. Phosphate - Buffered Saline (PBS):

## Basic Solutions:

#1	$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	134 gms/liter deionized water (heat in a 37C water bath to dissolve)
#2	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	69.1 gms/liter deionized water

- II. Buffer Stock: pH 7.15: M - 0.25 (one liter lasts approximately one month and doesn't have to be sterilized if kept refrigerated. If crystals form after refrigeration, warm in a 37C water bath).

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (or $.12 \text{ H}_2\text{O}$ )	386 ml	M/2	(.5M)
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	114 ml	M/2	
Deionized water	500 ml		

III. Phosphate Buffered Saline: pH 7.2

NaCl	17 gms
Buffer stock	80 ml
Deionized water	1920 ml

If desired, autoclave at 15 lbs. for 15 minutes.

A STUDY OF SPONTANEOUS ROSETTE FORMATION  
IN THE BOVINE FEMALE DURING THE IMMEDIATE  
POSTESTRAL PERIOD

by

HISHAM AHMAD FAHMI

B.V.M.S., University of Baghdad, 1972

---

ABSTRACT OF THESIS

submitted in partial fulfillment of the  
requirements for the degree

MASTER OF SCIENCE

Department of Surgery and Medicine

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1981

An investigation on the lymphocyte spontaneous rosette formation was conducted on 64 lymphocyte samples obtained from the peripheral blood of 44 cows within four days following breeding and/or estrus. The investigation involved three experiments concerned with rosette formation and the effect of pregnancy on rosette formation.

The first trial was conducted on lymphocyte rosette formation of 12 cows within four days following breeding and 12 cows within four days after estrus. Significant rosette inhibition of lymphocytes was found in cows tested following breeding. The cows had an average of 96.75 rosettes in 400 lymphocyte samples compared to an average of 152.83 rosettes in cows tested after estrus.

In the second and the third trials, the effect of pregnant cow serum on lymphocyte spontaneous rosette formation was conducted on ten cows within four days following breeding and on ten cows following estrus.

In trial number 2, the serum effect on rosette formation was compared to the effect of non-pregnant cow serum on lymphocytes obtained from the same cows. A significant inhibition of rosette formation of lymphocytes was found with lymphocytes incubated with pregnant cow serum. The average rosette formation was 222.8 in 400 lymphocyte samples with lymphocytes incubated in pregnant cow serum compared to an average of 253.1 rosette formations samples tested after incubation with non-pregnant cow serum. In the third trial, the effect of pregnant cow serum on spontaneous rosette formation was conducted on lymphocytes obtained from ten cows within four days after estrus and compared to the effect of non-pregnant cow serum on rosette formation on lymphocytes obtained from the same cows. The average rosette formation of the lymphocytes incubated with pregnant

cow serum was 267.8 compared to an average of 300.3 rosettes in 400 lymphocyte samples formed by the lymphocytes incubated with non-pregnant cow serum. The results represent a significant inhibitory effect on the rosette formation by pregnant cow serum. Inhibition of rosette formation in this investigation was thought to be due to a pregnancy factor detectable within the four days following breeding and in the pregnant cow serum. This factor was considered to be immunosuppressive and similar to the early pregnancy factor described earlier in sheep and in women.