

STUDY OF THE POSSIBILITY OF IMMUNOLOGICAL REJECTION  
OF EMBRYOS AS A CAUSE OF REPRODUCTIVE FAILURE

by

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## INTRODUCTION

For many years early embryonic mortality has been recognized as a significant factor in reproductive failure (Tanabe and Almquist, 1953). Various mechanisms causing early embryonic death have been investigated including abnormal gametes, abnormal embryonic growth, genetic abnormalities and placental failure (Olds, 1969). Abnormalities of the sperm and ova are readily demonstrated as well as the genetic abnormalities which cause failure of structures or enzyme systems to form at the proper time and in proper sequence. Placental failure can be blamed on a series of events: abnormal morphology of the uterus or oviducts, endometrial infections by either pathogens or non-pathogens (Singh, 1962), trauma to the uterus during placentation and blood group incompatibilities (Davis and Sheckler, 1957).

Fetal and placenta tissues have been shown to be antigenic by injecting or grafting them into animals of another species (Simmon and Russell, 1963). Pregnant female rats and rabbits have been shown capable of an immunological response (Woodruff, 1958). Singh (1965) injected intravenously a bovine fetal homogenate into heifers and had 75% conception on the first service. In the same study when the fetal homogenate was combined with a bacterin 66% of the heifers returned to estrus 40 to 42 days after the service.

The present study was designed to determine if (1) antigens can be effectively absorbed through the intact uterine epithelium (2) embryonic tissues within the uterus can produce isoantibodies, (3) bacterins may be stimulatory to production of antibodies against embryonic tissues, and (4) production of isoantibodies is detrimental to successful pregnancy.

## LITERATURE REVIEW

Work concerning production of antibodies that might be detrimental to pregnancy has been concentrated on studies using sperm, ovaries, embryos, or placental materials as antigens. Antibody production has been determined either by precipitin tests, hemagglutination tests, anaphylactic reactions, or ability to conceive and successfully carry embryos.

Male Factor. Katch (1959) cited Darwin in "Descent of Man" as the first to suggest sperm could be antigenic and might be involved with infertility in mammals. The specific reference was to the seeming infertility of prostitutes or permissive women. Proof of the antigenicity of sperm came when almost simultaneously Landsteiner (1899), Metchnikoff (1900), and Metalnikov (1900) reported sperm and testicular extracts caused the formation of specific heterologous antibodies when injected into experimental animals. During the next 40 years nearly all work related to this problem was concentrated on the development of antibodies against sperm and trying to produce infertility in either the male or female. Savani (1911) was one of the first to claim sterility of the female following the injection of sperm in rabbit and guinea pig. The induced sterility lasted for the first breeding following a series of injections; subsequent matings were fertile. Pommerinke (1928) made the following observations from injecting rabbits with homologous sperm: serum and vaginal secretions contained toxic antibodies; the time that sperm could be found in the female tract was decreased; a period of infertility lasting 6 to 25 weeks followed injections of homologous sperm; and apparently no ovarian dysfunction was involved in this infertility. Similar results were obtained if sperm were injected intra-vaginally, but infertility could not be demonstrated.

Not all investigators could demonstrate infertility from injections

of sperm. Parsons and Hyde (1940) showed antibodies to several species of sperm can be demonstrated either by systemic injections or by intra-vaginal injections. In the same study it was demonstrated that rabbits immunized intra-vaginally had titres as high as those injected systemically. They could find no prevention or delay of pregnancy due to systemic injections but when the vagina was injected with immune serum before insemination no pregnancy followed.

More recently Otani *et al.* (1963) reported reduced pregnancy rate and embryo count in actively immunized guinea pigs but observed no significant change in animals inseminated with sperm treated with immune serum. These results were in agreement with Isojima, Graham, and Graham (1959), and Katsh (1959) but not with Parson and Hyde (1940) and Brunner (1941), Lamoreux (1940), or Henle (1940). A circulating antibody could not always be detected but with Dale-Schultz reaction was always positive when antigen impregnated vaginal tampons were used. This has led some investigators to believe there is a tissue fixed antibody in the uterus or vagina (Behrman and Otani, 1963).

Franklin and Duker (1964) demonstrated antispermatozoa agglutinating antibodies in the serum of women. Menge, Protzman, and De Falco (1964) detected sperm agglutinins in the vaginal mucus sample following injections of semen into the fundus of the uterus for two months. Gershowitz, Behrman, and Neel (1958) reported hemagglutinins in uterine secretions and hypothesized because sperm may possess A and B blood group antigens (Landsteiner and Levine, 1926; Gullbring, 1957) it may increase the frequency of abortion. McLaren (1966) reported antibodies produced against spermatozoa are species specific but not strain specific.

Leucocytic response to the injection of spermatozoa into the uterus

is greater under the influence of estrogen than under progesterone treatment. This appears not to be a species specific response nor dependent on the motility of spermatozoa in the golden hamster (Marcus, 1966). Mahajan and Menge (1964) reported when a sample of mixed sperm was injected into ligated uteri of ovariectomized rabbits there was a differential leucocytic response to sperm type but no differential response to sperm due to the hormonal state of the uterus.

The Ovary. It is obvious that ova of an individual would not be recognized as foreign protein. The success obtained in transferring ova of one breed in uteri of another breed for transport leaves doubt the ova are antigenic at all or if so a very weak antigen (Handcock and Hovell, 1961; Moore and Rowson, 1960). Lewis (1941) demonstrated antitestis serum reacted with the corpus luteum but claimed the injection of homogenized whole ovary reduced fertility and inferred that steroid hormones present in the homogenate were responsible.

In cattle the frequency of fertilized ova dying before 34 days has been estimated between 50% and 70%. In a summary of 299 repeat breeders studied, 6.4% animals failed to ovulate, 5.4% had obstruction, 13.4% lost ova, and 4.7% abnormal ova were observed. There was 23.7% unexplainable fertilization failures (Olds, 1969). In 74 first-service cows there was 2.7% oviducal obstruction, 1.4% lost ova, 8.1% abnormal ova, and 13.5% unexplained fertilization failures (Kidder, et al. 1954). In a Chicago hospital, karyotyping was done on 109 abortuses and 23% had abnormal chromosomes (Kadotani, 1966).

The Placenta. Studies on the antigenicity of the placenta and its part in immunity start back in the last century. Cardawil and Toy (1959) mentioned in their historical review of chorio-carcinoma an interest

in the role of placental tissues in allergic reactions following a report on syncytial deportation and clinical eclampsia in 1898 by Schmorr. In an effort to produce eclamptic convulsions or abortion by the injection of antiplacental serum, Dobrowski (1903) claimed success and others claimed failure (Ascoli, 1902; Wormser and Labhardt, 1904). Cohen and Nedzel (1940) injected six pregnant guinea pigs each with 1 ml of rabbit antiserum against guinea pig placenta over a six day period and observed abortion in 5 animals within 5 days. They noted the cast placenta had passive congestion and eosinophilia. They also reported the blood serum of two women, known to be habitual aborters, gave a positive precipitation reaction to full term human placenta. Seegal and Loeb (1940) reported that in 68 rats injected with anti-rat placenta antiserum two thirds of the embryos were resorbed. In 1943, Seegal and Loeb reported antiplacental serum was likely to produce fatal nephritis in rats. McCaughey (1955) reported that duck antisera to term rat placenta did not appear to jeopardize rat pregnancies. Lin (1947) injected, intraperitoneally, full term rat placenta to 10 virgin female rats then bred the animals. All carried to term with two animals having young born dead and one female in convulsions during parturition. Gray, Tupper, and Rowse (1958) reported significant correlation between visible villous lesions and positive latex-flocculation titres and agglutinations in spontaneous abortion cases. They concluded the substance responsible was a mucopolysaccharide. Kaku (1953) demonstrated both human and rabbit placental polysaccharides which stimulated antibody production.

The demonstration of systemic isoantibodies against fetal tissues has been difficult to quantitate. Brent (1961) found specific agglutination and lysis could be inhibited by specific absorption. Singh (1965) demonstrated a low hemagglutination titre isoantibody to bovine embryonic



homogenates. Chilgreen (1967) demonstrated isoantibody production to embryonic homogenate in rabbits by a passive hemagglutination test.

#### MATERIALS

Six month old, female, virgin, New Zealand rabbits were utilized in this experiment. Each animal was individually housed in either a Bussey hanging cage or Wahman enclosed cage. Each had an individual water and feed pan and was fed a 120 g. ration per day of a balanced feed prepared by the Department of Grain Science, Kansas State University. The temperature was held constant at 72F., the humidity at 70%, and the lights were controlled for 15 hours of light per day. There was 20% air exchange both winter and summer.

The cannula for intra-uterine injections were fabricated by the experimenter as a modification of the one used by Singh (1965). The tip of the cannula was made of a one inch length of vivosil tubing, O.D. .065 manufactured by Benton Dickinson, which had been heat-flared to three times its original diameter on one end. The other end was inserted in an 18 inch length of polyethylene, I.D. 0.060 tubing. The cannula was sterilized in tincture of Zephiran Chloride (Winthrop Laboratories, New York, N.Y.) for 24 hours prior to surgery.

Embryonic hemogenate to be used as antigen was obtained by breeding selected does and at 15-days gestation, a Caesarean section was performed and placental pads, extraembryonic membranes, and fetus removed intact without losing the extraembryonic fluids. This material was fast-frozen and stored at -25C. until used. An embryo was thawed, placed in a Thomas tissue grinder and homogenized with 1 ml of saline. An additional 1 ml of saline was used to flush residue from the grinder. The final volume

of brei was approximately 4.5 ml per embryo. Merthiolate was added to a final dilution of 1:10,000 as a preservative. Without centrifugation the homogenate was drawn into a syringe through a 23 ga. needle and used as such for the injections. If this homogenate was allowed to stand at temperatures above freezing there was possibility of recombination of cells. Recombined cells were re-ground without additional saline.

The bacterins were prepared by growing the specific organism in broth and formalizing them for 24 hours. A mineral salts broth without peptones was used to grow Pseudomonas aeruginosa.<sup>1</sup> Staphylococcus aureus<sup>2</sup> and Escherichia coli<sup>3</sup> were cultivated in a mineral salts broth containing peptones. Aeromonas hydrophilia<sup>4</sup> was grown in heart infusion broth. The cultures of P. aeruginosa and A. hydrophilia were incubated for 24 hours while the cultures of S. aureus and E. coli were incubated for 10 days. The formalin-killed bacterins were brought to pH 7.0 with ammonium hydroxide which forms methenamine (urotropin) which is non-toxic to tissues and readily excreted from the body. For convenience the bacterins were adjusted to approximately  $10^8$  organisms per milliliter by direct count and utilized in the broths. All bacterins used were tested for sterility.

Homogenized samples of uterine tissue were prepared from frozen samples by first weighing the tissue then mincing and placing the total sample in a Thomas tissue grinder with 1 ml of sterile saline. By the use of a Sargent variable speed stirring motor the tissues were agitated until only the myometrial tissues remained intact. This sample was centrifuged and the volume of supernate noted so the reactivity could be expressed

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<sup>1</sup>Thanks to M. Crumrine for this culture.

<sup>2</sup>Thanks to Dr. J. Iandolo for this culture.

<sup>3</sup>Thanks to A. Ling for this culture.

<sup>4</sup>Thanks to Dr. C. Hibbs for this culture.

as mg/ml sample. Merthiolate (1:10,000) was added to the samples to be used for agglutination studies as a preservative then the preparation was frozen. The samples for the peroxidase study were homogenized at the time of the determinations.

Sheep blood was collected in an EDTA treated blood donor unit and washed three times in phosphate buffered saline, pH 7.2, and incubated for 24 hours in a 36C. water bath with occasional agitation. The formalized cells were washed three times in buffered saline to remove the excess formalin. If any hemolysis occurred the cells were discarded. Due to the stickiness and tendency to settle, the cells were centrifuged at 1000 RPM for 8-10 minutes to avoid packing them too tightly. The formalized cells were re-suspended to make a 10% suspension, and an equal volume of a 1:20,000 dilution of tannic acid was added and the mixture incubated for 10 minutes at 37C. The cells were then washed twice with saline. The formalized, tannic acid treated cells were diluted to a 10% suspension in a 1:200 dilution of normal rabbit serum absorbed with sheep erythrocytes diluted in buffered saline, pH 7.2. The formalized, tanned cells (FTC) were sensitized by mixing one volume of supernate fetal antigen, two volumes buffered saline pH 6.4, and one volume of a 10% FTC's suspended in buffered saline, pH 6.4, and incubated for 30 minutes in a 37C. water bath with agitation. The FTC's were then washed once in 1:100 normal rabbit serum, pH 7.2 and used for passive hemagglutination studies. Normal rabbit serum (NRS) for the diluent was absorbed with 1/10 volume of washed, packed, formalized, tanned cells by incubating at 37C. for 30 minutes. The cells were packed by centrifugation and the supernate was inactivated at 56C. for 30 minutes.

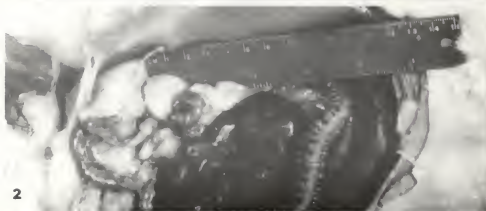
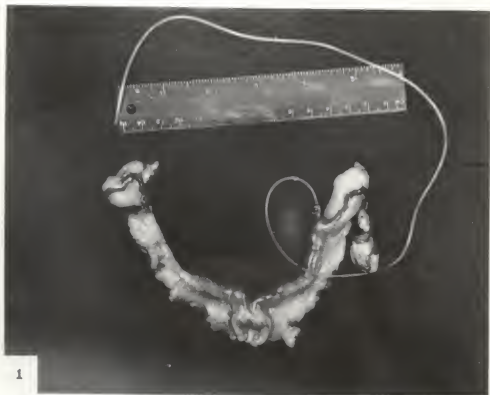
## METHODS

For surgery the rabbits were anesthetized by injecting pentobarbital sodium, 30 mg. per kg. of body weight, intravenously in the marginal ear vein. An experimental laparotomy was performed. If the ovaries appeared to have normal follicular development, consistent with the animals age, the right uterine horn was cannulated. A small stab wound was made with a mosquito forcep at the anterior end of the uterus near the junction of the uterus and oviduct and between the bifurcations of the utero-ovarian artery. The flared silastic end of the cannula (Fig. 1) was introduced into the lumen of the uterus and held in place with a purse string suture of 5-0 mild chromic absorbable surgical suture manufactured by Ethicon, Inc. (Sommerville, New Jersey). By the aid of a small wire the free end of the cannula was directed through the lateral body wall (Fig. 2) and sub-dermal fascia to a point between the scapulae near the base of the neck where a second incision was made. The cannula was tied in a knot and sutured so a loop of tubing was exposed (Fig. 3). This allowed injection directly into the lumen of the uterus without further surgery or trauma and the rabbit was not able to maneuver its head into position to pull the tubing out during normal grooming. The peritoneum and skin were closed in separate layers with 3-0 chromic gut and 0 silk or 0 chromic gut, respectively, using an interrupted mattress suture. Prophylactically 1 ml of Pfizer's Combiotic per 5 kg. of body weight was given I.M. at the time of surgery.

One week following surgery 20 ml of blood was collected by cardiac puncture for pre-exposure control sera. Blood samples were drawn in glass syringes and collected in metal capped culture tubes which had been autoclaved with 10 ml saline in each. The saline was poured out of the blood collecting tubes immediately prior to the blood collection. The blood clot

EXPLANATION OF FIGURES 1-3

- Fig. 1 Rabbit uterus with implanted uterine cannula.
- Fig. 2 Dissection of rabbit abdomen with cannulated uterus and cannula passing through the abdominal muscles.
- Fig. 3 External end of uterine cannula at the base of the neck.



was loosened from the sides of the tube with a stick applicator within 30 minutes and the blood centrifuged (10 minutes at 1,000 G) in an International Model No. 2 centrifuge 2 to 3 hours after collection. The serum was drawn off and stored in screw capped culture tubes at -25C. until tested. Merthiolate was added to a concentration of 1:10,000 as a preservative. Blood serum samples were collected four weeks from the first exposure and at sacrifice.

The animals were separated into 7 groups of 3 each on the basis of what antigen they would receive and by what route they would receive that antigen. Group 1 received the following doses of embryonic homogenate intraperitoneally. One animal received a total of 8.5 ml embryonic homogenate in a two week period and when no antibody was demonstrable a second series totaling 12.5 ml homogenate was given. Four months later four 1.5 ml booster injections were given. The second animal received eight injections of 1.5 ml each of embryonic homogenate at the rate of two injections per week. The third animal received 7.5 ml at the rate of one injection every 6 days.

Group 2 received bacterin via uterine cannula. Each of three animals was injected with 2 ml of bacterin twice a week for two weeks. One animal received equal parts Staphylococcus and E. coli. Another animal received E. coli and another Staphylococcus. One week following the last Staphylococcus injection, 1.5 ml of embryonic homogenate was given via the uterine cannula.

Groups 3-7 were allowed a one week rest period following the cardiac puncture before the start of injections. Group 3 received saline; 4 received the pooled bacterin described in materials; 5 received a mixture of equal parts of pooled bacterin and embryonic homogenate; 6 were injected for

two weeks (6 injections) with pooled bacterin followed by one week (3 injections) of embryonic homogenate; and Group 7 received only embryonic homogenate. Three injections were given per week for 3 weeks, a total of 9 injections. Each injection consisted of 2 ml of suspension by use of a 25 ga. needle to pierce the polyethylene tubing of the cannula and injecting through the cannula into the lumen of the uterus. Injection dosage was established experimentally by determining the maximal volume of fluid a uterine horn would hold without becoming distended.

One week following the last injection, 20 ml blood was collected for serum testing and one animal from each group sacrificed. Samples of the uterus were stored in a liquid nitrogen refrigerator for preparation of tissue homogenate and for enzyme studies. All animals in Group 2 were sacrificed while only one animal per group in Groups 3-7 was sacrificed.

The remaining animals of Groups 3-7 were given a one week rest period following the cardiac puncture. They were anesthetized and by laparotomy the cannula was removed. The fibrous tissue at the site of cannulization was debrided and the uterine wall was not sutured, to lessen scar tissue formation.

Three weeks were allowed for post-surgical recovery and subsidence of pseudopregnancy if induced by trauma of surgery. The animals were then bred to a male of known fertility. The two remaining animals in Group 1 were re-bred each month until they conceived and parturated.

#### DETERMINATIONS

The ability to agglutinate each species of bacteria was tested separately with each sample of serum and homogenized tissue. This was accomplished by serial dilution of the sera from 1:10 to 1:5120 in 10x100mm. tubes and



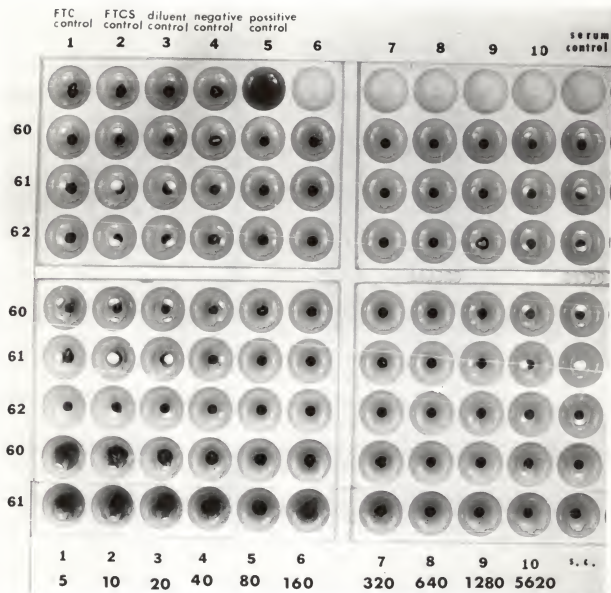
the addition of an equal volume of a  $10^8$  suspension of cells. The tests were well mixed and incubated in a water bath for 24 hours at 37C. Positive tests showed definite aggregates that were not easily dispersed by gentle tapping at the base of the tube. Negative tests produced a vortex of cells on tapping of the tube, demonstrated uniform turbidity, and usually formed a discrete button in the bottom of the tube. There was no attempt to grade bacterial agglutinations other than positive or negative; a weak reaction was considered negative. Saline controls were run concurrently with all tests.

In an attempt to demonstrate the presence of antibody specific for homogenized rabbit fetus and associated membranes, Ouchtolony gel diffusion plates and micro-tube precipitation were tried, unsuccessfully. Anti-fetal antibodies could be demonstrated by using the highly sensitive method of hemagglutination utilizing formalized, tannic-acid treated, protein sensitized sheep erythrocytes (Daniel, Weyand, and Stavitsky, 1963; Whitman and Hetrick, 1965; Chilgreen, 1966). Serial dilutions from 1:50 to 1:2560 were made in Limbo Dispo trays, with a final volume of 0.5 ml per well. To this 0.5 ml of a 1% suspension of sensitized cells were added. The tests were incubated for 4 to 6 hours then photographed and held for 24 hours to observe any possible change in reaction titre. Diluent for the test was a 1:100 dilution of absorbed normal rabbit serum (page 8) in phosphate buffered saline. The criteria for reading the reaction was after Daniel, et al. (1963) as follows:

- +++ A compact granular aggregate
- +++ A smooth mat on the bottom of the well with folded edges
- ++ A smooth mat, edges somewhat ragged
- + A dark ring around the edge of a smooth mat
- + A thick dark ring around edge of mat covering small area
- A discrete button in bottom of the well

#### EXPLANATION OF FIGURE 4

Fig. 4 Passive hemagglutination test using formalized tannic acid treated sheep erythrocytes - sera tested are from Group 5. Controls are in the top row. The next 3 rows are pre-exposure sera from animals 60, 61 and 62. All pre-exposure sera were non-reactive. The next 3 rows are week 4 sera from the same animals. Week 4 sera 60 has a titre of 1:10; sera 61 has a titre of 1:20; sera 62 was non-reactive. The last 2 rows are week 8 sera from animals 60 and 61. Sera 60 had a titre of 1:160 and sera 61 had a titre of 1:320. Note: the last row has reactions of +4, +4, +3, +2, +2, +2, +1.



The end point dilution was the last + of the serial dilution. A serum control using the end dilution plus unsensitized formalized tanned cells was run on each serum. System controls consisted of (1) a known positive serum which was produced in chickens, (2) a negative serum plus sensitized cells, (3) diluent plus sensitized cells, (4) diluent plus unsensitized cells, and (5) saline plus sensitized cells (Fig. 4).

The method used for peroxidase determination was that of Nickel (1966) which is a modification of the method of Smith, Robinson, and Stoz (1959). The method is based on the reoxidation of reduced (leuco) 2,3'6-trichloro-indophenol (TIP) dye by peroxidase. The method was convenient and reliable, involving the action of thiosulfate producing the leuco substrate. The dye turns from bright blue to pale yellow during reduction. In the presence of hydrogen peroxide and a uterine extract the rate of blue color formation due to reoxidation of leuco TIP is proportional to the enzyme concentration. The rate of the reaction was measured at 10 second intervals spectrophotometrically at 675 mu. A unit of enzyme is that amount causing a change of one absorbance unit per minute.

The protein concentrations were determined by Yonetani's (1949) modification of the method of Gornall (1949). For a sample of 8 mg. or less 0.5 ml of a 6%  $H_2O_2$ , 2.0 ml of biuret reagent and water were incubated at room temperature then read spectrophotometrically at 540 mu. Crystallin bovine albumen was used for the protein standard curve.

## RESULTS AND DISCUSSION

Evaluation of Methods. This study was undertaken in an effort to determine the immunologic capabilities of the uterus to foreign protein. The rabbit was chosen as the experimental animal because of the duplex

nature of the uterus. The cervical canals are separate and have separate internal and external os, thus, precluding any cross-over of injected materials. Any immunologic response had to originate from the uterine tissue through an intact epithelium and any similarity in response between one uterine horn and the other would necessarily be a humoral antibody response. Similarly any fixed or tissue bound antibodies would be shown as a higher titre in the uterine tissue homogenate.

A uterine cannula (Fig. 1) was selected as a means of introducing the antigenic material because it was impossible to reach the os cervix through the vagina even with a specially designed speculum and transluminator. The cannula used was one described by Singh (1965). This consisted of a vivosil tip attached to a length of polyethylene tubing. The vivosil tip was not ejected; fibrous tissue surrounded and adhered to it. The polyethylene tubing was totally inert, not stimulating fibrous tissue to adhere. The placement of the external end of the tubing between the shoulders (Fig. 3) was made necessary by grooming habits of the animal. If the rabbit grasped the tubing she would dislocate or cut off the end necessitating another surgical procedure. In all cases in which the cannula was damaged beyond use, the animal was rejected and replaced with another. There was apparently no adverse effect of cannulization on the reproductive capacity in the 10 animals which were bred. Six of the animals had normal appearing embryos at 15 days gestation on gross observation. The treated uterine horn had an average of 4.6 embryos and the nontreated horns had 4.5 embryos.

The tests used in determinations of antibody to embryonic homogenates were found to be highly sensitive and extremely subject to malfunction. The micro tube precipitation and gel diffusion tests were found to be useless. Others also found this to be the case (Singh, 1965; Chilgreen,

1967). The passive hemagglutination test of Boyden (1951) as modified by Weinbach (1958) and used by Chilgreen (1967) to detect anti-embryonic antibodies in rabbits was the most sensitive test available. The test, if not working, will give false negatives but not false positives. Spontaneous agglutination occurs but has a different pattern and occurs in controls also. Chilgreen (1967) reported some nonspecific hemagglutination in sera collected prior to immunization but after pregnancy and considered that this hemagglutination was due to embryonic fluid or tissues lost into the abdominal cavity during removal of the embryos. I suggest possibly this was due to previous pregnancy.

The bacterial antigens used are found as contaminants in the uteri of animals: *Staphylococcus* (Dennis, 1966); *Escherichia coli* (Hawk, 1958); *Pseudomonas* (Black et al. 1964); *Aeromonas hydrophila* (Hibbs, Merker, Kruckenberg, unpublished). Killed bacteria were used because an active purulent infection was not considered necessary to sensitize the animal.

The age of the embryo was considered critical and the antigens that may be involved in embryonic loss would be present during the establishment of the placenta but might not be present later in gestation. Fifteen days gestation embryos were selected so reasonable amounts of tissue would be available and the placentation process would be nearing completion (Houston, 1963). Rowson and Moor (1966) obtained a differential response with different aged embryos in sheep uteri perfused with sheep embryos. There was an increased cycle length from materials collected while placentation was in progress but no change when materials were collected after the placenta was well established. They reported the effects obtained were dependent on the stage of development of the embryo in relation to the uterus. They were also unable to obtain any response with intramuscular

injections, but determined this response is species specific.

Peroxidase tests. The peroxidase enzyme test was undertaken to determine if enzyme activity would reflect eosinophilia as a response to foreign proteins in the uterus. Singh (1965) reported an influx of eosinophils and mast cells to the response of induced uterine infection repeatedly. These cells contain large numbers of granules which stain positive for peroxidase (Wachstein and Meisel, 1964). The suggestion has been made that tissue eosinophils are the source of uterine peroxidase (Llebanoff, 1965; Paul, 1966). The leuco 2,3'6-trichloroindophenol (TIP) test for peroxidase was used<sup>1</sup> (Nickel, 1968) to assay the uterine samples. Treated and untreated uterine samples from 10 animals representing every group were tested.

The TIP peroxidase activity ranged (Table 1) in units of activity per gram of tissue sample from 17.1 to 0.7 units from the highest to lowest. The activity between the treated and non-treated uterine horn was about three units except for one animal in which the values were .01 units for the treated to 1.4 units in the non-treated. This 100 fold difference is unexplainable. The difference between treated and non-treated uteri was not uniform. In groups 3-7 there were four animals with more peroxidase activity in the untreated than the treated horn and two with the non-treated higher. Nickel (1966) reported the average peroxidase activity for rabbit uterus was 2.6 units per gram tissue. The samples tested in this study averaged 5.1, higher than Nickel's average because of the presence of one animal with excessive peroxidase (Table 2).

Archer (et al. 1965) concluded that eosinophil peroxidase is different

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<sup>1</sup>Thanks to K. Nickel for her help in conducting these determinations.

Table 1. Results of peroxidase assay of uterine extracts, in TIP units from both treated (R) and untreated (L) uterine horns of each animal tested.

GROUP	ANIMAL #	UTERUS	TIP UNITS GRAM TISSUE
1	3	R	10.7
		L	7.7
2	8	R	1.4
		L	1.0
	26	R	16.9
		L	17.1
	36	R	10.7
		L	7.7
3	40	R	.9
		L	1.3
4	50	R	2.7
		L	.8
5	62	R	.9
		L	.7
6	72	R	5.4
		L	3.8
7	82	R	1.2
		L	1.0



from neutrophile peroxidase. Neutrophile peroxidase is more commonly known as myeloperoxidase. Lucas (1964) reported the cyclic variations in peroxidase activity was accounted for by the number of eosinophil granulocytes occurring in the uterus. Nellor and Brown (1966) reported the leucocytic cells of the uterus are cyclic. Estrogens increase the number of eosinophil in the uterus and the peroxidase activity while progesterone suppressed this activity. Estrogen influence on a progesterone primed uterus causes a subsequently higher degree of eosinophilia and peroxidase activity than estrogens alone (Baker, Bergman, and Paul, 1967). Since there was no appreciable difference between the treated and non-treated uterine horn the tests were discontinued.

Antibody Reactions. In Group 1 injections of embryonic homogenate failed to produce a demonstrable antibody (Table 2). Chilgreen (1967) was able to produce a titre with 10 subcutaneous injections of embryonic homogenate incorporated in adjuvants. The use of adjuvants is probably necessary to build a titre against systemic injections unless daily injections are given.

Group 2, which utilized the uterine cannula to permit injection directly into the uterus without repeated surgical procedures, had bacterial agglutination titres up to 1:640. This experiment demonstrated an immunological response without erasing the uterine epithelium with sodium carbonate or similar substance as was practiced in earlier work in cattle (Singh, 1965). The blood serum titre to the bacterin was not as high as demonstrated by 9 subcutaneous injections of bacterins. The antibody titre present in the uterine tissue homogenate was less than that of blood serum, indicating primarily response rather than a tissue bound response.

Table 2. Antibody response to serum drawn pre-exposure, 4 weeks and 8 weeks, and to extracts of each uterine horn. Antibody tests to bacterin (B) are reported as 1:10 through 1:5120 tube dilution and those against embryonic antigen; (E) as 1:5 through 1:2560 tray dilutions.

GROUP	PRE-EXPOSURE	WEEK 4	WEEK 8	RIGHT	LEFT
ANTIGEN	SERA	SERA	SERA	UTERUS	UTERUS
1					
E	NR	NR	NR	NR	NR
2					
B	NR	4	-	3	2.25
3					
B	NR	1.16	2.41	1.83	2.0
E	NR	NR	NR	NR	NR
4					
B	.42	3.85	5.0	3.33	2.41
E	NR	1	4.5	2	3.3
5					
B	.08	4.41	6.12	2.30	3.50
E	NR	2	6.5	2.3	2.
6					
B	.08	5.25	4.62	3.25	1.87
E	NR	3.3	5.5	.3	1
7					
B	NR	3.16	3.62	2.62	2.25
E	NR	NR	5	NR	NR

Group 3 had bacterial agglutination titres which progressed from non-reactive, pre-exposure, through 1:20 at week 4 to 1:40 at week 8 sacrifice. These animals were injected with sterile saline but were subjected to 2 surgical procedures and at least 9 manipulations of the tubing where it tranverses the skin, giving ample opportunity for contamination with dead or viable organisms. Subclinical infection was eliminated by the animal's natural body defenses and the prophylactic injection of Combiotic administered the day of surgery. The tissue homogenate titre was 1:20 for both horns. Group 7 which received only embryonic homogenates, had comparable bacterial agglutination response but one dilution higher. Both the week 4 and week 8 serus titres averaged between 1:40 and 1:80. Explanation for the higher titre in the embryonic homogenate was proteinaceous content acting synergistically with the contaminating organisms, the organisms being from the same sources as those used in Group 3. Considering the bacterial agglutination titre of these 2 groups, it was assumed that any bacterial titre of 1:80 or less was from the manipulations of the animal, not from any treatment. Group 3 was completely non-reactive to embryonic homogenate. Therefore all passive hemagglutination titres to embryonic homogenate were considered a response to the treatments.

Group 4 received 9 injections of bacterin. The pre-exposure titre was less than the first tube dilution of 1:10. At week 4 the titre was 1:80, then 1:160 at week 8. The uterine homogenate titre was 2 dilutions below the week 8 serum titre. The treated uterine horn was one dilution higher than the non-treated uterine horn. The treated uterus had a titre of 1:40 and the non-treated uterus titre was 1:20. No significance can be placed on such a small difference.

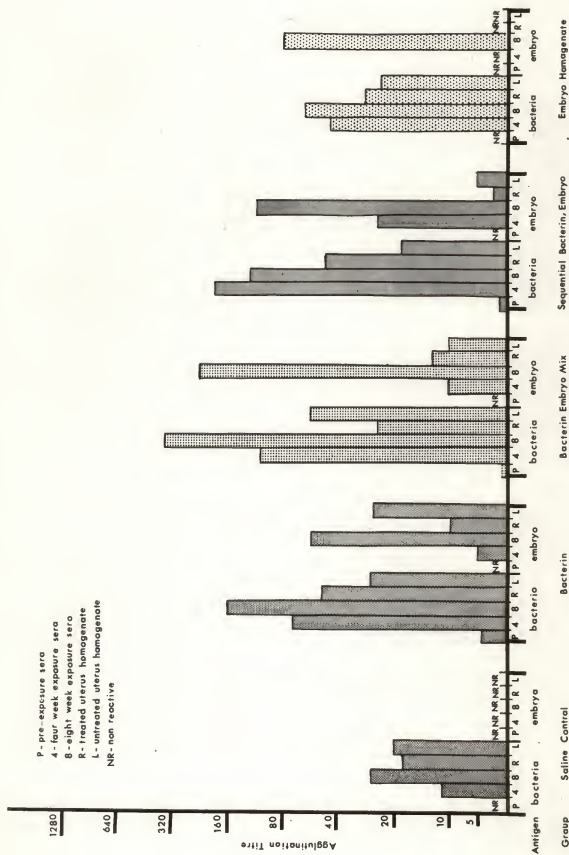
Group 4 response to embryonic antigen was hard to explain. The pre-exposure serum was non-reactive. The week 4 blood serum titre was 1:5 and the week 8 titre exceeded 1:40. These reactions were not of the strong type (+4) but were intermediate reactions being +2 and +1 reactions. The uterine homogenates of this group produced the highest reaction of any uterine homogenate test. Both the treated and non-treated had 1:20 titres. Chilgreen (1967) reported this type non-specific reaction in his pre-immunized serum. All of the rabbits in his study had been pregnant and I suggest that this non-specific type reaction was due to the previous pregnancy. In this study at least 2 of the "virgin" does purchased for this work were pregnant at the time of purchase. No animal known to be pregnant was used in the experiments but the possibility exists one may have been included. The most probable explanation for the reaction to embryonic homogenate in Group 4 is a common antigen between the embryonic material and one of the bacterins.

Group 5 pre-exposure serum (Fig. 5) was essentially non-reactive to bacteria. At week 4 the titre rose to 1:160 and at week 8 the average was in excess of 1:320. The bacterial agglutination titre was 1:40 in the treated uterus homogenate and 1:80 in the non-treated uterus. The blood serum titres were one dilution higher than in Group 4. The embryonic passive hemagglutination titre was non-reactive in pre-exposure serum and 1:10 in week 4 serum. The week 8 serum was higher than any other group, with a titre of 1:160. The uterine homogenates were both near 1:10. The higher titre to the embryonic homogenate was possibly due to the synergistic action between the bacterins and the protein of the embryonic homogenate.

Group 6 received bacterin for 6 injections followed by 3 injections

EXPLANATION OF FIGURE 5

Fig. 5 Graphic representation of bacterial agglutinations and embryonic passive hemagglutination reactions averaged by group.



of embryonic homogenate (Fig. 5). The pre-exposure serum was essentially non-reactive to bacterial agglutination. The 1:160 titre at week 4 was maximal for this group. At the end of week 8 the bacterial agglutination titre was down to 1:80. This group received bacterial antigens one week less than the other groups, which may account for the lowered response. The uterine homogenate titres were equivalent to those of Group 4, which received bacterins. Group 6 anti-embryonic homogenate titres were: non-reactive for the pre-exposure serum, 1:20 for the week 4 serum, and 1:160 for the week 8 serum. The uterine homogenate average was 1:5 or below. Even with the reduced volume of embryonic antigen stimulation there was a relatively high titre with a pre-exposure or sensitization to bacterial antigens.

Group 7 received only embryonic homogenate and consequently received more volume of embryonic homogenate than any other group. The pre-exposure and week 4 serum were non-reactive. At week 8 there was a 1:80 reaction to embryonic homogenate. The uterine homogenates were non-reactive. Group 7 bacterial agglutination reactions ranged from 1:40 at week 4 and 1:80 at week 8. These were discussed with Group 3.

The small differences found in titres between the treated and non-treated uterine horns, which in some cases were higher in the non-treated horns, was explained by the possibility of more lymphatic tissue included in one random sample than another. This can not be proven since fixed tissue has little if any demonstratable antibody present and the satisfaction of one aim would defeat the other.

#### CONCLUSIONS

It is concluded that the immunologic response to the embryonic homo-

EXPLANATION OF FIGURE 6

Fig. 6 Rabbit uterus at 15 days gestation from animal 52 from Group 4. There were 4 apparently normal viable embryos in the non-treated uterine horn. The whitish areas on the 5 resorption sites on the treated uterine horn.





genate is facilitated by the presence of or pre-exposure to bacterins. The blood serum titre to embryonic homogenate was consistently higher in the week 4 serum when treatment included a bacterin. The tissue homogenate titre to embryonic antigen was dependent on the presence of a bacterin and there was a correlation to the amount of embryonic material administered as shown in Groups 4 and 5. Group 7 which received only embryonic materials had a late blood serum response but no response against embryos in the uterine homogenates. The antibody against the embryonic materials does not depend entirely upon the injection of embryonic material's into the body. Group 3 which was injected with only bacterins had a higher titre against embryonic antigens than any other group of uterine homogenates. This response was not as complete as the others but was of a high titre indicating possibly a cross reaction antibody or one of low specificity.

There was no adverse effect on the reproductive capacity due to the cannulization or the antigenic treatment in any of the 10 animals bred. Only one animal gave any indication that the treatment affected viability of the embryos. This animal in Group 4 which received only bacterins, had conceived on both sides but the embryos in the treated horn had undergone degeneration and all that remained were resorption sites (Fig. 6). Due to the small number of animals per treatment, this event is not statistically significant but is more likely the result of the treatment than the cannulization. Further study in this area is warranted to determine what specific treatment can cause unilateral response. The normal appearing pregnancies gave no indication of a reduction in numbers of embryos in the treated horn. This is in agreement with Ketchel, et al. (1966) who found no reduction in the number of fetuses in rats after injection with placenta.

## REFERENCES

- Archer, G.T., G. Air, M. Jackas and D.B. Morell. 1965. Studies on rat eosinophil peroxidase. *Biochem. Biophys. Acta.* 99:96.
- Ascoli, A. 1902. Zur experimentellen pathogenese der eklampsie. *Zentr. Gybakol.* 26:1321. In Bardawil, W.A. and B.L. Toy. 1959. *The Natural History of Chorio-Carcinoma: Problems of Immunity and Spontaneous Regression.* New York Academy of Sciences. 80:197.
- Baker, A.P., F. Bergman and K.G. Paul. 1967. Studies of eosinophil granulocytes III. Steroid hormones and number of eosinophils in the uterus of the rat and mouse. *Acta Endocrinologica* 54:696.
- Behrman, S.J. and Y. Otani. 1963. Transvaginal immunization of the guinea pig with homologous testis and epididymal sperm. *Internat. J. Fert.* 8:829.
- Black, W.G., J. Simons, H.E. Kidder and J.W. Wiltbank. 1954. Bacterial activity in the uterus of rabbit and cow. *Am. J. Vet. Res.* 15:247.
- Boydén, S.V. 1951. The absorption on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J. Exp. Med.* 93:107.
- Brent, R.L., P.B. Medawar and M. Ruskiewicz. 1961. Serological methods in the study of transplantation antigens. *British J. Exp. Path.* 42:464.
- Brunner, E.K. 1941. An experiment in temporary immunization against pregnancy. *Human Fert.* 6:10.
- Bardawil, W.A. and B.L. Toy. 1959. *The natural history of chorio-carcinoma: Problems of immunity and spontaneous regression.* New York Academy of Sciences. 80:197.
- Cohen, H.R. and A.J. Nedzel. 1940. Specific action of an antiserum for placental proteins on placenta and normal progress of pregnancy. *Proceedings of the Society for Experimental Biology and Medicine.* Vol. 43, No. 2, pp. 249.
- Chilgreen, Donald R. 1967. Studies to detect antibodies in rabbits following the injection of embryo-brei. Master's Thesis. Kansas State University, Manhattan, Kansas.
- Daniel, T.M., J.G.M. Weyand, Jr. and A.B. Stavitsky. 1963. Micromethods for the Study of Proteins and Antibodies IV. Factors involved in the preparation and use of a stable preparation of formalinized, tannic acid-treated, protein-sensitized erythrocytes for detection of antigen and antibody. *J. Of Immuno.* 90:171.

- Davis, M.E. and C.E. Sheckler. 1957. De Lee's Obstetrics for Nurses 16th Ed. W.B. Saunders Company, Philadelphia, Pa.
- Dennis, S.M. 1966. Perinatal staphylococcal infections of sheep. *Vet. Record.* 79:38.
- Dobrowlski, M.S. 1903. O Cytotoksynie Lozyskowij. *Bull. Intern. Acad. Sci. Cracovie* No. 5:256. In Bardawil, W.A. and B.L. Toy. 1959. The natural history of chorio-carcinoma: Problems of immunity and spontaneous regression. *New York Academy of Sciences.* 80:197.
- Franklin, R.R. and C.D. Dukes. 1964. Antispermatozoal antibody and unexplained infertility. *Am. J. Ob. Gyn.* 89:6.
- Gershowitz, H., S.J. Behrman and J.V. Neel. 1958. Hemagglutinins in uterine secretions. *Science* 128:719.
- Gornall, A.G., C.J. Virdawill and M.M. David. 1949. Determination of serum proteins by means of the biuret reagent. *J. Biol. Chem.* 177:751.
- Gray, J.D., C. Tupper and J.A. Rowse. 1958. The problem of spontaneous abortion V. The genesis of spontaneous abortion. *Am. J. Ob. Gyn.* 75:43.
- Gullbring, B. 1957. Investigations on occurrence of the blood group antigens in spermatozoa in man and serologic demonstration of the segregation of the characters. *Acta Med. Scand.* 159:169.
- Handcock, J.L. and G.J.R. Hovell. 1961. Transfer of sheep ova. *J. Reprod. Fert.* 2:295.
- Hawk, H.W. 1958. The influx of leucocytes and presence of bactericidal substances in inoculated uteri of estrous and pseudo-pregnant rabbits. *J. Animal Sci.* 17:416.
- Henle, W. and G. Henle. 1940. Spermatozoal antibodies and fertility. II. Attempts to introduce temporary sterility in female guinea pigs by active immunization against spermatozoa. *J. Immuno.* 38:105.
- Houston, M. 1963. Placentation in the rabbit. Master's Thesis. Kansas State University, Manhattan, Kansas.
- Isojima, S., R.R. Graham and J.B. Graham. 1959. Sterility in female guinea pig induced by injection with testis. *Science* 129:44.
- Kadotani, T. 1966. In "Medical News". *J.A.M.A.* 197:41.
- Kaku, M. 1953. Placental polysaccharide and the aetiology of the toxæmia of pregnancy. *J. Ob. Gyn. British Empire* 60:148.
- Katsh, S. 1959. Immunology, fertility, and infertility: A historical survey. *Am. J. Ob. Gyn.* 77:946.

- Katsh, S. 1959b. Infertility in female guinea pigs induced by injection of homologous sperm. *Am. J. Ob. Gyn.* 78:276.
- Ketchel, M.M., U.K. Bank, E. Minassian and L. Galassi. Normal pregnancy and litter size in rats injected with fetal placentas. *Transportation* 4:131.
- Kidder, H.E., W.G. Black, H.N. Wiltbank, L.C. Ulberg and L.E. Casida. 1954. Fertilization rates and embryonic death rates in cows bred to bulls of different levels of fertility. *J. of Dairy Sci.* 37:691.
- Lamoreux, W.F. 1940. Spermatozoal antibodies and infertility in the fowl. *J. Exp. Zool.* 85:419.
- Landsteiner, K. 1899. William Young Ed. *Sex and Internal Secretions*. 2nd Vol. Williams and Wilkins, Baltimore. 1961.
- Landsteiner, K. and P. Levine. 1926. On group specific substances in human spermatozoa. *J. Immuno.* 12:415.
- Lewis, J.H. 1941. The antigenic relationship of alcohol soluble substances of corpus luteum to those of testis and brain. *Am. J. Path.* 17:725.
- Linn, H.A.C. 1947. Is toxemia of pregnancy an allergic reaction? *Am. J. Ob. Gyn.* 54:97.
- Lucas, F.V., V.M. Carnes, H.J. Schmidt, D.R. Sipes and D.G. Hall. Uterine peroxidase activity in human endometrium. *Am. J. Ob. Gyn.* 88:965.
- McCaughey, W.T.E. 1955. The nephrotoxic action of antiplacental serum in rats. *J. Ob. Gyn. British Empire* 62:863.
- McLaren, A. 1966. Studies on the isoimmunization of mice with spermatozoa. *Fert. and Ster.* 17:493.
- Mahajan, S.C. and A.C. Menge. 1964. Hormonal control of rabbit and rat uterine response to sperm. *J. of Animal Sci.* 23:909.
- Marcus, S.L. 1966. Influence of ovarian hormones on the leucocytic response to spermatozoa in the uterus of the golden hamster. *Fert. and Ster.* 17:212.
- Menge, A.C., W.P. Protzman and R.J. DeFalco. 1964. Immunoreactions and fertility and infertility: A historical survey. *Am. J. Ob. Gyn.* 77:946.
- Metalnikov, S. 1900. *Ann. Inst. Pasteur.* 14:577. In Katsh, S. 1959. Immunology fertility and infertility: A historical survey. *Am. J. Ob. Gyn.* 77:946.
- Metchnikoff, K. 1900. *Ann. Inst. Pasteur.* 14:1. 1900. In Katsh, S. Immunology, fertility and infertility: A historical survey. *Am. J. Ob. Gyn.* 77:946.

- Moore, N.W. and L.E. Rowson. 1960. Egg transfer in sheep. Factors affecting the survival and development of transferred eggs. *J. Reprod. Fert.* 1:332. .
- Nellor, J.E. 1965. The leucocyte-like cells of the oviducts during the normal estrous cycle and their modification by progesterin and estrogen treatment. *The Anatomical Record* 151:171.
- Nellor, J.E. and J.E. Brown. 1966. Leucocyte-like cells of the vagina and uterus, and their modification during the normal estrous cycle and by progesterin and estrogen treatment. *Anatomical Record* 155:591.
- Nickel, Karen L. 1966. The demonstration, characterization and partial purification of a peroxidase and NADase in uterine tissue. Master's Thesis, Kansas State University, Manhattan, Kansas.
- Olds, D. 1969. An objective consideration of dairy herd fertility. *J.A.V.M.A.* 154:253.
- Otani, Yoshihiki, S.J. Berhman, C.W. Porter and M. Nakayama. 1963. Reduction of fertility in immunized guinea pigs. *Internat. J. of Fert.* 8:835.
- Parsons, E.I. and R.R. Hyde. 1940. An evaluation of spermatoxins sera in the prevention of pregnancy. *Am. J. Hyg.* 31:89.
- Paul, K.G., A. Kumlien and S. Brody. 1966. Studies on eosinophil granulocytes. II The effect of cytostatics on the uterine response to oestrogen. *Experientia* 22:799.
- Pommerenke, W.T. 1928. Effects of sperm injection into female rabbits. *Physio. Zool.* 1:97.
- Rowson, L.E.A. and R.M. Moor. 1967. The influence of embryonic tissue homogenate infused into the uterus on the life span of the corpus luteum in the sheep. *J. Reprod. Fert.* 13:511.
- Savani, E. 1911. *Compt. Rend. Sve. Biol.* 71:22. In Katsh, Immunology, fertility and infertility: A historical survey. *Am. J. Ob. Gyn.* 77:946.
- Siegal, B.C. and E.N. Loeb. 1940. Effect of antiplacenta serum on development of foetus in the pregnant rat. *Proc. Soc. Exp. Bio. and Med.* 45:248.
- Simmons, R.L. and P.S. Russell. 1963. Immunologic problems of pregnancy. *Am. J. Ob. Gyn.* 85:583.
- Singh, N.P. 1962. Histopathological studies of the bovine uterus. Master's Thesis. Kansas State University, Manhattan, Kansas.
- Singh, N.P. 1965. Certain immunological bacteriological factors producing embryo loss in dairy cattle. Ph. D. Dissertation. Kansas State University, Manhattan, Kansas.

- Smith, F.G., W.B. Robinson and E. Stotz. 1949. A colorimetric method for the determination of peroxidase in plant material. *J. Biol. Chem.* 179:881.
- Tanabe, T.Y. and J.D. Almquist. 1953. Some causes of infertility in dairy heifers. *J. Dairy Sci.* 36:586.
- Wachstein, Max and E. Meisel. 1964. Demonstration of peroxidase activity in tissue sections. *J. Histochem. and Cytochem.* 12:538.
- Weinbach, R. 1958. Use of formaldehyde treated erythrocytes as antigen carrier in indirect hemagglutination test. *Schweiz. A. Allg. Path.* 21:1043.
- Whitman, James E. and F.M. Hetrick. 1965. An indirect hemagglutination test for detecting antibody to infectious bovine rhinotracheitis virus. *The Cornell Vet.* 55:613.
- Woodruff, M.F.A. 1958. Transplantation, immunity, and the immunological problems of pregnancy. *Pro. Roy. Soc. B.* 148:68.
- Wormser, E. and A. Labhardt. 1904. Weitere Untersuchungen Zur modernen Lehre der Eklampsie. *Munch Med. Wochschr.* 51:2285. In Bardawil, W.A. and B.L. Toy. 1959. The natural history of chorio-carcinoma: problems of immunity and spontaneous regression. *New York Academy of Sciences.* 80:197.
- Yonetani, T. 1961. Studies on cytochrome oxidase III Improved preparation and some properties. *J. Biol. Chem.* 236:1680.

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STUDY OF THE POSSIBILITY OF IMMUNOLOGICAL REJECTION  
OF EMBRYOS AS A CAUSE OF REPRODUCTIVE FAILURE

by

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#### ABSTRACT

This study was devised to investigate the possibility that immunological rejection of embryos is a cause of reproductive failure.

Sexually mature, virgin, female rabbits in seven groups of three animals each constituted the basis of the tests. Groups 1 and 2 provided tests for production of isoantibodies without an adjuvant. In (1) 15-day fetal homogenate was administered intraperitoneally and in (2) bacterins were injected into an intact uterus. Only the bacterin (2) gave positive reactions. All animals of Groups 3 to 7 were anesthetized and a cannula implanted into the right uterine horn through which antigens were administered in nine successive injections at three-day intervals. The injected materials, per group, consisted of (3) 2 ml physiological-saline solution: (4) 2 ml of pooled bacterin composed of killed cultures of Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, and Aeromonas hydrophila, in approximately equal amounts: (5) a mixture of pooled bacteria and 15-day fetal homogenate: (6) pooled bacteria for 6 injections followed by 3 injections of embryonic antigen: (7) 15-day fetal homogenate alone.

After the 9th injection, one animal of each group was sacrificed, and the uterine cannulas were removed from the other two. After 15-days recovery period, these animals were bred by known fertile bucks, then sacrificed on the 15th day after breeding.

Blood was drawn for antibody determinations before injections and at the end of weeks 4 and 8 of treatment. Uterine horns were removed at 8 weeks and homogenized and tested for anti-bacterial and anti-fetal antibodies and for peroxidase activity. Tests for antibacterial antibodies were agglutination dilutions performed separately for each species of

bacteria. The test for anti-fetus antibodies was a passive hemagglutination test using formalized, tannic acid treated, protein sensitized, sheep erythrocytes. Assay for uterine peroxidase was the reoxidation of reduced (leuco) 2,3'6-trichloroindophenol (TIP) dye by peroxidase. The peroxidase tests revealed no difference between treated and non-treated horns.

All species of bacteria used resulted in production of blood serum antibody titres of 1:40 to 1:320 in 4 weeks and up to 1:1200 in 8 weeks indicating the bacterial antigens were absorbed through the uterine epithelium in sufficient quantity to be effective in stimulating antibody production but not as much as animals injected intraperitoneally. Uterine homogenates agglutinated bacteria generally at one dilution below that of the serum, but both horns responded alike, indicating a circulating rather than a localized or fixed antibody.

Both blood sera and uterine homogenates gave positive tests to 15-day fetal antigens with the magnitude of response increasing from none in Group (3), progressively through Groups (4), (7), (6), to (5). The reactions indicate (a) fetal antigens can elicit a response through the uterine epithelium (b) the response to fetal homogenate is greatly enhanced by the presence of or pre-exposure to bacterial antigens, and (c) the response to fetal antigens is not totally specific.

Neither the surgical procedures nor the antigenic stimulation had an adverse effect on the reproductive capacity of the animals, since 60% conceived at first breeding with at least one of each group pregnant at sacrifice. The treated horns had an average of 4.67 embryos, while the untreated horns averaged 4.50. One animal from Group (4) had resorption of all embryos from the treated horn and 4 viable embryos on the untreated side.