The Effect of Pseudorabies (Aujeszky's) Virus Infection on Young Mature Boars and Boar Fertility

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ABSTRACT

This study was designed to determine the effects of experimental inoculation with pseudorabies virus on the reproductive tracts of young adult boars. Pseudorabies virus was inoculated intranasally into 12 boars and intrapreputially into four boars.

All animals seroconverted after nasal or preputial inoculation. Semen abnormalities were observed 21 days postinoculation with partial recovery by 50 days postinoculation. Virus was isolated from the preputial sheath of two intrapreputially inoculated boars 12 days postinoculation. It was concluded that pseudorabies virus infection can be established via preputial inoculation and that decreased spermatogenesis and infertility can result.

Key words: Pseudorabies, Aujeszky's disease virus, boar fertility.

RÉSUMÉ

Cette étude visait à déterminer les effets de l'inoculation du virus de la pseudo-rage sur les organes génitaux de jeunes verrats. Les auteurs inoculèrent à cette fin le virus précité dans les voies nasales de 12 verrats âgés de cinq à huit mois et dans le prépuce de quatre autres du même âge. Tous ces animaux développèrent ultérieurement des anticorps neutralisants. Des anormalités séminales apparurent au bout de 21 jours, pour disparaître toutefois presque complètement au bout de 50 jours. Au bout de 12 jours, les auteurs réussirent à isoler le virus précité de la gaine préputiale de deux des quatre verrats inoculés dans le prépuce; ils en conclurent que l'inoculation à cet endroit peut réaliser l'infection par le virus de la pseudo-rage et qu'il peut en résulter une diminution de la spermatogénèse, ainsi que de l'infertilité.

Mots clés: pseudo-rage, virus de la maladie d'Aujeszky, fertilité des verrats.

INTRODUCTION

In the past decade, Aujeszky's disease (pseudorabies) virus (PRV) has been implicated in a broad range of clinical syndromes. Reproductive disorders associated with pseudorabies and its possible venereal transmission have generated considerable interest.

The reproductive tracts of man and a variety of animal species are a natural route of entrance and site of latency and excretion of several viruses. Many viruses found in domestic animals are reported to have been transmitted venereally and pathogenic viruses have been isolated from the reproductive tracts of clinically normal animals (1-6).

The susceptibility of the porcine male reproductive tract to viral infections has been demonstrated by Phillips *et al* (1) and others (7).

Historically, various experimental and field observations have suggested that PRV may be in boar semen. Investigators have noted testicular degeneration and necrotic foci (8,9) in the testicles of experimentally infected boars and have observed scrotal edema in some naturally infected boars (10).

Recently, some investigators apparently have overcome the obstacles which have prevented the isolation of PRV from boar semen. Gueguen and Aynaud (11) were able to reisolate PRV from the prepuce. They felt that the excretion of virus reflected an increase in activity of virus in the prepuce, since virus was isolated in the last fractions of the ejaculate eight days after the experimental inoculation. Hungarian researchers (12) reported the first case of PRV isolation from semen of naturally infected boars. They demonstrated that the isolate was different from their vaccine strain.

Larsen *et al* (9) described the changes in the semen of boars experimentally infected with PRV. They observed an increase in numbers of proximal cytoplasmic droplets in sperm. Increased numbers of proximal cytoplasmic droplets have frequently been associated with infertility in boars (13,14). Based on several surveys, an increased incidence of proximal droplet and head shape abnormalities appear to be the most important morphological findings in reduced fertility (15-19).

The principal objectives of this study were: to determine the effects of PRV infection on the reproductive tract of boars after intranasal (IN) and intrapreputial (IP) inoculation; to evaluate the effects of PRV infection

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on semen morphology and to determine if the Iowa strain of PRV can be recovered from experimentally inoculated boars.

MATERIALS AND METHODS

Sixteen young mature boars ranging in age from five to eight months old were purchased from a commercial source. The source herd was free of serological evidence of pseudorabies, leptospirosis, transmissible gastroenteritis and clinical evidence of atrophic rhinitis. All pigs were tested for PRV antibody using the microtitration serum neutralization (SN) test (20). Housing consisted of total confinement in isolation units with concrete floors and automatic drinkers. The boars were fed a commercial balanced diet which met with the minimum National Research Council requirement for boar maintenance (21).

EXPERIMENTAL DESIGN AND EXPOSURE

Twelve boars were inoculated intranasally, 4 of the 12 boars received 2 mL of 1 x 10^5 plaque forming units (PFU) per mL and eight of the boars received 4 mL of 1 x 10^6 PFU per mL of a virulent Iowa (S-62) strain of PRV (22). Five mL of the 1 x 10^5 inoculum were perfused into the preputial sheaths of four boars by the use of a sterile 5 mL syringe. The inoculum was then further spread in the preputial sheath by manual manipulation. Four boars were used as control animals.

A control ejaculation preceded inoculation with PRV (Table I). After inoculation, boars that did not die and that were not killed were electroejaculated six more times, at weekly intervals for four times and subsequently at two week intervals until they were killed (see necropsy section).

A pharmacological stress treatment followed the viral inoculation by 50 days in four boars inoculated IN and in four inoculated IP. On the first two treatment days, boars were given 375 IU doses of ACTH (D-M Pharmaceuticals, Inc., Rockville, Maryland) intramuscularly. Simultaneously, intramuscular injections of 37.5 mg of dexamethasone (Schering Corporation, Kenilworth, New Jersey) were given twice daily. On the following three days, after ACTH treatment was discontinued, four daily doses of 75 mg of dexamethasone were given intramuscularly.

TABLE I. Control Semen Evaluation

Boar no.	Vol mL	% Motile	% Abnormal Heads	% Abnormal Tails	Total Sperm Billion
76	55	75	5[a]	5[a]	23,280
77	12	55	5[a]	7	15,000
78	42	80	5[a]	5[a]	11,930
79	37	70	5[a]	10	10,150
80	25	50	5[a]	5[a]	15,950
81	64	70	5[a]	5[a]	11,440
82	40	75	5[a]	5[a]	22,000
83	68	80	5[a]	5[a]	18,540

[a]Denotes true value is less than stated

VIRAL PROPAGATION

A field isolate of a virulent Iowa strain of PRV at the ninth tissue culture passage containing 1.0 x 10^5 PFU per mL was inoculated into a 48 hour old monolayer of porcine kidney (PK 15) cells. The cells were contained in 150 cm tissue culture flasks. The virus was allowed to adsorb to the cells for 60 minutes at 37°C in a 5% CO_2 atmosphere. Following adsorption, the supernatant fluids were removed and minimum essential medium (MEM) containing 2% fetal bovine serum was added to the flask. The flask was reincubated until 90% of the cells developed cytopathic effect (CPE). The flask containing the virus was frozen and thawed twice at -70°C and 37°C respectively. The cells and supernatant were then centrifuged at 1500 x g for 20 minutes in a refrigerated centrifuge. The supernatant was used as the infective virus.

SEMEN EVALUATION

Semen was collected into prewarmed plastic-lined insulated bottles and the semen gel was removed by gauze pads as the semen entered the flask (23). From the total ejaculate a sample was obtained for laboratory evaluation. Wet preparations of semen under coverslips, were used for mortality (live-dead) estimates and sperm cell concentration was determined by spectrophotometry at 525 nm in a 1 to 50 dilution with 2.9% sodium citrate (24). An aliquot of semen was negatively stained (eosin-nigrosin) [Stain was provided by Society for Theriogenology (eosin-nigrosin), 9th and Minnesota, Hastings, Nebraska] and smeared on a glass slide for morphological evaluation. One hundred spermatozoa were randomly selected from the stained preparation and categorized as either morphologically normal or abnormal. Primary (serious) abnormalities involved abnormal heads or acrosomes, coiled tails and proximal cytoplasmic droplets. Secondary abnormalities were detached heads, bent tails and cytoplasmic droplets.

VIRUS ISOLATION

The technique used to demonstrate infectious virus from the trigeminal ganglia was the same as described in a recent publication (25). One mm^3 sections of trigeminal ganglia were collected by the use of sterile scissors and forceps and were placed in 2 mL of MEM containing 2% fetal bovine serum and antibiotics. Tissue fragments were placed on a six well plate containing a monolayer of Madin Darby Bovine Kidney (MDBK) cells and on PK15 cells for seven days. Cells were incubated at 37°C in a 5% CO_2 atmosphere. Nutrient media was removed from these monolayers on day 1, 3 and 6 and was inoculated onto a 24 well plate containing cell monolayers of MDBK or PK15 cells respectively. Fresh nutrient media was used to replenish media removed. The 24 well plates were examined for seven days for CPE. If no CPE was observed, the cell monolayers were freeze-thawed and centrifuged at a low speed to remove cellular debris, the supernate was inoculated onto a new 24 well plate of MDBK or PK15 cells. This procedure was repeated two more times over a 21 day period.

Tissue fragments from the original

monolayer were removed after seven days and placed on a new set of six well plates. This procedure was repeated two more times over a 21 day period. The original cell monolayers were freeze-thawed once and were inoculated onto a new 24 well plate containing monolayers of MDBK or PK15 cells. These were examined for seven days for CPE. If no CPE was observed this procedure was repeated once and the plate was examined for seven more days. Fluorescent antibody tests were used to confirm PRV in cells having CPE suggestive of herpes virus infection. Testicular tissue was examined in the same manner described above.

VIRUS ISOLATION ON SEMEN SAMPLES

The technique used to demonstrate virus in the semen was the same as described by Kahrs *et al* (26). Antitrypsin (Sigma Chemical Company, St. Louis, Missouri) was added to fresh semen samples in a 1:1 ratio. The samples were incubated for one hour at room temperature, and freeze-thawed three times at -70°C. The semen was then centrifuged at 1500 x g for 15 minutes in a refrigerated centrifuge. The supernatants were used to inoculate PK15 cells. The cell cultures were observed for seven days then carried through one blind passage and observed for seven additional days.

Control samples, taken from similarly aged animals, were run with the test samples. For a positive control 4.5 mL of semen was inoculated with 0.5 mL of PRV with a titer of 1×10^3 PFU per mL. The positive control semen and the negative control semen were treated similarly to the experimental semen samples.

VIRUS ISOLATION FROM NASAL AND PREPUTIAL SECRETIONS

Cotton swabs were utilized for swabbing of nasal cavity and preputial membranes. After the swab was taken, it was immediately immersed in 1 mL of sterile Earle's medium and frozen at -70°C. Each sample was prepared for virus isolation by thoroughly mixing swab and media and then wringing all liquid from the swab. The liquid was passed through a 0.45 μm filter onto bovine turbinate cell monolayers and the cultures were assessed daily for specific CPE for seven days. All suspected isolates were passed one additional time to confirm that CPE was not due to toxicity. Fluorescent antibody tests were used as a confirmation test.

CLINICAL SIGNS

Following exposure of the boars to PRV, the animals were observed for clinical signs. Rectal temperatures were taken daily.

NECROPSY

The eight boars which received 4 mL of 1×10^6 PFU of PRV per mL intranasally were killed on successive days after elevation of body temperatures which occurred five days postinoculation (PI). One boar died seven days PI.

Two boars inoculated IN with 2 mL of 1×10^5 PFU per mL and two boars inoculated IP were killed nine days and 29 days PI. The two additional animals that were treated with ACTH and dexamethasone were killed 54 days and 72 days PI respectively. Necropsies were performed on all the boars and gross lesions were recorded.

All representative tissue sections were fixed in 10% neutral buffered formalin, except tissues from the reproductive tract which were fixed in Bouin's fixative.

HISTOTECHNIQUE

Tissues taken for histopathological examination were: central nervous system — trigeminal ganglia, olfactory bulb, pituitary gland, cerebral cortex, optic chiasma, pons, cerebellum, medulla, cervical spinal cord taken at the level of the fifth and sixth cervical vertebrae, thoracic spinal cord taken at the level of the seventh and eighth thoracic vertebrae and lumbar spinal cord taken at the level of the fourth and fifth lumbar vertebrae; reproductive system — the testicle, epididymis, seminal vesicles, prostate gland, bulbo-urethral gland, penis and preputial sheath. Other tissues taken were turbinates, tonsils, spleen, liver, kidney, heart and submandibular lymph nodes.

ELECTRON MICROSCOPY

Utilizing the technique described by Halvorsen (27), selected sections from the hematoxylin and eosin stained sections were processed for electron microscopy to demonstrate the presence of viral particles.

RESULTS

The boars were depressed, reluctant to move, anorectic, ataxic, and had signs of severe respiratory distress characterized by dyspnea and sneezing, four days PI. Constipation was evident in the boars during the febrile period. All animal temperatures were in excess of 40.5°C.

One boar inoculated IN died seven days PI. The death was attributed to PRV.

GROSS LESIONS

All of the boars had a mucopurulent nasal discharge and a cloudy exudate which was tightly adherent to the nasal turbinates. The tonsils of 9/12 boars innoculated IN contained multiple small abscesses throughout the parenchyma. Tonsillar abscesses were also present in two of the four boars inoculated IP that were killed at 9 and 29 days PI.

The lungs were edematous and congested in 8/12 boars inoculated IN and in the boar inoculated IN that died seven days PI. The lungs had multiple abscesses and cranial ventral consolidation. The consolidated areas were dark red and contained fibrin on the pleural surface. The interlobular septa were edematous. On section, purulent exudate could be squeezed from the bronchioles.

Pasteurella multocida was isolated from the pulmonary abscesses. The remainder of the viscera and the reproductive tracts of all the boars were grossly normal.

VIROLOGY

All inoculated animals developed serum neutralizing antibody titers against PRV ranging from 1:4 to 1:64. Pseudorabies virus was isolated from the nasal passage of both groups of boars at least once after inoculation. In two boars inoculated IN and killed five days PI, the tonsil and trigeminal ganglia were positive for viral isolation (VI). Similar results were obtained with specimens from the boar that died seven days PI. In the two boars inoculated IN and killed eight days PI, the tonsil of one was positive with the fluorescent antibody test (FAT) while

the trigeminal ganglia were FAT and VI negative. In the boar that was killed nine days PI the tonsils were FAT positive and the trigeminal ganglia were FAT and VI negative. The tonsils and trigeminal ganglia of the boar inoculated IN and killed ten days PI were FAT and VI negative.

Virus was isolated from the prepuce of two of the four boars inoculated IP at 12 days PI and from the turbinates and brain of one of the boars inoculated IP and killed 29 days PI. Pseudorabies virus was also isolated from the kidney and spleen of one of the four boars inoculated IN (killed nine days PI) before treatment with ACTH and dexamethasone and from the kidney of another IN inoculated boar killed 72 days PI (22 days posttreatment). The remainder of the group was negative for virus isolation. No virus was isolated from the semen at any time during the experiment. Reproductive tissues from all boars were negative on VI and FAT.

MICROSCOPIC LESIONS

In the reproductive system changes were observed in the preputial sheath and penis of the boars inoculated IP. These changes were characterized by edema of the connective tissue in the submucosa of the preputial sheath and the penis. The endothelium of vessels in and around lymphoid nodules in these tissues was swollen and nearly occluded the lumen. Some of the reticuloendothelial cells in the lymphoid nodules were swollen and had displaced chromatin. These changes were not observed with lymphoid nodules in the control or intranasally inoculated boars.

The boars inoculated IN also had mild degeneration of the seminiferous tubules (nine days PI and 29 days PI). Similar lesions were observed in the reproductive tracts of boars inoculated IP (nine days PI and 29 days PI). One boar inoculated IP (nine days PI) had multiple lymphohistiocytic aggregates in the interstitium of the spermatic cord. This lesion was not observed in the control or boars inoculated IN. No lesions were found in the bulbo-urethral gland, prostate gland or seminal vesicle.

The central nervous system, regardless of the route of inoculation, was characterized by a diffuse nonsuppurative meningoencephalomyelitis and ganglioneuritis. Spinal cord lesions were present in two of the four boars inoculated IP. These lesions essentially were confined to the lumbar region where lymphohistiocytic perivascular cuffs were consistently seen nine days PI. There was demyelination and axonal swelling in the lumbosacral plexus. None of the boars inoculated IN had spinal cord lesions.

Eosinophilic intranuclear inclusion bodies were commonly observed in the attached stratified squamous epithelial cells in the tonsils. Necrotic material and exudate extended into the adjacent lymphoid follicles and surrounding reticuloendothelial tissue.

The submandibular lymph nodes contained multiple areas of coagulative necrosis which were associated with germinal centers in the cortical areas.

SEMEN RESULTS

The volume of semen varied over a wide range (12 mL to 105 mL), throughout the study, although each individual boar maintained a uniform volume from one ejaculate to the next. Pseudorabies virus did not have an effect on total sperm production. The first recognizable changes in sperm morphology were coiled and bent tails, which were observed in three of the boars inoculated IP and one of the boars inoculated IN 10 to 14 days PI. The more severe changes observed in the tails were noted 21 days PI (Table II). Four boars (two IP and two IN inoculated) had marked increase in the percentage of reversed, coiled and bent tails, and retained distal cytoplasmic droplets at this time. Head abnormalities were manifested four weeks after inoculation (Table III). The changes observed were knobbed cystic acrosomes, detached heads and double heads. The tail changes mentioned above also remained at this time. A few spheroids (tail-less spermatids) were present as well. Treatment with ACTH and dexamethasone did not have any notable effect on sperm morphology (Table IV). By the sixth week, semen values were normal and the head and tail abnormalities had disappeared in all except one boar inoculated IP which still had minor tail defects.

ELECTRON MICROSCOPY

Virus particles were not demonstrated in any of the selected sections

TABLE II. Semen Evaluation Twenty-one Days Postpseudorabies Virus Inoculation. Intranasal Group Numbers 76-79; Intrapreputial Groups Numbers 80-83

Boar no.	Vol mL	% Motile	% Abnormal Heads	% Abnormal Tails	Total Sperm Billion
IN					
76	25	70	5[a]	5[a]	10,000
77	40	20	5[a]	57	22,000
79	26	20	5[a]	33	9,100
IP					
80	45	15	5[a]	26	15,750
81	15	5	5[a]	72	3,750
82	26	75	5[a]	5[a]	7,800
83	12	15	5[a]	8	3,000

[a]Denotes true value is less than stated

TABLE III. Semen Evaluation Thirty-five Days Postpseudorabies Virus Inoculation

Boar no.	Vol mL	% Motile	% Abnormal Heads	% Abnormal Tails	Total Sperm Billion
IN					
76	45	75	5[a]	5[a]	18,000
77	35	10	34	41	13,125
79	75	15	20	57	18,750
IP					
80	65	55	5[a]	43	14,625
81	60	5[a]	8	72	10,200
82	55	75	5[a]	5[a]	6,875

[a]Denotes true value is less than stated

TABLE IV. Semen Evaluation Fifty-one Days Postpseudorabies Virus Inoculation and Following ACTH and Dexamethasone Treatment

Boar no.	Vol mL	% Motile	% Abnormal Heads	% Abnormal Tails	Total Sperm Billion
IN					
76	24	35	5[a]	6	4,200
77	35	50	5[a]	5[a]	8,750
79	20	20	5[a]	5[a]	3,000
IP					
80	45	70	5[a]	5[a]	22,500
81	22	20	5[a]	13	2,750
82	25	25	5[a]	6	4,000

[a]Denotes true value is less than stated

from the hematoxylin and eosin stained sections.

DISCUSSION

All of the boars in this study had evidence of pseudorabies. Detectable quantities of PRV were not found in the semen of either IN or IP inoculated boars. Several animals were ataxic after IN and IP inoculation. There were severe respiratory signs observed in the IN inoculated group. The interstitial changes produced in the lungs of these animals were similar to those described previously with the exception of intranuclear inclusion bodies in the lung (8,28,29). The lesions observed in the tonsils were similar to those described in previous reports (30-34). Absence of gross lesions in the testicles and epididymis of the boars in this study is in agreement with workers from Taiwan (35) who observed no gross lesions in these organs during an outbreak of PR. This disagrees with one report (10) of observed scrotal edema in boars during an outbreak of PR.

A higher dose of virus was used for the boars inoculated IP in order to establish infection which was verified by seroconversion and isolation of virus from preputial swabs and brain. Though virus was isolated from the kidney of one IN-ACTH/dexamethasone treated boar and from the spleen of another boar from this group, microscopic lesions were not present in these organs.

Microscopic changes in the reproductive tissues were associated with the lymphoid nodules in the submucosa of the preputial sheath and penis. Dogs infected with canine herpesvirus developed vesicular lesions which regressed to firm lesions resembling lymphoid follicles (36). Cattle infected with infectious pustular vulvovaginitis virus, a herpesvirus, develop lymphoid follicles in the submucosa. Many of the small vessels in these follicles have swollen endothelial cells protruding into the lumen (37). Vesicular lesions were not observed in young swine inoculated with herpesvirus intrapreputially.

The presence of swollen reticuloendothelial cells with displacement of chromatin and swollen vascular endothelial cells in the lymphoid nodules of the submucosa of the preputial sheath and penis in IP inoculated boars may be suggestive of viral localization and replication sites. Virus was isolated from preputial swabs 12 days PI indicating that virus had replicated in that area.

Corner (8) reported focal necrosis of the testicular tissues in piglets inoculated with PRV. He observed intranuclear inclusion bodies in cells at the periphery of necrotic foci and in endothelial cells of blood vessels in the necrotic foci. These changes are in disagreement with the findings of this study and are possibly related to the age of the animals used in his study which were considerably younger than the animals used here.

The initial changes observed in the semen were tail abnormalities which were first detected 10-14 days PI. That change was attributed to the acute stages of the infection and elevated body temperature because the subsequently affected spermatozoa were in the seminiferous tubules at the time of the inoculation. Other investigators have demonstrated that increasing testicular temperature to the level of the body temperature for only a few hours can cause infertility (38). The two samples taken prior to and during the initial temperature rise were not abnormal because mature spermatozoa in the epididymis are less sensitive to temperature elevation. The duration of infertility depends on the magnitude and duration of elevated testicular temperature (38). Subsequent developmental changes, head abnormalities and spheroids, indicated damage to the spermatocytes and sertoli cells in the seminiferous tubules (39). Infertile boars have on the average a significantly higher incidence of droplets than do fertile boars. Indications from the current study suggest that production and maturation defects in the seminiferous tubules and epididymis prevent sperm cells from releasing droplets. The inability to isolate PRV from the testicular tissues and semen may suggest that this virus does not cross the blood-testes barrier. This finding differs from European (12,13) reports of viral isolation from semen. Their finding may be due to differences of strain pathogenicity or contamination of semen at the time of collection with PRV from preputial secretions.

The necrotic foci seen in the submandibular lymph nodes are the result of lymphocytic necrosis due to the administration of immunosuppressant drugs. Steroids have been shown to result in lymphopenia and lympholysis in lymph nodes (39-41). The lack of isolation of virus from tissues other than those discussed, after steroid treatment, indicates the lack of virus or that virus was present in undetectable quantities.

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