Research Note—

Avian Infectious Bronchitis Virus: A Possible Cause of Reduced Fertility in the Rooster

David A. Boltz, Masaaki Nakai, and Janice M. Bahr

Department of Animal Sciences, University of Illinois at Urbana-Champaign, 1207 West Gregory, Urbana, IL 61801.

Received 8 April 2004; Accepted 29 July 2004

SUMMARY. The formation of epididymal stones in the rooster epididymis is a widespread problem that has detrimental effects on sperm production and fertility. The cause of epididymal stones is unknown, but an infectious agent, the avian infectious bronchitis virus (AIBV), has been implicated. The goal of this study was to determine if administering the live attenuated AIBV vaccine to male chicks increases the incidence of stones in the epididymal region of the adult rooster. Specific pathogen free (SPF) Leghorn roosters were divided into two groups: a vaccine-free group ($n = 7$) and a group vaccinated with AIBV ($n = 12$). The vaccine was administered orally at 2, 4, 10, and 14 wk of age. Blood was drawn weekly to monitor antibodies to AIBV. At 26 wk of age, blood was obtained to determine testosterone concentrations, and reproductive tracts were removed to analyze daily sperm production and to detect epididymal stones. Nine of 12 vaccinated roosters developed stones, whereas those not given the vaccine did not develop stones. Serum testosterone concentrations were significantly ($P < 0.05$) reduced in vaccinated roosters with epididymal stones (3.6 ± 0.30 ng/ml) when compared with nonvaccinated roosters that did not have epididymal stones (7.0 ± 1.63 ng/ml). Testis weight was significantly ($P < 0.05$) reduced in vaccinated roosters with epididymal stones (12.1 ± 0.76 g), as compared with nonvaccinated roosters without epididymal stones (15.2 ± 0.81 g). Daily sperm production was significantly ($P < 0.05$) decreased in vaccinated roosters with epididymal stones (5.03 ± 0.31 × 10⁸ sperm/testis/day) when compared with nonvaccinated roosters without epididymal stones (7.43 ± 0.52 × 10⁸ sperm/testis/day). Comparing daily sperm production on a per gram basis, vaccinated roosters with epididymal stones had 4.38 ± 0.14 × 10⁷ sperm/g of testis, which was significantly ($P < 0.05$) smaller than nonvaccinated roosters without epididymal stones, which had 5.17 ± 0.17 × 10⁷ sperm/g of testis. We conclude that the use of a live attenuated AIBV vaccine increases the incidence of epididymal stones in roosters, resulting in decreased sperm production and decreased serum testosterone concentrations.

RESUMEN. Nota de Investigación—Virus de la bronquitis infecciosa aviar: Posible causa de la reducción de la fertilidad en machos reproductores.

La formación de cálculos en el epidídimo de machos reproductores es un problema difundido que tiene efectos adversos en la producción de esperma y en la fertilidad. Se desconoce la causa de los cálculos en el epidídimo, sin embargo, el virus de la bronquitis infecciosa ha sido implicado. El objetivo de este estudio fue determinar si la administración de una vacuna viva atenuada del virus de la bronquitis infecciosa a pollitos machos aumenta la incidencia de cálculos en el epidídimo en el macho reproductor. Se dividieron pollitos machos tipo Leghorn libres de patógenos específicos en dos grupos: un grupo no vacunado ($n = 7$) y un grupo vacunado con el virus de la bronquitis infecciosa ($n = 12$). Se administró la vacuna por vía oral a las 2, 4, 10 y 14 semanas de edad. Se tomaron muestras semanales de sangre para evaluar la presencia de anticuerpos contra el virus de la bronquitis infecciosa. Se tomaron muestras de sangre a las 26 semanas de edad para determinar las concentraciones de testosterona y se removieron los tractos reproductivos para analizar la producción diaria de esperma y para detectar la presencia de cálculos en el epidídimo. Se desarrollaron cálculos en el epidídimo en nueve de los 12 machos reproductores vacunados,

^ Corresponding author.

909
Avian infectious bronchitis virus (AIBV) is a coronavirus known to cause an acute respiratory infection in chickens. AIBV not only grows in respiratory tissue, but also in nonrespiratory organs such as the kidney, the female reproductive tract, intestine, and spleen of chickens (11). The virus has received attention because of its negative economic impact on poultry. AIBV causes economic loss to the poultry industry, not only as a result of high morbidity and, in some instances, mortality, but also from the debilitating nature of the disease resulting in poor use of feed by young chickens. Several AIBV strains have been isolated that are known to infect the kidney. In the kidney, AIBV causes tubule dilation and kidney lesions of varying degrees (1,2,3). The major economic loss is from ovarian damage and the precipitous and prolonged decrease in egg production in laying flocks (5). AIBV infects the oviduct and causes lesions, degeneration of the epithelial lining, and the formation of cysts (4). The epithelial lining of the female reproductive tract contains numerous ciliated cells, which are a target for AIBV. Similarly, the male reproductive tract contains ciliated epithelial tissue and is a possible target for AIBV.

Pathologic changes can occur in the epididymal region that adversely alter the production of fertile spermatozoa. A recently discovered dysfunction of this region is the presence of stones composed of calcium carbonate in the efferent ductules of the rooster (8). The epithelial lining of the efferent duct was eroded in roosters with stones. This erosion could be a cause of cell death that results in the formation of stones, or the stones could be the cause of the epithelial cell erosion. Roosters with stones had decreased testis weight and decreased sperm production per day. The presence of epididymal stones reduced fertility 40% and 35% with natural mating and artificial insemination, respectively.

The cause of epididymal stones is unclear, but two possible explanations have been explored. It was first suggested that the stones could be caused by diet. Roosters were fed the laying hen diet, which contains high levels of calcium, the primary component of the stones, and vitamin D3, which are well above the daily requirements for a rooster. An experiment to determine if high dietary intake of calcium and vitamin D3 was the cause of the epididymal stones indicated that diet was not the cause for the stones (unpublished data). A second possibility is that vaccination with AIBV may cause epididymal stone formation (J. Kirby, University of Arkansas, pers. comm.). This virus has an affinity for tissues with ciliated cells. The epididymal region of the rooster contains many ciliated cells, and stone formation was observed in that region. Therefore, we hypothesized that AIBV is the causative agent responsible for the formation of epididymal stones. To test this hypothesis we used specific pathogen free (SPF) Leghorn roosters. We vaccinated one group with the AIBV vaccine, whereas the second group (control) was not vaccinated. At 26 wk of age, the reproductive tract was removed and
analyzed for the presence of epididymal stones and sperm production.

**MATERIALS AND METHODS**

**Animals.** Commercial white Leghorn specific pathogen-free (SPF) eggs, purchased from Charles River Laboratories (North Franklin, CT), were hatched in the Animal Sciences Laboratory and reared in the animal facility in the Edward R. Madigan Laboratory at the University of Illinois, Urbana-Champaign, IL. Roosters were separated into two groups and raised in a brooder until 4 wk of age. The roosters were moved into chicken cages until 16 wk of age. After 16 wk of age, roosters were placed in individual rooster cages. Vaccination with the attenuated live AIBV vaccine began at 2 wk of age. The vaccinated group (12 roosters) were isolated from the nonvaccinated group (7 roosters). Roosters were fed a commercial laying hen diet. Feed and water were provided ad libitum. All animal use and procedures were approved by the University of Illinois Animal Care and Use Committee.

**Vaccine virus.** The vaccine used in the study contained the live attenuated AIBV. The commercial IBVac-H® Mass type vaccine was used for this study and is available from Intervet (Millsboro, DE). Serial 0100001 IBVac-H® titer is 10^4.5 EID50 viral particles per dose. The roosters were administered one dose in a volume of 0.2 ml orally using a 1-ml syringe at ages 2, 6, 10, and 14 wk.

**Sample collection.** Blood was drawn from each bird beginning at 3 wk of age at 1-wk intervals continuing until 19 wk of age. At 19 wk of age, blood was drawn at 2-wk intervals through 26 wk of age. Blood was taken from the basilic vein, and serum was removed from each sample and stored at −80°C for determination of antibody titer to AIBV. Serum was taken at 26 wk of age was stored at −20°C for determination of testosterone concentrations by radioimmunoassay. At 26 wk of age, roosters were euthanatized with an ELISA plate reader set at 405 nm. Microtiter plates were read with an ELISA plate reader set at 405 nm.

**Estimation of sperm production.** One testis from each 26-wk-old bird was processed for the determination of sperm production, as described by Kirby et al. (9). The epididymis was removed, and the weight of the thawed testis was recorded with and without the capsule. After weighing the testis, it was homogenized in 10 volumes (w/v) of saline-triton-merthiolate buffer (150 mM of NaCl, 0.05% (v/v) Triton X-100, and 0.25 mM of merthiolate (Sigma Chemical Co., St. Louis, MO)) in a kitchen blender. Homogenization was achieved by subjecting the tissue and buffer mixture to five 30-sec pulses. Elongated spermatids that survived homogenization were counted. To count the elongated spermatids, 0.2 ml of the sample homogenate was diluted with 0.8 ml of a saline solution containing 0.4% trypan blue. Ten microliter aliquots were counted on a hemocytometer in quadruplicate to determine the average number of spermatids per sample. Daily sperm production estimates per testes and per gram of testis were determined by dividing the number of resistant nuclei by 4.5, the average number of days elongated spermatids remain in the testis before their entry into the excurrent duct system.

**Stone analysis.** The epididymis of the frozen testis was removed before homogenization of the testis to determine if stones were present in the epididymis. The epididymis was weighed and then placed in 5 N of NaOH overnight. The tissue was vortexed periodically to ensure total tissue disruption. Once the stones were no longer observed in the tissue, any remaining tissue was rinsed away, and the solution containing the stones was filtered through Whatman® 541 filter paper (Whatman, Clifton, NJ) to remove excess water. The filter paper and stones were placed in a drying oven overnight. The stones were then weighed to determine the total amount of stones present in the epididymis. The weight of stones per gram of epididymis was compared to determine the percentage of stones present in the epididymis.

**Histology.** Testes were fixed in 10% neutral-buffered formalin for 3 days and then processed for paraffin embedding. Sections were cut at 4 μm and stained with hematoxylin and eosin. They were evaluated under a light microscope to determine structural differences between groups.

**Serum testosterone measurements.** Roosters were bled and serum obtained at 26 wk of age just before euthanatization. Testosterone concentrations were determined in serum after ethyl ether extraction of serum samples using the procedure of Janssen et al. (8).

**Antibody titers to AIBV.** Antibody titers to AIBV were monitored using the ProFLIK® IBV ELISA (enzyme-linked immunosorbent assay) Kit from Synbiotics Corporation (San Diego, CA; Catalog number 96-6506). Samples were analyzed according to the test-kit instructions. Microtiter plates were read with an ELISA plate reader set at 405 nm.

**Statistical analysis.** A Student t-test was performed to determine the effects of treatments. Differences were considered statistically significant when \( P < 0.05 \).

**RESULTS**

Epididymal stones were observed in 75% (9/12) of the roosters vaccinated with AIBV, whereas nonvaccinated roosters did not develop stones (0/7) (Table 1). The weight of the epididymis was similar between roosters given the vaccine (576.6 ± 0.07 mg) and roosters not given the vaccine.
The mass of stones present in roosters vaccinated with AIBV ranged from less than 0.1 mg to 20.4 mg. As a percentage of total epididymal weight, the stones accounted for between 0.01% and 3.4% of the epididymal mass. Subsequent data analyses were performed comparing data of nonvaccinated roosters without epididymal stones (n = 7) and data of vaccinated roosters with epididymal stones (n = 9). These comparisons are presented in Results and the tables.

Testis weight was reduced (11.9 ± 0.6 g) in roosters given the vaccine compared with roosters not given the vaccine (15.7 ± 0.6 g) (Table 1). Daily sperm production (5.0 ± 0.3 × 10^8 sperm per testis per day) was lower (P < 0.05) in vaccinated roosters when compared with roosters not given the vaccine (7.7 ± 0.5 × 10^8 sperm per testis per day) (Table 1). Comparing daily sperm production on a per gram basis, roosters given the vaccine produced 4.5 ± 0.13 × 10^7 sperm/g of testis, whereas roosters not given the vaccine produced 5.3 ± 0.18 × 10^7 sperm/g of testis (P < 0.05) (Table 1). Radioimmunoassay of serum testosterone indicated that vaccination with AIBV and subsequent epididymal stone formation adversely affected serum testosterone concentrations. Serum testosterone concentrations were significantly (P < 0.05) reduced in roosters given the vaccine and having stones (3.5 ± 0.3 ng/ml) when compared with roosters not given the vaccine and not having stones (7.0 ± 1.9 ng/ml) (Table 1). Control roosters did not develop antibodies, whereas vaccinated roosters developed antibodies to AIBV, as determined by ELISA (Table 1). No correlation could be established between the levels of serum antibody titers and stone formation.

Efferent ductules of nonvaccinated roosters were normal, consisting of ciliated and nonciliated columnar cells, and were highly folded (Fig. 1A, C). In roosters vaccinated with AIBV, the presence of stones made it difficult to examine the histologic structures of the affected efferent ductules (Fig. 1B). The columnar epithelium of affected efferent ductules was shorter than that of stone-free efferent ductules. There was an infiltration of plasma cells and lymphocytes surrounding the affected ductules (Fig. 1D). There was no difference in the histology of the testes between roosters vaccinated and those not vaccinated (Fig. 1E, F).

**DISCUSSION**

Based on previous data, we hypothesized that vaccination with attenuated AIBV is the causative agent responsible for the development of epididymal stones and ultimately impaired fertility in roosters. To test this hypothesis, SPF roosters were either vaccinated with attenuated AIBV or not vaccinated. At 26 wk of age, 75% of the roosters vaccinated with AIBV had epididymal stones, decreased testis weight, decreased daily sperm production, and decreased serum testosterone concentrations. Nine of 12 males vaccinated with the attenuated AIBV vaccine developed epididymal stones, whereas those that were not vaccinated did not have stones. AIBV vaccinated roosters developed antibodies, whereas those that were not vaccinated did not develop antibodies. These data are strong evidence that vaccination against AIBV with a live attenuated AIBV vaccine results in the formation of epididymal stones. The process through which epididymal stones are formed is unclear. Initially, it was speculated that stone formation was related to a high dietary intake of calcium. However, a nutritional study tested the effects of dietary calcium on the presence of epididymal stones, and no relationship between the presence of stones and the concentration of calcium

Table 1. Effect of vaccination and subsequent epididymal stone formation on testis weight, sperm production, serum testosterone concentrations, and antibody titers to AIBV.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Epididymal stones</th>
<th>Testis weight (g)</th>
<th>Sperm production (sperm/testis/day)</th>
<th>Sperm production (sperm/g of testis)</th>
<th>Testosterone (ng/ml)</th>
<th>AIBV antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND^B</td>
<td>15.7 ± 0.6^*</td>
<td>7.7 ± 0.5^C</td>
<td>5.3 ± 0.2^D</td>
<td>7.0 ± 1.9^*</td>
<td>ND^B</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>75%</td>
<td>11.9 ± 0.8</td>
<td>5.0 ± 0.3^C</td>
<td>4.5 ± 0.1^D</td>
<td>3.5 ± 0.3</td>
<td>3314^E</td>
</tr>
</tbody>
</table>

^*Values given are mean ± SE.
^ND = not detectable.
^CMultiply by 10^8.
^DMultiply by 10^7.
^EAverage titer of vaccinated roosters at 23 wk of age.

^ Indicates that mean is significantly different (P < 0.05) from that of the vaccinated group.
Fig. 1. Testes and epididymal regions of 26-wk-old nonvaccinated roosters and vaccinated roosters with stones. (A) The lumen of the efferent ductules of a nonvaccinated rooster is folded with sperm in the efferent ductule lumen (ED). The seminiferous tubules (ST) of the testes are normal. (B) Epididymal stones are present in two efferent ductules (arrows) of a rooster vaccinated with AIBV. Adjacent to the ducts with stones are normal ducts without stones and with sperm in the lumen (L). Seminiferous tubules of the testes are normal. (C) The epithelium of a proximal efferent ductule is columnar with ciliated cells (arrows) and nonciliated cells (arrow heads). (D) The efferent ductule with a stone is surrounded by lymphocytes, as seen by the darker stain (arrows). A normal efferent ductule is adjacent to the affected duct with sperm present in the lumen (L). (E) The seminiferous tubules of a testis from a 26-wk-old nonvaccinated rooster are normal with the germinal epithelium present and sperm in the lumen. (F) The seminiferous tubules of a testis from a vaccinated rooster with stones are normal.
and vitamin D in the diet was found (unpublished data).

The second possibility that AIBV vaccination may be the cause of epididymal stone formation was based on the tropism of AIBV for ciliated epithelium. AIBV may have a tropism for the ciliated cells in the epididymal region. The virus has a tropism for ciliated epithelial cells, which are found in the respiratory tract, oviduct, and the intestine (11). AIBV has been shown to decrease egg production in the female, thus reducing fertility. The effects of vaccination on the male reproductive tract have not been previously investigated.

Testis weight was decreased in vaccinated roosters with epididymal stones compared with nonvaccinated roosters without stones. A regression in testicular size is associated with a decrease in androgen levels and atrophy of the seminiferous tubules (6). The histology of the seminiferous tubules and spermatogenesis was normal in vaccinated roosters as compared with nonvaccinated roosters. Therefore, the decrease in testis size cannot be attributed to abnormalities in spermatogenesis, even though a reduction in daily sperm production was observed.

Daily sperm production was reduced in vaccinated roosters with stones. This reduction in daily sperm production was also observed on a per gram basis, so the reduction was not only attributed to the decrease in testis size. The presence of stones may cause a decrease in daily sperm production at 26 wk of age by decreasing testosterone production. Testosterone is required for the maintenance of daily sperm production. A decrease in testosterone adversely affects reproduction in birds by decreasing or arresting sperm production (10). Also, in the absence of testosterone, the testis will regress.

Vaccination against AIBV and subsequent epididymal stone formation resulted in a reduction of serum testosterone concentrations. This decrease in serum testosterone concentration could be due to an inflammation, as noted by the infiltration of lymphocytes into the stroma of the epididymis. Lymphocytes secrete interleukins in response to an infection. An interleukin, IL-1, linked with inflammation will decrease the activity of the P450/c17 lyase, the enzyme that converts progesterone to androgens and will decrease testosterone production (7). It is difficult to determine what effect the presence of stones has on the Leydig cells because they are so few in number in both vaccinated and nonvaccinated roosters.

The presence of stones has been shown to adversely affect fertility (8). In our previous study, we found that fertility was reduced 35% in hens artificially inseminated with equal numbers of sperm from roosters with epididymal stones as compared with hens inseminated with equal numbers of sperm from roosters without epididymal stones. These results suggest that sperm collected from roosters with epididymal stones were defective. No tests were performed to determine whether there were significant differences in seminal plasma constituents, sperm concentration, motility and morphology, and sperm survival in the female reproductive tract. The rooster does not have any accessory glands; therefore, the seminal plasma is epididymal fluid. A change in seminal plasma constituents would be an indicator of an epididymal dysfunction.

We have described a reproductive impairment caused by vaccinating roosters with live attenuated AIBV. This investigation provides convincing evidence that AIBV is involved in the formation of epididymal stones and reduced daily sperm production and serum testosterone concentrations. Future research will be performed to determine whether vaccination with AIBV and subsequent epididymal stone formation are directly or indirectly related.

REFERENCES


