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Epididymal Stone Formation and Decreased Sperm Production in Roosters Vaccinated with a Killed Strain of Avian Infectious Bronchitis Virus

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SUMMARY. Our objective was to determine if vaccination with killed avian infectious bronchitis virus (AIBV) causes epididymal calcium stones in the rooster as is seen following vaccination with live attenuated AIBV. Specific-pathogen-free roosters were divided into three groups: nonvaccinated (NONVAC), live attenuated AIBV–vaccinated (LVAC), and killed AIBV–vaccinated (KVAC) groups. Roosters were vaccinated at 2, 6, 10, and 14 wk of age and the epididymal region was observed at 27 wk of age. Epididymal stones were present in 13% of NONVAC, 50% of KVAC, and 64% of LVAC roosters. Histologically, immune cells were seen in the interstitium of efferent ductules containing stones. We conclude that use of a killed vaccine does not reduce the incidence of epididymal stones.

RESUMEN. Formación de cálculos y disminución de la producción de esperma en gallos vacunados con una cepa inactivada del virus de bronquitis infecciosa aviar.

Nuestro objetivo fue demostrar si la vacunación con virus inactivado de bronquitis infecciosa aviar causa cálculos epididimales de calcio en gallos, de la misma manera como se observan posterior a la vacunación con virus vivo atenuado de bronquitis infecciosa aviar. Gallos libres de patógenos específicos se dividieron en tres grupos: no vacunados, vacunados con virus vivo atenuado de bronquitis infecciosa aviar y vacunados con virus inactivado de bronquitis infecciosa aviar. Los gallos fueron vacunados a las 2, 6, 10 y 14 semanas de edad y la región del epidídimo se observó a las 27 semanas de edad. Se encontraron cálculos epididimales en el 13% de las aves no vacunadas, en el 50% de las aves vacunadas con virus inactivado de bronquitis infecciosa aviar y en el 64% de las aves vacunadas con virus vivo atenuado de bronquitis infecciosa aviar. Histológicamente se observaron células inmunes en el intersticio de los conductos eferentes que contenían cálculos. Se concluye que el uso de vacunas inactivadas no disminuye la incidencia de cálculos epididimales.

Key words: killed AIBV, epididymis, stones

Abbreviations: AIBV = avian infectious bronchitis virus; ELISA = enzyme-linked immunosorbent assay; KVAC = killed AIBV–vaccinated; LVAC = live attenuated AIBV–vaccinated; NONVAC = nonvaccinated; SPF = specific-pathogen-free

Avian infectious bronchitis virus (AIBV) is a member of the *Coronaviridae* family and the genus *Coronavirus* (5). AIBV is an enveloped virus with a single-stranded positive-sense RNA genome of 28 kb (3,17,25), and is most commonly associated with respiratory disease because the epithelial cells of the trachea are the primary site of AIBV replication. Clinical signs include breathing difficulty, coughing, sneezing, and lethargy (10,11). In addition, AIBV has been associated with pathological changes in other organs such as the kidney and oviduct (6,12). Nephritis is characterized by clinical signs of excess water intake, rapid weight loss, and possible mortality (26). Replication of AIBV in the epithelial cells of the oviduct results in decreased egg production, eggs of smaller size, and decreased egg shell quality (20,24).

To alleviate these clinical signs and to control the economic losses associated with poor weight gain and loss of egg production, AIBV vaccines are routinely administered. The type of vaccine, inactivated or live attenuated, and AIBV strains differ by location and operation. Although inactivated vaccines contain killed AIBV that is unable to replicate, they provide adequate protection against homologous serotypes when used alone. However, they are not cost effective in commercial production because of the high labor cost for administration (21) and, therefore, are used in addition to live attenuated AIBV, which is easier and less costly to administer than killed AIBV.

This combination vaccine strategy has been successfully used in preventing infectious bronchitis in poultry. However, a recent study from our laboratory reported that there is an association of live AIBV vaccination with epididymal stone formation and reduced sperm production (2). AIBV has also been isolated from semen of infected roosters 2 wk postinfection (7). Because the rooster does not have accessory sex glands and because live attenuated AIBV has been shown to replicate in epididymal tissue in culture (unpubl. data), we hypothesized that epididymal stones were a direct result of an AIBV infection to the epididymal region. The administration of killed AIBV, which is unable to infect tissue, could verify that live AIBV infects the epididymal region *in vivo* ultimately causing stones. The present experiment was conducted to determine if vaccination with killed AIBV (KVAC) induces the formation of epididymal stones as induced by vaccination with live AIBV (LVAC) in sexually mature white Leghorn roosters.

MATERIALS AND METHODS

Animals. Commercial single-comb 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. Roosters were separated into three groups: nonvaccinated (NONVAC, $n = 8$), KVAC ($n = 10$), and LVAC ($n = 11$). Roosters were raised in brooders until 4 wk of age. The roosters were then moved into chicken cages until 16 wk of age. After

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16 wk of age roosters were placed in individual rooster cages. The NONVAC, KVAC, and LVAC groups were isolated from each other prior to vaccination. Strict measures were taken to prevent cross contamination among groups. Vaccination with the live attenuated AIBV vaccine and killed AIBV vaccine began at 2 wk of age. Vaccines were administered at 2, 6, 10, and 14 wk of age. 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 live infectious bronchitis vaccine, IBVac-H® Mass-type vaccine, was used (serial number 0102002, Intervet, Millsboro, DE). IBVac-H® titer was 10 4.3 EID_{50} viral particles per dose. Roosters were administered one dose in a volume of 0.2 ml orally using a 1-cm^3 syringe on weeks 2, 6, 10, and 14 of age. The killed infectious bronchitis vaccine (INACTI/VAC® IB1) Massachusetts type, manufactured by Maine Biological Laboratories, Inc. (Waterville, ME) was provided by Lohmann Animal Health International (Waterville, ME). The killed vaccine was administered subcutaneously in the lower neck region using a dosage of 0.25 ml/kg according to the manufacturer's instructions on weeks 2, 6, 10, and 14 of age.

Sample collection. At 27 wk of age roosters were euthanatized with CO_2. The testes were removed and weighed; one testis was placed in 10% neutral buffered formalin for histological evaluation and the other was placed in dry ice and stored at −20 C for determination of sperm production. Prior to freezing the testis, the epididymis was removed and placed in 5 N NaOH to digest the tissue for stone analysis.

Presence of epididymal stones. The epididymis in 5 N NaOH was vortexed periodically to ensure total tissue disruption. Once the epididymis was digested and stones were no longer observed in the epididymis, the remaining epididymis was rinsed away and the solution containing the stones was filtered through Whatman® 541 filter paper to remove excess water. The stones were then weighed to determine the total amount of stones present in the epididymis.

Estimation of sperm production. One testis from each 27-wk-old bird was processed for the determination of sperm production as described by Kirby *et al.* (16). 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 NaCl, 0.05% [v/v] Triton X-100, and 0.25 mM merthiolate [Sigma Chemical Co., St. Louis MO]) in a Waring 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 testis 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 prior to their entry into the excurrent duct system (9).

Histology. Testis and epididymis 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 for histological examination.

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

Statistical analyses. One-way analysis of variance was performed for comparison among groups. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Presence of epididymal stones. Epididymal stones were observed in 64% (7/11) of LVAC and 50% (5/10) of KVAC roosters. In NONVAC, 13% (1/8) developed epididymal stones (Fig. 1A). The mass of stones present in roosters vaccinated with AIBV (live-attenuated and killed) ranged from less than 0.1 mg to 28.2 mg. Subsequent analyses were performed on all roosters in the vaccinated groups, KVAC ($n = 10$) and LVAC ($n = 11$). The rooster with epididymal stones in the NONVAC group was removed from subsequent analysis.

Testis weight and daily sperm production. Testis weights in the NONVAC (12.3 ± 0.7 g), KVAC (12.7 ± 1.1 g), and LVAC roosters (12.1 ± 0.9 g) were not significantly different (Fig. 1B). Daily sperm production per testis was reduced in LVAC ($3.9 \pm 0.3 \times 10^8$, $P < 0.05$) and in KVAC ($4.4 \pm 0.7 \times 10^8$) roosters when compared to NONVAC roosters ($4.9 \pm 0.4 \times 10^8$) (Fig. 1C). Daily sperm production per gram testis was reduced in LVAC ($3.5 \pm 0.2 \times 10^7$, $P < 0.05$) and KVAC ($4.1 \pm 0.3 \times 10^7$, $P < 0.05$) roosters when compared to NONVAC roosters ($4.7 \pm 0.1 \times 10^7$) (Fig. 1D).

Histology. Efferent ductules of NONVAC roosters were normal, consisting of ciliated and nonciliated columnar cells, and were highly folded (Fig. 2A). Efferent ductules of KVAC and LVAC roosters without stones resembled those of NONVAC roosters (not shown). In KVAC and LVAC roosters, the presence of epididymal stones made it difficult to examine the histological structures of the affected efferent ductules. There was an infiltration of immune cells, specifically lymphocytes and plasma cells surrounding affected ductules (Fig. 2B). In epididymal tissue from KVAC roosters, aggregations of sperm, macrophages, and cell debris were observed in the efferent ductules (Fig. 2B, inset). Sloughed epithelial cells were also present in the lumen of an efferent ductule associated with the erosion of epithelial cells. The epididymal tissue from LVAC roosters with stones was similar to epididymal tissue from KVAC roosters with stones (Fig 2C). Dense sperm aggregation was observed on the perimeter of a recently developed epididymal stone in the lumen, indicating that calcium deposition occurred within the sperm aggregation (Fig. 2C, inset). In the rete testis, fibrous connective tissue and immune cells were surrounding extravasated sperm typical of a spermatic granuloma (Fig. 2D). Seminiferous tubules were normal in all groups (data not shown).

Antibodies. At 27 wk of age, NONVAC roosters did not develop antibodies, whereas KVAC and LVAC roosters developed antibodies to AIBV, as determined by ELISA. Antibody titers were higher in KVAC roosters (7571 ± 2617) when compared to LVAC roosters (2984 ± 1003) at 27 wk of age. No correlation could be established between serum antibody titers and stone formation.

DISCUSSION

The present experiment was designed to determine if vaccination with killed AIBV induces the formation of epididymal stones as is induced by vaccination with live attenuated AIBV. The results showed that 50% of roosters in the KVAC group and 64% of roosters in the LVAC group developed epididymal stones. In addition to the presence of epididymal stones, we observed a decrease in daily sperm production in KVAC roosters when compared to NONVAC roosters. These results indicate that not only live AIBV but also killed AIBV can induce epididymal stones in the rooster, and suggest that stone formation is not caused by AIBV entering and replicating in the epithelium of the epididymal region.

Based on our earlier study (2), the development of epididymal stones in 64% (7/11) of LVAC roosters was expected. The virus has a tropism for ciliated epithelium, which is found in the trachea, oviduct, and epididymal region. Furthermore, AIBV has been shown to infect the epididymal region *in vitro* (8, unpubl. data). Accordingly, we did not expect to observe epididymal stones in

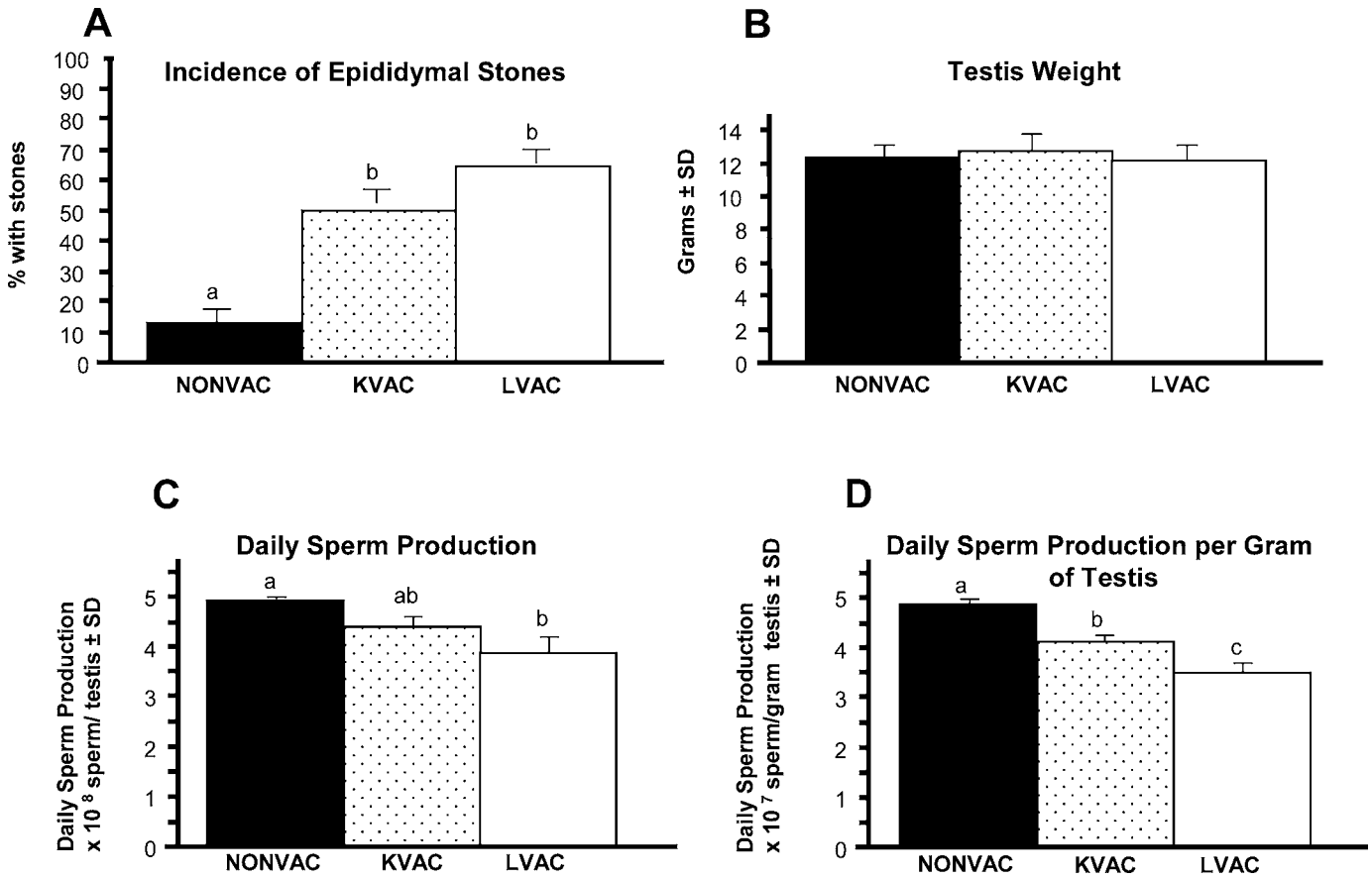

Fig. 1. Analysis of rooster reproductive organs at 27 wk of age. (A) Incidence of epididymal stones. Stones are seen in 13% of NONVAC roosters, 50% of KVAC roosters and 64% of LVAC roosters. (B) Testis weight. There is no statistical difference among groups. (C) Daily sperm production. LVAC roosters show a significant decrease compared to NONVAC ($P < 0.05$). Different letters indicate significant differences. (D) Daily sperm production per gram of testis. A significant decrease is seen between NONVAC and KVAC groups ($P < 0.05$). A significant decrease is also present between KVAC and LVAC groups. Different letters indicate significant differences.

KVAC roosters. However, vaccination with the killed AIBV resulted in a 50% (5/10) incidence of epididymal stones in the present study. These results indicate that the presence of a live virus and an active infection are not necessary for the development of epididymal stone formation. This idea is supported by the fact that epididymal stones developed in 13% (1/8) of the NONVAC group.

The presence of epididymal stones in KVAC roosters suggests an alternative mechanism for the development of epididymal stones. Antibodies to AIBV may be responsible for the development of epididymal stones in KVAC and LVAC roosters. We have identified antibodies in the seminal plasma of KVAC and LVAC roosters that react with AIBV and sperm membrane proteins (unpubl. data). Antibodies bound to sperm have been associated with sperm clumping in the excurrent duct of the rooster epididymal region (15). Homology of sperm proteins to AIBV proteins has not been characterized. However, it is known that antibodies against infectious agents such as viruses, bacteria, and parasites may share epitope homology with self-protein (molecular mimicry; 23). This phenomenon results in various diseases including multiple sclerosis, diabetes mellitus type I, Lyme disease, and rheumatoid arthritis (1,18,20,22). Therefore, it is possible that AIBV proteins have some homology with sperm membrane proteins.

Testis weight did not differ among treatment groups. The histology of the seminiferous tubules was normal in NONVAC, KVAC, and LVAC roosters in the present study. These observations contradict those previously reported; however, the roosters used in this experiment were younger (27 wk of age) than those used in the other study (2). Epididymal stones, decreased testis weight, and atrophy of the seminiferous tubules were reported in roosters 62 wk of age and older (14,19). The formation of epididymal stones begins between 16 and 20 wk of age with calcifications apparent at 26 wk of age (13). Pathological changes in the testis are likely to be caused by the persistence of epididymal stones with age as previously observed in older roosters with epididymal stones. Therefore, absence of abnormalities in the testis in this study could possibly be attributed to the young age of the roosters.

Daily sperm production was reduced in the KVAC group when compared to the NONVAC group. A greater reduction of daily sperm production was observed in LVAC roosters when compared to NONVAC roosters. The decrease in daily sperm production is more pronounced among groups when compared on a per-gram-testis basis. This decrease in daily sperm production correlates with the incidence of epididymal stones among groups. The decrease in daily sperm production cannot be attributed to decreased testis weight or abnormalities in spermatogenesis in this study. Previous studies have reported a decrease in serum testosterone concentrations which could account for the decreased daily sperm production (2,14). The infiltration of immune cells into the reproductive tract could be responsible for a decrease of testosterone via the production of inflammatory cytokines because inflammatory cytokines have been shown to inhibit Leydig cell steroidogenesis (4). However, in the present study no significant differences in serum testosterone

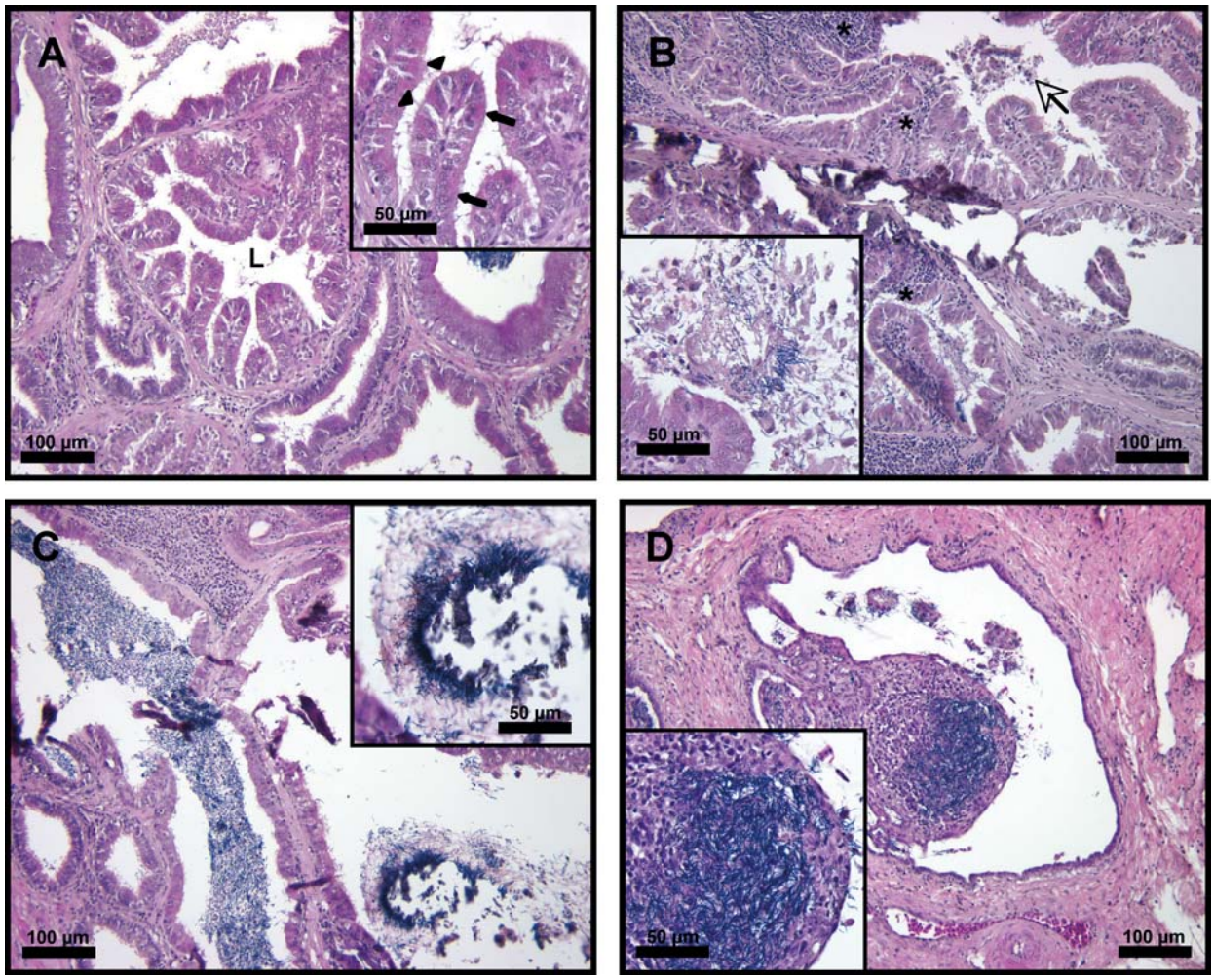

Fig. 2. Histology of epididymal regions in 27-wk-old roosters. (A) NONVAC group. The efferent ductules are lined by folded epithelium with few sperm present in the lumen (L). Inset: The epithelial cells consist of ciliated (arrow heads) and nonciliated cells (arrows). (B) KVAC group. A tear in this section is because of the stone. Sloughed epithelial cells are present in the lumen (arrow and inset). Immune cells are in the interstitium surrounding the efferent ductules (*). (C) LVAC group. A tear in this section is because of the stone. An aggregation of sperm is observed in the lumen (inset). (D) In the rete testis connective tissue and immune cells are surrounding sperm. Lymphocytes and plasma cells are present indicative of chronic inflammation.

concentrations were detected (data not shown), suggesting an alternative cause for the reduction in sperm production.

The formation of epididymal stones resulted in histological abnormalities in the epididymal tissue in KVAC and LVAC roosters. The presence of epididymal stones was identified by tears in histological sections. Tissue surrounding the ductule with an epididymal stone had an infiltration of immune cells, primarily lymphocytes and plasma cells. The presence of these immune cells is indicative of a chronic inflammation. In ductules with epididymal stone development at earlier stages, there is an aggregation of sperm and cellular debris in the lumen, sloughing of epithelial cells, and immune cells surrounding sperm in the interstitium. Because the epithelium is still intact in ductules with stones forming, it is unlikely that a loss of epithelium is the cause of epididymal stones as previously believed (14).

The formation of epididymal stones appears to occur even in nonvaccinated roosters but at a lower incidence. In the present study we observed epididymal stones in 13% (1/8) of NONVAC roosters at 27 wk of age. Previous studies have also reported the presence of epididymal stones in nonvaccinated roosters. In nonvaccinated roosters, 25% (2/8) at 26 wk of age and 100% of roosters examined at 82 wk of age developed epididymal stones (13,19). We also observed epididymal stones in roosters at 52 wk of age that were from an SPF flock (unpubl. data). This observation suggests an increase in the incidence of epididymal stones with age regardless of treatment.

In conclusion, the present study demonstrated that vaccination of the rooster with killed AIBV does not reduce the incidence of epididymal stones that have been observed when roosters were vaccinated with the live attenuated AIBV. Therefore, entrance into and replication of AIBV in the reproductive tract is not required for stone formation. The pathogenesis of epididymal stone formation remains unclear. It is possible AIBV proteins resemble those of the sperm resulting in an autoimmune response. Therefore, an autoimmune disease may be responsible for the initiation of sperm aggregations and stone formation.

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