Interaction of porcine circovirus type 2 and Mycoplasma hyopneumoniae vaccines on dually infected pigs

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A B S T R A C T

The objective of this study was to determine the effects of porcine circovirus type 2 (PCV2) and Mycoplasma hyopneumoniae vaccinations on disease severity in an experimental PCV2-M. hyopneumoniae dual challenge model. Vaccine effectiveness was evaluated using microbiological (PCV2 viremia and M. hyopneumoniae nasal shedding), immunological (neutralizing antibodies and interferon-γ-secreting cells), and pathological (gross lung lesions, histopathological pulmonary and lymphoid lesions, and the presence of PCV2 antigen and M. hyopneumoniae DNA within the lesions) evaluations. Although M. hyopneumoniae potentiates the severity of PCV2-associated lesions and lesion-associated PCV2 antigen in dually challenged pigs, vaccination against M. hyopneumoniae alone did not reduce PCV2 viremia, PCV2-induced lesions, or PCV2 antigen in dually challenged pigs. In addition, vaccination against PCV2 did not reduce the nasal shedding of M. hyopneumoniae, the M. hyopneumoniae-induced pulmonary lesions or the lesion-associated M. hyopneumoniae DNA in dually challenged pigs. Dual challenge with PCV2 and M. hyopneumoniae did not interfere with the induction of active immunity induced by a previous single vaccination for either PCV2 or M. hyopneumoniae. The results of this study demonstrated that (i) vaccination against M. hyopneumoniae alone did not decrease the potentiation of PCV2-induced lesions by M. hyopneumoniae and (ii) vaccination against PCV2 alone decreased the potentiation of PCV2-induced lesions by M. hyopneumoniae in dually challenged pigs.

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1. Introduction

Porcine circovirus type 2 (PCV2) is the primary etiological agent for several diseases and syndromes, which are collectively referred to as porcine circovirus-associated disease (PCVAD) [1]. Among these conditions, postweaning multisystemic wasting syndrome (PMWS) and porcine respiratory disease complex (PRDC) are the most important. Mycoplasma hyopneumoniae is the primary pathogen causing enzootic pneumonia, which is characterized by a dry, non-productive cough, reduced growth rate and poor feed conversion efficiency [2]. Co-infection with PCV2 and M. hyopneumoniae plays a primary role in the PRDC and continues to have a major economic impact on the global swine industry [3].

Several studies based on experimental dual infection have been conducted to better understand the interaction between PCV2 and M. hyopneumoniae [4–6]. In a sequential challenge model, M. hyopneumoniae potentiated the severity of PCV2-associated lung and lymphoid lesions, and increased the incidence of PMWS in pigs that were first inoculated with M. hyopneumoniae and then inoculated with PCV2 2 weeks later [4]. In contrast, in a concurrent infection model, pigs that were inoculated with both M. hyopneumoniae and PCV2 at 6 weeks of age did not produce the synergistic clinical outcomes observed when using the sequential challenge model [5].

Since dual infection of pigs with M. hyopneumoniae and PCV2 results in increased severity of PCV2-induced lesions and incidence of PMWS using the sequential challenge model [4], one possible way to minimize the effect of the M. hyopneumoniae-associated enhancement of PCV2 replication may be the use of a M. hyopneumoniae-based vaccine. Surprisingly, however, it has been reported that M. hyopneumoniae vaccination alone actually increased the incidence of PMWS under experimental and field conditions [7,8]. These unexpected results make difficult to understand the interaction between M. hyopneumoniae vaccination and incidence of PMWS. Hence, it is necessary to conduct experimental studies to elucidate the effects of a single vaccination for either PCV2 or M. hyopneumoniae on dually infected pigs. Currently, commercial PCV2 and M. hyopneumoniae vaccines are widely used in swine production worldwide. Therefore, the objective of this

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study was to determine the effects of single PCV2 and/or M. hyopneumoniae vaccinations on pigs in an experimental PCV2 and M. hyopneumoniae dual challenge model.

2. Materials and methods

2.1. Commercial vaccines

The inactivated chimeric PCV1-2 vaccine (Foster & Partners, Madison, NJ, USA) and the inactivated M. hyopneumoniae vaccine (RespiSure-One, Zoetis) were used in this study. Vaccines were administered according to the manufacturer’s instructions (1 dose via the intramuscular route).

2.2. Animals

A total of 88 colostrum-fed, cross-bred, conventional piglets were weaned and purchased at 18 days of age from a porcine reproductive and respiratory syndrome virus (PRRSV)- and M. hyopneumoniae-free commercial farm based on serological testing of breeding herd, and long term clinical and slaughter history. Pigs were all negative for PCV2, PRRSV, and M. hyopneumoniae according to routine serological testing. PCV2 and PRRSV were not detected in the sera samples by the real-time polymerase chain reaction (PCR) [9,10]. M. hyopneumoniae was not detected in the nasal swab samples by real-time PCR [11].

2.3. Experimental design

A total of 72 pigs were randomly divided into 11 groups (8 pigs per group): 5 vaccinated challenged (VC), 3 unvaccinated challenged (UVC), 2 vaccinated unchallenged (VUC), and 1 unvaccinated unchallenged group (Table 1). At 7 days of age (−42 days post challenge (dpc)), pigs in groups 1, 2, 5, and 9 were injected intramuscularly in the right side of the neck with 2.0 mL of the M. hyopneumoniae vaccine (RespiSure-One, Zoetis). At 21 days of age (−28 dpc), pigs in groups 3, 4, 5, and 10 were injected intramuscularly in the left side of the neck with 2.0 mL of the PCV2 vaccine (Foster & Partners, Zoetis). An equal volume of phosphate buffered saline (PBS) (2.0 mL) was injected in the same anatomical location in the positive and negative control pigs (groups 6–8, and 11) at 7 and 21 days of age.

At 35 days of age (−14 dpc), pigs in the VC groups (1, 2, 4, and 5) and UVC (groups 6 and 8) were intratracheally administered with a 10 mL dose of frozen lung homogenate of M. hyopneumoniae strain SNUJ98703 (1:100 dilution in Fris medium) at a final concentration of 10^6–10^5 color changing units (CCU)/mL as previously described [12]. At 49 days of age (0 dpc), pigs in the VC (groups 2–5) and UVC (groups 7 and 8) groups were intranasally administered with a 3 mL dose of PCV2b (strain SNUVR000463 (GenBank no. KF871068), 5th passage) containing 1.2 × 10^5 50% tissue culture infective dose (TCID50)/mL (Table 1).

Blood samples and nasal swabs were collected at −42, −28, −14, 0, 7, 14, 21, and 28 dpc. Pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 28 dpc as previously described [13]. Tissues were collected from each pig at necropsy. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

2.4. Quantification of PCV2 DNA in blood

DNA was extracted from serum samples using the QIAamp DNA Mini Kit (QIAGEN Ltd, Crawley, UK) to quantify PCV2 genomic DNA copy numbers by real-time PCR [8].

2.5. Quantification of M. hyopneumoniae DNA in nasal swabs

DNA was extracted from nasal swabs using the QIAamp DNA Mini Kit (QIAGEN Ltd, Crawley, UK) to quantify the M. hyopneumoniae genomic DNA copy numbers by real-time PCR with primers based on the putative ABC transporter [11].

2.6. Serology

The serum samples were tested for antibodies to PCV2 and M. hyopneumoniae using the commercial PCV2 ELISA (Synbiotics, Lyon, France) and M. hyopneumoniae ELISA (IDEXX Laboratories Inc, Westbrook, ME, USA). Serum virus neutralization (SVN) test for PCV2 was performed as previously described [14].

2.7. Enzyme-linked immunosorbent assay

PCV2 and M. hyopneumoniae antigens were prepared as previously described [15,16]. The numbers of PCV2- and M. hyopneumoniae-specific interferon-γ-secreting cells (IFN-γ-SCs) were determined in peripheral blood mononuclear cells (PBMCs) as previously described [17,18].

2.8. In situ hybridization and immunohistochemistry

In situ hybridization for M. hyopneumoniae and immunohistochemistry for PCV2 was performed as previously described [15,19,20].

2.9. Gross lung lesion scores

The total extent of gross lung lesions was estimated and calculated as previously described [21]. The frequency distribution of the lung lesion scores for each lung lobe was calculated by treatment. The percentage of total lung with lesions was calculated using the following formula: 100 × [(0.10 × left cranial) + (0.1 x left middle) + (0.25 × left caudal) + (0.10 × right cranial) + (0.10 × right middle) + (0.25 × right caudal) + (0.10 × accessory)].

2.10. Morphometric analyses

For the morphometric analyses of the microscopic pulmonary and lymph lesion scores, tissue sections were blindly examined by two veterinary pathologists (Seo and Cha) [4,22]. The morphometric analyses of in situ hybridization for M. hyopneumoniae

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. hyopneumoniae (−42 dpc)</td>
<td>PCV2 (−28 dpc)</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>11</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* There were eight animals in each groups, and necropsy was performed at 28 days post challenge (dpc) in all cases.
and immunohistochemistry for PCV2 was performed as previously described [4,20].

2.11. Statistical analyses

Summary statistics were calculated for all of the groups to assess the overall quality of the data, including normality. The continuous data for the quantification of PCV2 and M. hyopneumoniae DNA, the PCV2 and M. hyopneumoniae serology, IFN-γ-SCs, and PCV2 antigen by immunohistochemistry were analyzed using an ANOVA for each time point. When a one-way ANOVA revealed a significance of $P<0.05$, the Bonferroni adjustment procedure was used to determine the significance of individual between group differences.

Discrete data (histopathological lung and lymphoid lesions, and M. hyopneumoniae DNA scores) were analyzed by the Chi-square and Fisher’s exact tests. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Quantification of PCV2 DNA in blood

At the time of challenge, no genomic copies of PCV2 were detected in any of the sera samples from all 11 groups of pigs. Pigs that received the M. hyopneumoniae vaccine followed by a dual challenge (group 2) had a significantly higher number of genomic copies of PCV2 in their sera than did pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), and pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5) at 7, 14, 21, and 28 dpc ($P<0.05$). The rest of the results are summarized in Fig. 1A. Throughout the experiment, no genomic copies of PCV2 were detected in any of the sera samples from pigs that were not challenged with PCV2 (groups 1, 6, 9–11).

3.2. Quantification of M. hyopneumoniae DNA in nasal swabs

At the time of challenge, no genomic copies of M. hyopneumoniae were detected in any of the sera samples from all 11 groups of pigs. Unvaccinated pigs challenged with M. hyopneumoniae alone (group 6) and dually challenged pigs (group 8) had a significantly higher number of genomic copies of M. hyopneumoniae in their nasal swabs than did pigs that received the M. hyopneumoniae vaccine followed by a challenge with either M. hyopneumoniae alone (group 1) or a dual challenge (group 2), and pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5) at 14, 21, and 28 dpc ($P<0.05$). The rest of the results are summarized in Fig. 1B. Throughout the experiment, no genomic copies of M. hyopneumoniae were detected in any of the sera samples from pigs that were not challenged with M. hyopneumoniae (groups 3, 7, 9–11).

3.3. Anti-PCV2 IgG antibodies

The results of anti-PCV2 IgG antibodies are summarized in Fig. 2A. Pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5), and pigs that received the PCV2 vaccine only (group 10) had significantly higher anti-PCV2 IgG antibody levels than did non-PCV2-vaccinated pigs that were challenged with PCV2 (groups 2, 7, and 8) from 0 to 28 dpc ($P<0.001$). No anti-PCV2 IgG antibodies were detected in pigs from groups 9 and 11.

3.4. PCV2-specific neutralizing antibodies

The results of PCV2-specific neutralizing antibodies (NA) are summarized in Fig. 2B. Pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), and pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5), and pigs that received the PCV2 vaccine only (group 10) had significantly higher PCV2-specific NA titers than did non-PCV2-vaccinated pigs that were challenged with PCV2 (groups 2, 7, and 8) at 14, 21 and 28 dpc ($P<0.05$). No PCV2-specific NA titers were detected in pigs from groups 9 and 11.

3.5. PCV2-specific interferon-γ-secreting cells

The results of PCV2-specific IFN-γ-SCs are summarized in Fig. 2C. Pigs that received the PCV2 vaccine followed by either a PCV2-only challenge (group 3) or a dual challenge (group 4), and pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5) had a significantly higher...
numbers of PCV2-specific IFN-γ-SCs than did non-PCV2-vaccinated pigs that were challenged with PCV2 (groups 2, 7, and 8) at −14, 0, 7, 14, 21, and 28 dpc (P < 0.05). No PCV2-specific IFN-γ-SCs were detected in pigs from groups 9 and 11.

3.6. Anti- M. hyopneumoniae IgG antibodies

The results of anti- M. hyopneumoniae IgG antibodies are summarized in Fig. 3A. Each pig in all 11 groups was seronegative for M. hyopneumoniae at 21 dpc. Pigs that received the M. hyopneumoniae vaccine followed by a challenge with either M. hyopneumoniae alone (group 1) or a dual challenge (group 2), and pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5) had significantly higher anti-M. hyopneumoniae IgG antibody levels than did pigs in groups 4, 6, and 8 at 21 and 28 dpc (P < 0.05). No anti-M. hyopneumoniae IgG antibodies were detected in pigs from groups 10 and 11.

3.7. M. hyopneumoniae-specific interferon-γ-secreting cells

The results of M. hyopneumoniae-specific IFN-γ-SCs are summarized in Fig. 3B. Pigs that received the M. hyopneumoniae vaccine followed by a challenge with either M. hyopneumoniae alone (group 1) or by a dual challenge (group 2), and pigs that received both the M. hyopneumoniae and PCV2 vaccines followed by a dual challenge (group 5) had a significantly higher numbers of M. hyopneumoniae-specific IFN-γ-SCs than did non-M. hyopneumoniae-vaccinated pigs that were challenged with M.
3.8. Lung lesion scores

Lung lesion scores are summarized in Table 2. Gross lung lesions were observed in the challenged group, including varying degrees of red-to-purple consolidation in the lung tissues. Pigs that received the PCV2 vaccine followed by a dual challenge had significantly higher gross lung lesion scores than did pigs in the other groups (1, 3, 5, and 7).

3.9. Histopathologic lesion scores

The results of lymphoid and pulmonary lesion scores are summarized in Table 2. The typical granulomatous inflammatory reaction and lymphoid depletion that is associated with PCV2 infection in pigs and is consistent with the histopathologic lesions in PCVAD were observed in the lungs from dually challenged pigs (group 8). Pigs that received the M. hyopneumoniae vaccine followed by a dual challenge had significantly higher lymphoid lesion scores than did pigs in the other 4 groups (3–6). The pulmonary histopathologic lesion scores were characterized by moderate peribronchial lymphoid tissue hyperplasia and thickened alveolar septa (interstitial pneumonia) in the dually challenged pigs (group 8). Pigs that received the PCV2 vaccine followed by a dual challenge had significantly higher lesion scores for peribronchial lymphoid tissue hyperplasia than did pigs in the other 4 groups (1, 3, 5, and 7) (Table 2).

3.10. Immunohistochemistry of PCV2 antigen

The results of immunohistochemistry of PCV2 antigen are summarized in Table 2. PCV2 antigen was detected in lymph nodes and lungs from PCV2-challenged pigs (groups 2 (Fig. 4A), 3–5, 7 (Fig. 4B), and 8 (Fig. 4C)). Pigs that received the M. hyopneumoniae vaccine followed by a dual challenge had a significantly higher number of PCV2-positive cells per unit tissue in their lungs than did pigs in groups 3–5 and 7 as well as a higher number of PCV2-positive cells per unit tissue in their lungs than did pigs in groups 3 and 5 (Table 2).

3.11. In situ hybridization of M. hyopneumoniae DNA

The results of in situ hybridization of M. hyopneumoniae DNA are summarized in Table 2. M. hyopneumoniae DNA was associated with the surface of epithelial cells of bronchi and bronchioli in lungs from pigs challenged with M. hyopneumoniae (groups 1, 2, 4 (Fig. 5A), 5, 6 (Fig. 5B), and 8 (Fig. 5C)). Unvaccinated dually challenged pigs (group 8), unvaccinated challenged only with M. hyopneumoniae (group 6), and pigs that received the PCV2 vaccine followed by a dual challenge had a significantly higher number of M. hyopneumoniae-positive cells per unit tissue in their lungs than did pigs in groups 1, 2, and 5 (Table 2).

4. Discussion

In this study, dually challenged pigs had significantly increased PCV2-viremia, more severe PCV2-induced pulmonary and lymphoid lesions, and higher levels of lesion-associated PCV2 antigen than did pigs that were challenged only with PCV2. These results are in agreement with previous findings where M. hyopneumoniae potentiates the severity of PCV2-associated lesions and PCV2 antigen levels within the lesions in pigs [4]. In contrast, dually challenged pigs did not significantly potentiate the nasal shedding of M. hyopneumoniae or the M. hyopneumoniae-induced pulmonary lesions compared with pigs that were challenged only with M. hyopneumoniae. Enhancement of PCV2 replication by M. hyopneumoniae is clinically significant because the clinical signs of PCVAD

Fig. 4. Immunohistochemistry of PCV2. Positive signals (arrows) were detected in the macrophages in the lungs from different groups; pigs that received the M. hyopneumoniae vaccine followed by a dual challenge (group 2, A), unvaccinated pigs challenged only with PCV2 (group 7, B), and pigs that received a dual challenge (group 8, C).

Fig. 5. In situ hybridization of M. hyopneumoniae. Positive signals (arrows) were detected in the surface of bronchiolar epithelium in the lungs from different groups; pigs that received the PCV2 vaccine followed by a dual challenge (group 4, A), unvaccinated pigs challenged only with M. hyopneumoniae (group 6, B), and pigs that received a dual challenge (group 8, C).
are dependent on the levels of PCV2 viremia [23,24]. Although it may vary from laboratory to laboratory based on the standards used for quantification, the PCV2 load in the blood, as quantified by real-time PCR, is used to categorize PCV2-infected pigs as subclinically infected (<10^6 DNA copies/mL) or PCR-positive (>10^7 DNA copies/mL) [25–27].

Given that *M. hyopneumoniae* exacerbates PCV2-induced disease, one possible way to minimize the effect of the *M. hyopneumoniae*-associated enhancement of PCV2 replication may be the use of a *M. hyopneumoniae*-based vaccine for pigs in a PCV2/*M. hyopneumoniae*-co-infected herd. Interestingly, however, in this study, vaccination with *M. hyopneumoniae* alone did not reduce the PCV2 viremia or the PCV2-associated lymphoid lesions in pigs that were dually challenged. Although our experimental condition cannot be generalized as such to co-infected herds, a vaccination with *M. hyopneumoniae* alone may not be sufficient for reducing PCV2 viremia, PCV2-associated lung and lymphoid lesions, or lesion-associated PCV2 antigen in pigs from PCV2/*M. hyopneumoniae*-co-infected herds. Moreover, the types of strains used, the infection doses, the timing of infection, the types of vaccines may also largely influence the outcome.

The induction of protective immunity by a PCV2 vaccination results in reduced PCV2 viremia [24,28–30]. In this study, PCV2 vaccination elicits PCV2-specific NA and IFN-γ-SCs, even in pigs that received the PCV2 vaccine followed by a dual challenge. These results provide swine producers and practitioners with clinically significant information. *M. hyopneumoniae* is highly prevalent (ranging between 38% and 100%) in almost all swine production areas worldwide [2] and has a modulating effect on the immune system [31]. Nevertheless, the efficacy of PCV2 vaccines may not be affected by a subsequent infection with *M. hyopneumoniae*.

Vaccination is still considered the most effective tool for controlling infection by *M. hyopneumoniae* although mycoplasmal infections can be controlled by other practices such as improved management, pig flow, biosecurity measures, and housing conditions, as well as the use of antibiotics [2,32]. Cell-mediated immunity induced by vaccination is important for the control of mycoplasmal pneumonia in pigs [18,33]. In this study, a single vaccination against *M. hyopneumoniae* induced *M. hyopneumoniae*-specific IFN-γ-SCs, even in pigs that received the *M. hyopneumoniae* vaccine followed by a dual challenge. Although PCV2 is able to hamper the development of immune responses by suppressing Th1 responses [34], the efficacy of *M. hyopneumoniae* vaccines do not appear to be affected by subsequent infection with PCV2 and *M. hyopneumoniae*. These results suggest *M. hyopneumoniae* vaccines may be effective under field conditions where PCV2 is widespread in the swine population.

There are two different co-infection models (sequential vs. concurrent infection) to determine the interaction between PCV2 and *M. hyopneumoniae* [4,5]. Under Korean field conditions, *M. hyopneumoniae* from nasal swabs is most commonly detected in pigs that are 4–7 weeks old while PCV2 from sera samples is most commonly detected in pigs that are 6–9 weeks old, based on diagnostic samples from Seoul National University (C. Chae, personal observation). Therefore, the sequential infection model rather than the concurrent infection model more closely mimics the Korean field situations. The results of this study provide swine producers and practitioners with efficient vaccination regimens for controlling PCV2 and *M. hyopneumoniae* infections in co-infected swine herds.

**Conflicts of interest**

No conflicts of interest were declared in relation to this article.

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**Table 2**

Scores (mean ± standard deviation) of gross lung lesions, histopathologic lesions, *M. hyopneumoniae* (MHP) DNA, and PCV2 antigen in different groups at 28 days post challenge (dpc).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Gross lung lesions</th>
<th>Histopathology</th>
<th>PCV2 antigen</th>
<th>MHP DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lungs</td>
<td>Lymph node</td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycoplasmal pneumonia</td>
<td>Interstitial pneumonia</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.69 ± 1.44a</td>
<td>0.43 ± 0.53a</td>
<td>0.29 ± 0.48ab</td>
<td>0b</td>
</tr>
<tr>
<td>2</td>
<td>4.50 ± 1.93ab</td>
<td>0.71 ± 0.65b</td>
<td>1.71 ± 0.49ab</td>
<td>0a</td>
</tr>
<tr>
<td>3</td>
<td>0.88 ± 0.83a</td>
<td>0.14 ± 0.37b</td>
<td>0.43 ± 0.53ab</td>
<td>0a</td>
</tr>
<tr>
<td>4</td>
<td>11.87 ± 6.69a</td>
<td>2.00 ± 0.57b</td>
<td>0.57 ± 0.78bc</td>
<td>1.29 ± 0.7b</td>
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<tr>
<td>5</td>
<td>3.25 ± 1.58a</td>
<td>0.57 ± 0.53b</td>
<td>0.57 ± 0.53bc</td>
<td>0a</td>
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<tr>
<td>6</td>
<td>10.13 ± 2.75b</td>
<td>2.29 ± 0.48b</td>
<td>1.28 ± 0.48c</td>
<td>0b</td>
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<tr>
<td>7</td>
<td>1.0 ± 1.20a</td>
<td>0.43 ± 0.53b</td>
<td>1.00 ± 0.58bc</td>
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<td>8</td>
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<td>2.57 ± 0.53b</td>
<td>2.43 ± 0.53d</td>
<td>19.85 ± 7.86a</td>
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<td>9</td>
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<td>10</td>
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<td>11</td>
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<td>0.14 ± 0.37a</td>
<td>0b</td>
<td>0a</td>
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</tbody>
</table>

Different letters (a, b, c, and d) indicate that the groups are significantly (*P<0.05) different from each other.

1 Peribronchial and perivascular lymphoid tissue hyperplasia.


