The Effects of Germanium Biotite Supplement as a Prophylactic Agent against Respiratory Infection in Calves

Myunghwan Jung1, Bock-Gie Jung2, Seung Bin Cha1, Min-Kyoung Shin1, Won-Jung Lee1, Seung Won Shin1, Jin-A Lee2, Yeon-Kwon Jung3, Bong-Joo Lee2 and Han Sang Yoo1*

1Department of Infectious Diseases, College of Veterinary Medicine, KRF Zoonotic Disease Priority Research Institute and Brain Korea 21 Program for Veterinary Science, Seoul National University, Seoul, 151-742, Korea; 2College of Veterinary Medicine, Chonnam National University, 300 Yongbong-dong, Buk-gu, Gwangju 550-757, Korea; 3Seobong BioBestech. Co., Ltd., Hyechon Building #401, 831 Yeoksam-dong, Kangnam-gu, Seoul, Korea

*Corresponding author: yoohs@snu.ac.kr

ABSTRACT

Germanium biotite, a natural mineral, is comprised of mainly silicate. This mineral showed activities of increase in feed efficiency and non-specific immunostimulation in previous studies. The aims of the present study were to evaluate the prophylactic effects of germanium biotite against respiratory diseases in calves as a feed supplement and investigate the possibilities of the substitution of antibiotics with germanium biotite as feed additive. To achieve these purposes, bovine herpesvirus-1 (BHV-1) and Mannheimia haemolytica serotype A1 were experimentally inoculated into the calves. After challenge, germanium biotite showed a lower cumulative clinical score (CCS) than the control group. In accordance with these clinical results, enhanced clearance of BHV-1, a low infection rate of Mannheimia haemolytica serotype A1, tempered superficial lesions, and moderated histopathological signs were observed in the germanium biotite group, compared with the control group. The results of the present study indicated that germanium biotite had prophylactic effects against bovine respiratory disease and could be a candidate for a new alternative feed supplement in calves, through its effects as a non-specific immune stimulator.

INTRODUCTION

The consumption of livestock products has been increasing along with increased per capita income. This growth in consumption has driven an increase in the importance of the safety of different food commodities. Antibiotics have been used as feed supplements to improve the rate of gain and feed efficiency and to prevent infectious diseases (Muhl and Liebert, 2007). However, due to concern about antibiotic residues and emerging antibiotic resistance (van den Bogaard and Stobberingh, 1999), use of antibiotics as feed additives has been banned in the European Union (Kamphues, 1999) and Korea. For this reason, studies seeking for substances which can activate the non-specific immune ability in place of antibiotics are actively in progress.

Biotite, a well known feed supplement, is a common phyllosilicate mineral containing potassium, magnesium, iron, aluminum, and silicate (Sarker et al., 2010; Vondruskova et al., 2010). It has been reported that immune cells stimulated by silicate were associated with the non-specific immunostimulating ability of biotite. Fibrogenic silicate (SiO2) activated proinflammatory macrophages (Holian et al., 1997), and aluminosilicate (Al2SiO5) improved immune-cell differentiation (Jung et al., 2010). These previous studies suggested that germanium biotite has potential as a new alternative feed supplement for non-specific immunostimulators, prophylactic agents, and remedial agents.

Bovine respiratory disease (BRD) is a major economic problem in the cattle industry around the world due to morbidity, mortality, low feed efficiency, prevention costs, and treatment (Fulton, 2009; Irsik et al., 2006). The disease is caused by not only multi-factorial pathogens but also environmental condition, and Bovine herpesvirus type 1 (BHV-1) and Mannheimia haemolytica serotype A1.
serotype A1 have been described as major etiologic agents (Fulton, 2009). *Mannheimia haemolytica* serotype A1 causing BRD in young calves has been detected in the respiratory tract of healthy cattle. Although the microorganism naturally exists as commensal of the respiratory tract, it is also considered the major bacterial agent of BRD in calves (Rice et al., 2007). As host immunity becomes weaker as a result of stress or infection with other pathogens, the microorganism may become infective and play an important role in BRD, also known as shipping fever in calves. BHV-1 is also a major etiological agent of BRD along with *Mannheimia haemolytica* serotype A1 (Autio et al., 2007). BHV-1 is an α-herpesvirinae subfamily member that causes significant economic problems in the cattle industry (Irsik et al., 2006; Fulton, 2009). Following infection with BHV-1, the virus could be latent in sensory ganglia and reactivated both by stressful environments and administration of glucocorticoids. In addition, BHV-1 depresses cell-mediated immunity in the host by repressing expression of major histocompatibility complex (MHC) class I that acts in association with CD8+ T-cell recognition of infected cells and transporter associated with antigen presentation (Jones and Chowdhury, 2007). The suppression of the immune function renders the host susceptible to secondary infection like pneumonic mannheimiosis, thereby causing BRD more easily.

The aims of the present study were to investigate characteristics of germanium biotite, known to the non-specific immune stimulating mineral, about prophylactic effect in BRD infected calves. A BHV-1 and *Mannheimia haemolytica* serotype A1 challenge experiment in calves was performed to examine the prophylactic effect of germanium biotite against BRD. Finally, an aim was to explore the possibilities of the substitution of germanium biotite for antibiotics as a feed supplement.

MATERIALS AND METHODS

Source of feed supplements: Germanium biotite (under solto Bio) provided by Seobong Biobestech (Seoul, Korea) was comprised of silicon dioxide (SiO2, 61.90%), aluminum dioxide (Al2O3, 23.19%), iron oxide (Fe2O3, 3.97%), calcium oxide (CaO, less than 2%), magnesium oxide (MgO, less than 2%), and titanium oxide (TiO2 less than 2%).

Calves and challenge experiment design: Korean native calves of three months of age were used for this study. The calves were randomized into two groups: the control group (n=3), fed with a normal commercial feed without any antibiotics; and the germanium biotite group (n=3), fed with the commercial feed supplemented with 0.5% germanium biotite. The calves of two groups were housed in individual rooms and allowed easy access to water and feed. Before the challenge, calves were confirmed as negative against respiratory pathogens to be challenged. After feeding with those fodders for two weeks, the calves were challenged with BHV-1 (5.0 × 109 PFU) and *Mannheimia haemolytica* serotype A1 (1.0 × 108CFU) three times at 24 hours intervals. To equalize natural infectious condition, all calves were challenged through nasal spray.

Preparation of pathogens: The BHV-1 used was isolated from a calf naturally suffering from shipping fever (Animal, Plant and Fisheries Quarantine and Inspection Agency, QIA; Anyang, Korea). Before challenge, the virus was propagated and titrated in MDDK cells (Abril et al., 2004). *Mannheimia haemolytica* serotype A1 used in the study was also provided by QIA. The identity of the bacterium was confirmed by using multiplex PCR as previously described (Alexander et al., 2008). The pathogen was grown on a blood agar plate (BAP) in a CO2 incubator at 37°C and subcultured in tryptic soy broth (TSB; Difco, MD, USA) to obtain a sufficient quantity of challenge agent. Before challenge, the cultured bacteria were pelleted by centrifugation, washed three times in sterile phosphate buffered saline (PBS), and resuspended in PBS. The final concentration of approximately 1.0 × 1010 CFU was confirmed according to the standard curve of CFUs versus optical density (Hanzlickel et al., 2010).

After each challenge, plate counts were used to confirm bacterial concentration.

Clinical monitoring after pathogens challenge: The body conditions and clinical signs of calves were recorded each 24 hr throughout the experiment period. Rectal temperature, degree of nasal discharge, cough frequency degree, and respiratory rate were scored using the criteria established by Hodgson et al. (1995) (Table 1). The measured scores of each calf were accumulated and considered as cumulative clinical score (CCS).

<table>
<thead>
<tr>
<th>Signs</th>
<th>Score</th>
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<tbody>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>38.0-39.5 ºC</td>
<td>0</td>
</tr>
<tr>
<td>39.5-40.0 ºC</td>
<td>1</td>
</tr>
<tr>
<td>40.0-40.5 ºC</td>
<td>2</td>
</tr>
<tr>
<td>40.51-41.0 ºC</td>
<td>3</td>
</tr>
<tr>
<td>41.0 ºC &gt;</td>
<td>4</td>
</tr>
<tr>
<td>Degree of nasal discharge</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>3</td>
</tr>
<tr>
<td>Cough frequency degree</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
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<tr>
<td>Respiratory rate</td>
<td></td>
</tr>
<tr>
<td>&lt; 50</td>
<td>0</td>
</tr>
<tr>
<td>50-60</td>
<td>1</td>
</tr>
<tr>
<td>60-70</td>
<td>2</td>
</tr>
<tr>
<td>70-80</td>
<td>3</td>
</tr>
<tr>
<td>80 &gt;</td>
<td>4</td>
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</table>

These criteria were modified from Hodgson et al. (1995).

Measurement of challenged pathogens clearance: After challenge, nasal swab samples were collected after every two days and each sample was resuspended in 1 ml PBS and diluted in 10 fold serial dilutions up to 10^-4. Aliquots (50µl) of each dilution were used for experiment samples. Real-time PCR was used in measurement of virus clearance. DNA extraction from each sample was performed using the Accuprep genomic DNA extraction kit (Bioneer, Seoul, Korea) according to the manufacturer’s instructions and used template for real-time PCR. Primer and probes for quantitative real-time TaqMan PCR, real-time PCR standard of BHV-1 genomes, and condition were developed and validated as TaqMan PCR, real-time PCR standard of BHV-1 genomes, and condition were developed and validated as
described previously (Abril et al., 2004). Multiplex PCR was used in confirmation of bacterial infection using specific primers (Table 2). DNA extraction from aliquots of sample was performed according to a previous study (Suh and Song, 2005). DNA extracts were used for the PCR template as previously described (Alexander et al., 2008). The total isolated bacteria counts were measured by CFU counting. Aliquots (50µl) of each sample dilution were applied to BAP and incubated at 37°C for 16 hr. The viable counts were determined and expressed as CFU/swab. The area under curve (AUC) of bacteria shedding graph was calculated for confirmation of total shedding bacteria throughout the experiment.

Necropsy: On day 12th post challenge, all calves were humanely euthanized and their lungs were collected. The percentages of superficial lesion area were calculated by comparison with total lung area. The lungs and trachea were collected from calves, and then representative samples were placed in 10% formal saline for histopathological examination using standard techniques. Microscopic lesion scores were calculated using a described scoring system (Opriessnig et al., 2004). For viral and bacterial examination, tissue samples (approximately 1 g) were homogenized in 9 ml PBS and diluted in 10-fold steps up to 10⁻⁴ and then aliquots (50µl) of each dilution were used for experiment samples for pathogen examination.

Statistical analysis: The data were expressed as mean ± standard deviation (SD), and statistical differences between the groups were analyzed with student’s t-test; whereas, for microscopic lesion scores, a nonparametric test (Mann-Whitney U test) was performed using SPSS version 17.0 software (SPSS, Chicago, IL, USA). Differences were considered significant when probability values of P<0.05 were obtained.

RESULTS

Clinical signs after challenge in experiment calves: The CCSs of all calves were 0 before challenge. The CCSs, however, appeared to increase after challenge and lower CCSs than those of the control group were observed in the germium biotite group throughout the experimental period (Fig. 1).

Viral and bacterial clearance: On D+2 following the challenge, BHV-1 was detected in all calves. After D+6, the BHV-1 genomic quantity of the germium biotite group decreased, showing a lower level than the control group (Fig. 2a). The viral genome load in trachea of the germium biotite group was lower than the control group at post-mortem, while higher level than in the control group was observed in lungs of the germium biotite group (Fig. 2c). Mannheimia haemolytica serotype A1 was detected on D+2 by multiplex PCR in all calves except for two of the germium biotite group (Fig. 3). Infection of two calves in the control group and one calf in the germium biotite group was confirmed until D+10. Mannheimia haemolytica serotype A1 was identified in lungs from a calf of the control group at post-mortem, but not identified in other calves. The numbers of total bacteria isolated from nasal discharge in the germium biotite and control groups showed no significant differences, but higher AUC than in the germium biotite group was observed in the control group. The AUC of control and germium biotite groups was 41.3±0.5 and 38.3±0.7, respectively (Fig. 2b).

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Fig. 2: Clearance of challenge pathogens and percentage of lesion area. (a) Clearance of BHV-1 in experimentally infected calves. (b) Clearance of total bacteria in experimentally infected calves. The higher AUC than in the germanium biotite group was observed in the control group. (c) Quantity of BHV-1 genomes in trachea and lung at post-mortem. While germanium biotite group showed higher quantity of BHV-1 genomes than control group in lung, a lower quantity than in the control group was observed in trachea of germanium biotite group. Mannheimia haemolytica serotype A1 was not detected at post-mortem, except for in a lung from a calf in the control group. (d) Percentages of superficial lesion area in experimentally infected calves. The germanium biotite group showed significantly lower percentages of lesion area than the control group. **Highly significant at P<0.01.

Table 3: Microscopic lesion scores in challenged calves

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Germanium biotite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scores (± SD)</td>
<td>5.00 ± 0.26</td>
<td>2.50 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>4.30 ± 0.34</td>
<td>1.75 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>4.36 ± 0.28</td>
<td>1.92 ± 0.38</td>
</tr>
<tr>
<td>Means (± SD)</td>
<td>4.55 ± 0.22</td>
<td>2.06 ± 0.23 **</td>
</tr>
</tbody>
</table>

The microscopic lesion scores were calculated using a previous scoring system (Opriessnig et al., 2004). The control group shows higher lesion scores than the germanium biotite group significantly (P<0.01).

DISCUSSION

BRD, along with diarrhea a major cause of calf deaths, is caused by multi-factors mainly immune depression. BHV-1 and Mannheimia haemolytica serotype A1 are usually isolated from calves suffering from BRD (Irsik et al., 2006; Fulton, 2009). In the cattle industry, antibiotics have been used as a feed supplement to prevent both BRD and secondary infections. Virus infection, however, cannot be prevented by using antibiotics, and the use of antibiotics has been gradually limited because of antibiotic residues and emergence of antibiotic resistance. In addition, there are restrictions on vaccination against viruses especially, live vaccines, in view of vaccine infection, latent infection, pathogen carrier and serological differentiation from wild infection (Bosch et al., 1996). For these reasons, activation of non-specific immunity has been considered for controlling BRD in place of antibiotics.

Silicate, the major constituent of germanium biotite, has been studied for its non-specific immune enhancing effects. It was reported that macrophages could be stimulated and release large amounts of TNF-α by silicate in vitro (Holian et al., 1997). It was also reported that relative mRNA expression levels of IFN-γ, IL-4 and TNF-α produced mainly by T cell and macrophages could increase significantly in splenocytes of aluminosilicate (Al₂SiO₅) orally primed mice. In addition, aluminosilicate (Al₂SiO₅) primed mice also showed high antibody production levels when they were exposed to formalin-killed Pasteurella multocida type A antigen. Moreover, oral ingestion of aluminosilicate (Al₂SiO₅) showed enhancing effects on reinforcing clearance of porcine circovirus type 2 in experimentally infected pigs (Jung et al., 2010). The results of these studies of immune activities of silicate make us postulate that germanium
biotite could have prophylactic effects on BRD occurred mainly with relevance to immunosuppression. However, a natural mineral like germanium biotite has not been studied for its prophylactic effect against BRD. In this study, the first analysis of the prophylactic effect of germanium biotite against BRD in experimentally infected calves was conducted.

To investigate exact clinical signs, the normal temperature limit (39-40°C) confirmed in a previous study (Hodgson et al., 1995) was modified because situations in the present experiment, like rearing environments and breed of calves, differed from those in the previous study. Based on this information, the normal temperature limit was modified in the present experiment according to the mean of temperatures measured in preinoculated calves.

Following the challenge, the germanium biotite group showed alleviated clinical signs compared with the control group. In accordance with these clinical results, the germanium biotite group showed enhanced clearance of BHV-1 and a low infection rate of Mannheimia haemolytica serotype A1 compared with the control group. The germanium biotite group also showed lower AUC of clearance of total bacteria in nasal swab samples. At post-mortem, however, the germanium biotite group showed a higher quantity of BHV-1 in lungs than the control group and a lower quantity in trachea than the control group. It appeared that there was a greater amount of virus shedding in the trachea in the control group, even though a high quantity of BHV-1 was detected in lungs of the germanium biotite group compared with the control group. Moreover, it was reported that severe clinical signs were usually accompanied by a high titer of virus shedding (Song et al., 2011). These clinical and clearance results correspond with superficial lesion area data and microscopic lesion analysis in the present study. Significantly higher percentages of normal area than the control group were observed in the germanium biotite group (P<0.01). In microscopic lung lesions, the germanium biotite group showed moderate signs of neutrophils filtration and bronchiolar exudates compared with the control group. Neutrophils filtration in alveoli and bronchial exudates are typical signs of bronchopneumonias, which are generally caused by bacteria. Therefore, it could be inferred that germanium biotite primed calves were protected from secondary infections caused mainly by bacteria like Mannheimia haemolytica serotype A1.

These results suggested that ingestion of germanium biotite had prophylactic effects against BHV-1 and Mannheimia haemolytica serotype A1 in calves. It can be presumed that the prophylactic effects of germanium
biotite against challenged BRD pathogens are associated with its stimulating activities on non-specific immune response. In conclusion, results of this study indicated that germanium biotite has the potential to activate innate immunity thereby could be a good alternative to antibiotics as a feed supplement for calves. Future research is needed focusing on the mechanism of absorption of germanium biotite in calves’ gastrointestinal tracts and the mechanism of non-specific immune stimulating effects.

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