Effects of *In vitro* Atmospheric Ammonia Exposure on Recovery Rate and Luminol-dependent Chemiluminescence of Bovine Neutrophils and Bronchoalveolar Macrophages

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ABSTRACT. The effects of atmospheric ammonia, a major pollutant in animal confinement facilities, on bovine neutrophils and bronchoalveolar macrophages were evaluated *in vitro*. Ammonia exposure at concentrations 50, 100 and 200 ppm for one hour impaired recovery rates of neutrophils dose-dependently but enhanced their chemiluminescence activity per cell at lower concentrations (50 and 100 ppm). Macrophages were resistant to the exposure. Their recovery rates and chemiluminescence remained unaffected even at 200 ppm exposure. The present results suggest that ammonia exposure is unfavorable for bovine neutrophils *in vitro*, and probably *in vivo* also, in light of causing cell damage and triggering wider inflammatory responses.—**KEY WORDS:** ammonia exposure, bovine bronchoalveolar macrophage, bovine neutrophil.

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Atmospheric ammonia in poultry and swine confinement facilities is known to be a major harmful pollutant in impairing the defense mechanisms of the respiratory tract against pathogens, thereby, aggravating the incidence of respiratory diseases [2, 5]. In cattle, however, to our knowledge, the effects of ammonia exposure on the animals have been poorly documented.

In a previous study using calves receiving repeated inhalation of ammonia mists [7], our group found a marked increase of chemiluminescence activity, a bactericidal parameter, of bronchoalveolar lavage cells. The cell activation was considered to be due to direct and/or indirect effects of ammonia but the mechanisms remained uninvestigated.

The purpose of the present study was to assess *in vitro* the effects of ammonia exposure on pro-inflammatory cells in the bovine lung. For this purpose, cell recovery rates and luminol-induced chemiluminescence were evaluated on bovine neutrophils and bronchoalveolar macrophages exposed to various concentrations of atmospheric ammonia.

Neutrophils and bronchoalveolar macrophages were isolated from healthy adult Holstein steers over one year of age ($n=4$) as previously reported [7, 8]. The cells were adjusted with an automatic cell counter (Celltac, Nihon-Koden, Japan) to a concentration of $1 \times 10^6/\text{ml}$ in Hanks' buffered salt solution (HBSS, pH 7.4).

Ammonia gas chambers were prepared as follows. In brief, 10 μl of 6.25, 12.5 or 25 w/v % of ammonia solution (Wako, Japan) were mixed with 1 ml of 1 M NaOH in a glass well with a 25 mm diameter and a 50 mm depth to emit 50, 100 or 200 ppm ammonia inside a 1.5 l glass chamber, respectively. The control chamber (0 ppm) used 10 μl of distilled water in place of ammonia solution. The chambers were kept at 37°C and their ammonia levels were

monitored with a gas detector (Gastec model 801, Japan) before and after the cell exposure. Preliminary experiments have confirmed that ammonia concentration can be kept at each designed level at least for 2 hr at 37°C.

Two ml of cell suspension were placed into a silicon-coated polypropylene V-bottom tube with a 25 mm diameter and a 50 mm depth in the chamber and exposed to each ammonia concentration for one hour at 37°C. After the exposure, pH values of the cell suspensions were measured with a pH meter (pHBOY-P2, Shin-Dengen, Japan). Ammonia concentrations dissolved in the suspension were estimated with a pH-ammonia concentration standard curve obtained preliminarily. Cell counting was made on each suspension and cell recovery rate at each concentration was calculated as a percentage of cell count ratio (Exposure/Control). Recovered cells were centrifuged and resuspended in HBSS (pH 7.4) at a concentration of $1 \times 10^6/\text{ml}$.

The chemiluminescence assay of the recovered cells was performed as previously described [7]. Briefly, photon emission levels of the cells (2×10^5 cells) in response to phorbol myristate acetate (PMA: Sigma, 1.6×10^{-6} M at final concentration) were evaluated in the presence of luminol (Sigma, 0.5 mg/ml at final concentration) with a photon-counting luminescence analyzer (Berthold LUMAT LB9505C, Germany). The peak chemiluminescence (cpm) per cell at each ammonia exposure was calculated.

Data were analyzed using analysis of variance and Student's *t*-test. A level of $p<0.05$ was regarded as significant.

Table 1 shows recovery rates of neutrophils and macrophages exposed to ammonia at concentrations of 50, 100 and 200 ppm for one hour. Neutrophils were sensitive to ammonia exposure and their recovery rates decreased dose-dependently. In contrast, macrophages remained resistant to the exposure. The pH values and estimated ammonia concentrations (parentheses) in the cell suspensions were at pH8.4–8.6 (1–2 mg/dl), pH8.7–8.9 (2–3 mg/dl) and pH9.1–9.2 (5–6 mg/dl) at 50, 100 and 200

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ppm exposures, respectively, in both cell groups.

Chemiluminescence per cell at each ammonia exposure is shown in Table 2. Ammonia exposure enhanced neutrophil chemiluminescence per cell by 21 and 14% on average at concentrations 50 and 100 ppm, respectively. At 200 ppm, the chemiluminescence level was suppressed by 17%. Macrophage chemiluminescence remained unaffected by the exposure.

Table 3 shows total chemiluminescence of cells at each ammonia exposure, calculated by a formula (chemiluminescence per cell $\times$ cell recovery rate) and expressed as a percentage of control value. It was evident that the total chemiluminescence of neutrophils was impaired in a dose-dependent manner due to ammonia exposure, whereas that of macrophages remained unaffected even at 200 ppm exposure.

In the present *in vitro* study, bovine neutrophils exposed to atmospheric ammonia decreased their recovery rates dose-dependently but enhanced chemiluminescence per cell at lower ammonia concentrations. The decrease of cell recovery rates suggests that numbers of the cells might have been destroyed due to cytotoxic or cytolytic effects of ammonia, such as disturbance of acid-base balance or interference with essential metabolic cycles. Ammonia has also been found to inhibit neutrophil function including chemotaxis, phagocytosis, degranulation and bactericidal activity by alkalizing the intralysosomal pH [1, 6]. The enhancement of chemiluminescence per cell at lower ammonia concentrations in this study, however, seems contradictory to those findings.

There is a possible explanation to interpret this

discrepancy. Luminol-dependent chemiluminescence is an indicator of myeloperoxidase(MPO)-H₂O₂-Cl⁻ respiratory burst system of phagocytes [3]. The system is mediated by NH₂Cl which has been yielded from NH₄⁺, i. e., dissolved NH₃ [4]. Suzuki *et al.* [9] have reported that the presence of NH₃ enhanced luminol-dependent chemiluminescence of neutrophils distributed in *Helicobacter pylori*-induced gastric mucosal injury. It is therefore supposed in this study that the MPO-H₂O₂-Cl⁻ system of bovine neutrophils might have been activated in the presence of NH₄⁺ supplied during ammonia exposure. Excessive NH₄⁺, however, seemed to be suppressive on the system as observed in 200 ppm cases.

As compared with neutrophils, bronchoalveolar macrophages were much resistant to ammonia exposure. Their recovery rates and chemiluminescence levels (per cell and total) remained unaffected. Targowski *et al.* [10] have reported that ammonia added to the culture of guinea pig alveolar macrophages significantly inhibited their phagocytic/bactericidal activities. In this study, however, no such ammonia-related inhibition was evident on bovine macrophages, probably due to the different species of animals or parameters employed. It is not known why bovine macrophages remained less sensitive to ammonia exposure. The cells, because of their longevity and particular role in cell-mediated immunity, such as antigen presentation, may possess stronger ammonia detoxication mechanisms than neutrophils do.

It is still uncertain how bovine pulmonary pro-inflammatory cells would respond to *in vivo* ammonia exposure. The present study, however, suggests that ammonia exposure is unfavorable for neutrophils *in vitro*,

Table 1. Recovery rates of bovine neutrophils and bronchoalveolar macrophages exposed to various concentrations of atmospheric ammonia (% of Control)

Cell	Ammonia concentration (ppm)			
	0 (Control)	50	100	200
Neutrophil	100	75 $\pm$ 11 ^{a,b)*}	70 $\pm$ 18 ^{b)*}	59 $\pm$ 11 ^{b)*}
Macrophage	100	94 $\pm$ 14	103 $\pm$ 5	103 $\pm$ 8

a) Values represent mean $\pm$ standard deviation (n=4).

b) * The mean is significantly different (p<0.05) from control level.

Table 3. Total chemiluminescence of bovine neutrophils and bronchoalveolar macrophages exposed to various concentrations of atmospheric ammonia (% of Control)

Cell	Ammonia concentration (ppm)			
	0 (Control)	50	100	200
Neutrophil	100	90 $\pm$ 20 ^{a)}	82 $\pm$ 23	48 $\pm$ 14 ^{b)*}
Macrophage	100	91 $\pm$ 11	106 $\pm$ 11	96 $\pm$ 13

a) Values represent mean $\pm$ standard deviation (n=4).

b) * The mean is significantly different (p<0.05) from control level.

Table 2. Chemiluminescence per cell of bovine neutrophils and bronchoalveolar macrophages exposed to various concentrations of atmospheric ammonia

Cell	Ammonia concentration (ppm)			
	0 (Control)	50	100	200
Neutrophil	130 $\pm$ 58 ^{a)} (100)	163 $\pm$ 90 (121 $\pm$ 19) ^{b)}	153 $\pm$ 91 (114 $\pm$ 22)	116 $\pm$ 74 (83 $\pm$ 26)
Macrophage	14.2 $\pm$ 5.5 (100)	13.8 $\pm$ 5.3 (98 $\pm$ 5)	14.9 $\pm$ 7.4 (103 $\pm$ 13)	13.1 $\pm$ 4.5 (94 $\pm$ 9)

a) Values represent mean cpm/cell $\pm$ standard deviation (n=4).

b) Values in parentheses represent % of control level (mean $\pm$ standard deviation, n=4).

and probably *in vivo* also, in light of causing cell damage, disturbing their bactericidal oxidation systems and increased inflammatory responses.

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