ORIGINAL ARTICLE

Observation of Adverse Effect on Level Ammonia through Expression of CD8 Lymphocyte in Mice

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ABSTRACT

Introduction: The production of ammonia has been increasing over the past few years. Unfortunately, the production does not follow the safety control of ammonia on workers. Indonesia still adopts chemical standard from other countries. Therefore, it requires an ammonia standard at the highest dose without effect or no observed adverse effect level (NOAEL) in the workplace. This research aims to determine standard at the highest dose of without effect through the expression of CD8 cells as well as analysis of histological alteration CD8 lymphocyte between exposed to ammonia group and control. **Methods:** The study was a laboratory experimental research with a post-test only control group design. The research used Rattus novergicus species as many as 24. NOAEL was determined by middle dose with a location between the smallest and the largest dose. The doses of ammonia were given through inhalation. The histological alteration of CD8 between ammonia in exposed and the control group were analyzed by using the Kruskal Wallis test. **Results:** NOAEL was found through CD8 located in group 3 with 0.0154 dose mg/kg body weight. There was a differential expression of CD8 lymphocyte cells in the white mice lung between exposed to ammonia group and control (p=0.042). **Conclusion:** The expression of CD8 lymphocyte cells in the white mice lung exposed to ammonia differs significantly with the number of the expression of CD8 lymphocyte cells in white mice lung at control group. NOAEL was 0.0154 mg/kg body weight of white mice.

Keywords: Ammonia, CD8, Histological, Lymphocyte, No Observed Adverse Effect Level, White Mice

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INTRODUCTION

In Indonesia, ammonia is produced by large companies such as PT Pupuk Kalimantan Timur with a capacity of 1.85 million tons per year and PT Petrokimia Gresik with a capacity of 445 thousand tons per year. Sulfuric acid is also produced in Indonesia by PT Petrokimia Gresik with a capacity of 600,000 tons per year. Risks from exposure to ammonia have already been reported at rubber gloves factory in Medan with complaints of dry throat (80.00%), respiratory symptoms (73.30%) such as dry breathing and eyes irritation (66.67%), nasal irritation and coughs (53.30%) and fainting (6.67%) (1). However, after evaluation, the threshold limit levels were still below the standard regulation of the Ministry of Manpower and Transmigration No. 13 the Year 2011 at 25 ppm (2).

The standard threshold of Indonesia is still adopted from other countries or other institutions in the world, such as the American Conference of Governmental Industrial Hygienist (ACGIH) and Occupational Safety and Health Administration (OSHA). If a country does not have standards for chemicals, those countries can use data research results in the work environment (3). Toxin standard in the environment, including the work environment is directly proportional to the weight of human beings whereby the greater is the weight, the higher is the standard of toxin (3). The average weight of workers in western countries is greater than the average weight of workers in Indonesia. Therefore, the ammonia standard in Indonesia should be lower compared to the ammonia standard in the western countries, including those issued by the ACGIH or OSHA.

Environmental Protection Agency (EPA) identified NOAEL (No Observed Adverse Effect Level) ammonia as 6.9 ppm (4.9 mg/m^3) and for LOAEL (Lowest Observed Adverse Effect Level) as 26.1 ppm (18.5 mg/m^3) based on the increased prevalence of respiratory symptoms and a decrease in lung function (4). EPA also reported that NOAEL of 13.6 mg/m^3 was adjusted for continuous exposure based on the volume ratio of 10 m^3 breathed during an 8-hour workday and respirator (human ambient default minute volume of 20 m^3 breathed

during the entire day) and an exposure of 5 days out of 7 days (5).

Determination of NOAEL can also be acquired through the reference of concentration (RfC). In Europe, NOAEL is RfC that is divided by 100. RfC ammonia subchronic is 0.1 mg/m^3, whereas RfC ammonia chronic is 0.5 mg/m^3 (6-7). Based on the formulation, NOAEL ammonia subchronic is 10 mg/m3 and NOAEL ammonia chronic is 50 mg/m^3 (8).

Before being tested in human, we recommend the highest dose standard determination is fully tested in animal experiments such as using white mice. Several studies had conducted research on the impact of ammonia against white mice, such as experimentation by exposing 100 ppm of ammonia concentration during the 2-6 week on Rattus norvegicus with conjunctiva irritation (9). Geometric mean LTs obtained at wet (181 ppm) or dry (172 ppm) conditions did not differ significantly (P = 0.19) and were within the range of those reported by previous studies, these results suggest that humidity is not a critical factor in determining sensory irritation thresholds for ammonia, and future studies will examine if these findings are transferable to sensory irritation thresholds for other chemicals (10). NH_3 exposure at 30 ppm for 25 weeks increases stress status and suppresses immunity of laying hens as indicated by the changes of H/L ratio and plasma IgM and C4 concentrations (11).

Another study showed the effects of ammonia (NH4Cl) to a member of the immune cell family, the dendritic cells (DCs) or CD80 on white mice tumor cells (12). Ammonia caused the inhibition of phagocytosis by DCs which induced DC swelling. Mice that were exposed to higher concentrations of ammonia had significantly higher histological scores (13). Effects of ammonia on white mice showed anatomical changes of pulmonary histology and aspects of its biomolecular i.e. the immune response by CD8 cell. CD8 cell immune response is the expression of cells observed from immunoreactive cell number and intensity of color. Previous work has only focused on ammonia impact to immune cell through expression immune cell, but this research focuses on how to determine NOAEL through CD8 expression cell, and the result is expected to be a standard ammonia without effect on *Rattus novergicus*. Therefore, this study aims to determine the highest dose of ammonia without effect or NOAEL on *Rattus novergicus* through CD8 cell and analysis of histological alteration of CD8 lymphocyte in an ammonia exposed group and control.

MATERIALS AND METHODS

Research Subject

The research subject was species *Rattus norvegicus* or white mice with free from exposure to ammonia comes from the Animal Laboratory, Faculty of Pharmacy, University of Airlangga. With mice-selected by gender males, a weight between 138-142 gram and 2-3 months. The maturity Rattus norvegicus expected will not experience a different weight (14). This research already approved by the Ethical Committee of Veterinary Faculty, Airlangga University with ethic number 212-KEK in 2012.

Research Design

This research was an experimental study with post test of only control group design that used animals as subjects. Treatment in the study was performed by giving ammonia gas through inhalation with varied doses to *Rattus novergicus*. Technics of the sample used of simple random sampling with 24 *Rattus novergicus*. Firstly, the 24 *Rattus novergicus* were put into 5 metabolite kits, each of its was inserted 4 *Rattus novergicus* and 4 controls. Ammonia gas was exposed to each Rattus novergicus for 14 days, which was exposed every day for 8 hours. Every 2 hours ammonia solution was replaced so that in 8 hours there was 4 times ammonia exposure(15). After the 14th day, all the *Rattus novergicus* were dissected and viewed anatomical and histological changes as well as the expression of CD8 immunoreactive cells (percentage number and intensity of cells). The dependent variable was histopathologist of *Rattus novergicus* lung and expression of CD8 lymphocyte and the independent variable was ammonia concentration. There were two research groups, namely the group that was given exposure to ammonia gas (group 1, 2, 3,4, 5), and the group that was without exposure to ammonia or the control group. Group exposure ammonia divided into five sub-groups. Group 1 with concentration of ammonia 0.0872 mg/m3, group 2 with concentration 0.1309 mg/m3, group 3 with concentration 0.1963 mg/m3, group 4 with concentration 0.2944 mg/m3, and the last in group 5 with concentration 0.4416 mg/m3.

Determination of NOAEL

Determination of the NOAEL was conducted on a test sub-acute (16-19). General protocol on how to test the toxicity of the subacute are: 1) the duration of the test was 14 days, 2) using 3 test doses and 1 control and 3) conducted a chemical analysis of the blood or urine, histopathology examination, and others if possible. Steps to determining NOAEL was:

1) Find smallest and largest ammonia doses.

Based on the preliminary study, the smallest and the largest doses of ammonia concentrations were 0.058159 mg/m^3 and 0.58159 mg/m^3 respectively. Both concentrations of ammonia with units of mg/m3 was a conversion from units of mg/l after adjusting for air pressure (759 mmHg) and temperature conditions (29.6°C) at the site of the research with helped by Occupational Health and Safety Surabaya. Meanwhile, the acquisition of ammonia gas units of mg/l was derived from NH_4OH application of stoichiometric chemical equation evaporation reaction, which produces NH_3 and H_2O based on the following formula:

$$1 \text{ mol } NH_4OH = 2.059 \text{ mol } NH_3$$

Meanwhile, the formula for determining doses in animal (mg/kg) was (3) :

$$\text{Dose} = \frac{(\alpha)(BR)(C)(t)}{(W)} \text{ (mg/kg)}$$

α = % lung absorption, = 100% if not known.
BR = breathing rate (the rate of respiration of animals try, unit m3/hour
t = time (long time work, unit) is 8 hours/day
C = concentration (the concentration of toxins in the air, mg/m3)
W = weight (weight of animals, kg)

Table I shows that the lowest concentration of ammonia was 0.1275 mg/l, and the highest concentration of ammonia was 0.6455 mg/l. Between the concentrations above, the highest dose of ammonia without effect in mice (NOAEL) could be obtained.

Table I: Relationship between $[NH_4OH]$ and $[NH_3]$

No	$[NH_3]$ (mg/l)	$[NH_4OH]$ (mg/l) = 2.059 $[NH_3]$	$[NH_3]$ (mg/m^3)
1	0.1275	0.2625	0.0872
2	0.1913	0.3938	0.1309
3	0.2869	0.5907	0.1963
4	0.4303	0.8860	0.2944
5	0.6455	1.3291	0.4416

2) Found middle doses
The middle doses were doses between largest and smallest doses whose often has multiple of 1,5 started small doses (here are 5 variations of the middle dose derived from the 5 variations of the middle concentration), consisting of 0.0872; 0.1309; 0.1963; 0.2944; 0.4416 (mg/m^3). From middle and small doses will be found the highest unproved NH_3 dose i.e the highest dose of ammonia in white mice indicated by the highest ammonia dose of IRS CD8 before IRS score decline. IRS (Remmele Scale Index) is a semiquantitative scale resulted by multiplication between percentage score of lymphocyte cell with color intensity in T lymphocytes CD8 cells.

Histopathology
CD8 expression in lymphocyte cells was a semiquantitative scale of IRS CD8 (Remmele Scale Index) which is the result of multiplication percentage of the immunoreactive cell (A) with color intensity score on the immunoreactive cell (B) according to the modified Remmele method (IHC staining, 1000 x magnification) (20).

$$\text{IRS CD8} = (A \times B)$$

A representation of the percentage of immunoreactive with score 0: no immunoreactive cell, score 1 for immunoreactive cell less than 10 %, score 2 for immunoreactive cell was 11-50%, score 3 if immunoreactive cell was 51-80% and score 4 if the immunoreactive cell has percentage more than 80%. B was represented for the color intensity of cell. Score 0 for no color, score 1 for moderate color intensity, score 2 for moderate color intensity and score 3 for strong color intensity (20). This picture was obtained by staining the HE; enlargement of the 400x; Olympus BX-50. Camera Digital Pentax Optio 230; 2.0 megapixels) with scale 600 µm.

Statistical analysis
Statistical tests using a Kruskal Wallis with α = 0.05 to know the difference between the number of IRS (immunoreactive score) on CD8 between control group with the group exposed to ammonia with program SPSS version 21 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

From Table II, it showed that ammonia dose could be calculated with a comparison between the percentage of ammonia absorbed (α), breathing rate (R), NH3 concentration (C), and longtime work, which was 8 hours/day with average weight (W). The highest average weight (W) and NH3 concentration are in group 5, but the highest breathing rate was in group 2 and 3.

Table II: Results of calculation average weight (W), NH_3 concentration (C), percentage of ammonia absorbed (α) and breathing rate (BR) in white mice

Group	Frequency	W (average weight) (kg)	NH_3 Concentration (mg/m^3)	The percentage of ammonia absorbed (α)	Breathing Rate Mean (m^3/hours)
Control	4	0.1405	0.0000	100%	1.3750 ± 0.0000816
Group 1	4	0.1405	0.0872	100%	1.3755 ± 0.0001633
Group 2	4	0.1410	0.1309	100%	1.3809 ± 0.0001633
Group 3	4	0.1410	0.1963	100%	1.3809 ± 0.0000816
Group 4	4	0.1395	0.2944	100%	1.3657 ± 0.0000816
Group 5	4	0.1405	0.4416	100%	1.3754 ± 0.0001633

Based on the results in Figure 1, the lowest and highest dose of ammonia in the body of the white mice are control group with 0 mg/kg body weight and group 5 with 0.0346 mg/kg body weight.

Comparison between Expression of CD8 Cells in Lung White Mice Post Exposure with Different Doses of Ammonia
Based on Figure 2, K represents the control group without exposure to ammonia and 1, 2, 3, 4 and 5 represent the group exposed to ammonia with a dose of ammonia 0.0068; 0.0103; 0.0154; 0.0231; 0.0346 (mg/kg), respectively. The result shows that the number of cells of immunoreactive lymphocyte (arrow) started to increase from the control group to group 1 and group 3 and decreased in group 4 and continued to decline until group 5.

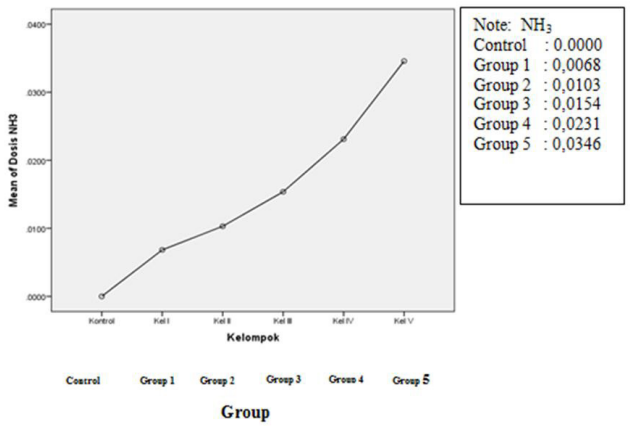

Figure 1: Dose of ammonia in white mice exposed to ammonia and control group

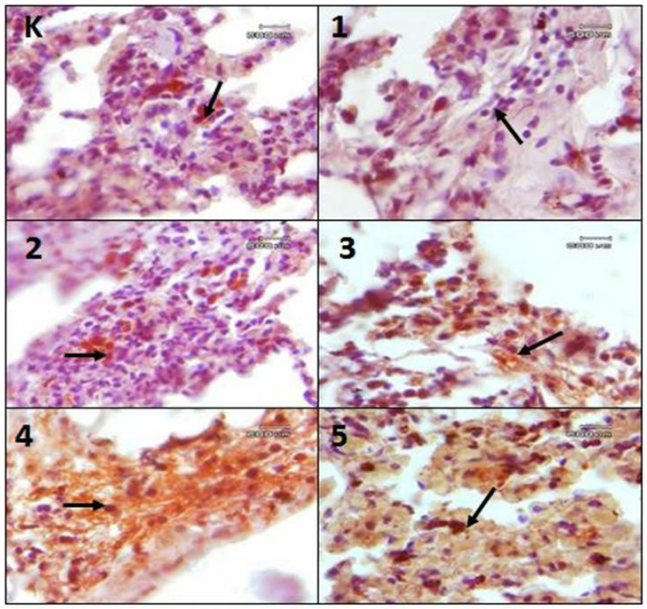

Figure 2: Comparison between expression of CD8 with different doses of ammonia

Figure 2 showed a comparison on expression as indicated by CD8 on lymphocyte cells in BALT lung white mice post exposure with different doses of ammonia. This picture is shown through the percentage of CD8 immunoreactive cell number and intensity of color, whereby the result of the multiplication between A and B is called the IRS score.

On the slide K (control), it is shown that there was a low CD8 expression as shown by the percentage of immunoreactive cell number which was still less than 10.00% and low color intensity. In Group 1 with a dose of 0.0068 mg/kg ammonia, immunoreactive cell counted less than 10% and moderate color intensity. In group 2 with 0.0103 mg/kg ammonia dose, the immunoreactive cell had a number 11-50% with moderate color intensity. In group 3 with a dose of 0.0154 mg/kg ammonia, immunoreactive cell number was 51-80% with strong color intensity. In Group 4 with a dose of 0.0231 mg/kg ammonia, immunoreactive cell number was 11-50% with strong color intensity. In group 5 with a dose of 0.0346 mg/kg ammonia immunoreactive cell was 10% with strong color intensity. Thus, IRS score on Group 4 is the lowest compared to other ammonia exposed group.

The result of Expression score CD8 Lymphocyte Cell Observation

From Table III, it can be concluded that the highest dose of ammonia without effects on the white mice was group 3 with 0.0154 mg/kg body weight. Analysis with Kruskal Wallis test mentioned that there was a significant difference between the number of IRS score on CD8 between control group with the group exposed to ammonia (group 1,2,3,4,5) (p=0.042).

From the Figure 3, it can be inferred that the highest dose of ammonia without effect (NOAEL) in white mice lies in group 3 with 0.0154 dose mg/kg body weight.

Table III. Expression score of CD8 Lymphocyte Cell

White Mice Group	NH_3 Dose (mg/kg)	IRS Score on CD8 Expression
Control	0.0000	2.00
Group 1	0.0068	4.00
Group 2	0.0103	5.50
Group 3	0.0154	6.75
Group 4	0.0231	4.00
Group 5	0.0346	2.75

p = 0.042 ; α = 0.05

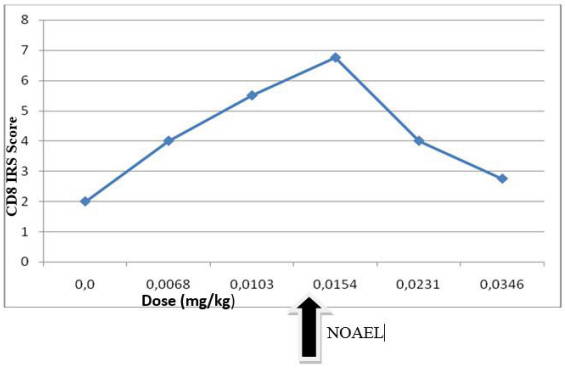

Figure 3: Relationship of NH3 dose and IRS score of CD8 cells white mice between ammonia exposed and control group

DISCUSSION

Dendritic cells which are non-specific immune cell play the role of innate immune system intermediaries (non-specific) to the adaptive immune system. Dendritic cells are nonspecific immune cells that present antigens to T cells to initiate the entire immune response that depends on T cells (21).

Ammonia is a polar molecule thus can be absorbed into a cell by passive diffusion with facility/carrier protein. When bound to a protein carrier, ammonia becomes immunogenic and sets into T cells, including CD8 (21). The interaction of toxins with receptors depends on the

suitability/ analog of the chemical structure of the toxin with its receptor. The suitability/ analog of chemical structure with its receptor is one of the determinants of the effectiveness of interactions between toxins and the body's own metabolism. CD 8 has an amine group (-NH +). The similarity/chemical structure analogs between ammonia (NH_3) and amine groups will cause strong reactions between the two molecules that accelerate CD8 damage (22).

The recipient's T cells or TCR (T cell receptor) has a typical domain structure that consists of a protein molecule (immunoglobulin superfamily) containing amine. Thus, T cells containing amine will easily absorb ammonia (21). Ammonia has a chemical formula analogous to the amine moieties on CD8 (21). The presence of this chemical compound similarities could absorb ammonia and reversible traits. CD8 also has a carboxylic cluster containing-OH that has a partial negative charge and bind to ammonia-containing positive charge. The ammonia absorption or ammonia bind cause the ammonia to easily tied to CD8. The bond between antigens and T cells in this case only involves CD8 non-covalent style and hence theoretically reversible in nature. However, in practice, most immune systems have a high affinity of atom O and N, so CD8 can bind strongly with ammonia which contains atom N. The interaction between ammonia (positive charge) and carboxylic group (negative charge) will cause electrostatic attractive forces. This forces is strong enough to cause damage of CD8 immune system (23).

NH3 gas with different doses will go to bronchioles and induces bronchioles in the lungs. After going through M cell, the cell will present the antigen (APCS), then ammonia gas entering through the M cells will be presented by APC in the MHC Class II and induced CD4 T helper cells – which will change morphology as Th1 (T-helper1) CD4 induce IL2 (Interleukin-2). IL2 will also induce CD4-Th2 CD4 Th2 and vice-versa would also induce IL2. Then, IL2 cells would induce CD8. Although both CD4 and CD8 induced by cell IL2, IL2 activate CD4 more than CD8 (21).

Based on the results, there is a meaningful difference in expression of CD8 lymphocyte cells in the lung white mice exposed to ammonia groups and control group. This difference can be seen from the IRS score group on exposed and control by using immunohistochemical color. By using statistical analysis, it can be described that IRS score on the group 1 (4.00) as indicated in the graph below did not differ markedly with groups 2, 4 and 5. But, another previous study did not have a similar result which mentioned that there is no significant meaning between expression CD8 lymphocyte between ammonia groups and control group (12). The difference can occur because it studied using tumor cells in mice, NH_4Cl (different with this research using NH_3), and different concentration of ammonia with 2.5 mM and 5 mM (12).

It can be stated that the highest dose of ammonia without effect (NOAEL) is in group 3 with 0.0154 dose mg/kg body weight. These findings are in contrast with the highest dose of ammonia with no effect on CD4 and IL2 in 0.0103 dose mg/kg body weight as reported by a previous study (24). This can happen because IL2 induces CD8, CD4 also induced CD8, therefore IL2 and CD4 were directly responded to ammonia. However, CD8 was being indirectly responded to ammonia. Thus, the highest dose of ammonia with no effect on CD8 was when the IRS CD8 scored position of 0.0154 dose mg/ kg body weight. We choose CD8 lung cell in *Rattus novergicus* as subject cause this research gave ammonia through inhalation which EPA showed that breathing ammonia at sufficiently high concentration can result in an effect on the respiratory system thus it was assumed that our subject (CD8 lung cell) is more sensitive to detect ammonia (5).

Another study gives another different result such as NOAEL ammonia exposure in workers in a soda ash plant was 13.6 mg/m^3 or developmental toxicity study on diammonium phosphate involving rats with doses of 0, 250, 750, and 1500 mg/kg/day which is resulted in NOAEL of 1500 mg/kg/day (25-27). Another result use strain F344 rat with dose 0,564, 0,650 and 1,371 mg/kg/ day have NOAEL of 1,288 (males) and 1,371 (females) (28). Based on the previous research, we assumed that difference of NOAEL result was determined by doses of ammonia, ammonia concentration, a subject which used for research, situation, and condition in the workplace such as temperature or pressure although NOAEL research still limited (29). Therefore, this NOAEL result (0.0154 dose mg/kg/ body weight) just can be applied in Indonesia workplace.

In addition, aspects of molecular weight (BM) were also influential(30). The molecular weight of IL2, CD4, and CD8 are 15 000, 60 000 and 75 000 respectively (30). The molecular weight of chemical has influenced the concentration and reaction rate(31). This is in accordance with the reaction rate law that mentions greater concentration would increase the reaction rate. Thus, the reaction rate of the CD4 and IL2 are greater than CD8 because reaction rate response of CD8 is smaller compared to the IL2 and CD4. Therefore, at the time of exposure dose 3 (0.0154), the effects of ammonia on white mice would appear.

The higher the dose ammonia, the higher is the tissue damage including congestion, edema, infiltration, degeneration, necrosis, hyperplasia and fibrosis. Our experiment is in line with the previous result, whereby ammonia can affect immune cell especially dendritic cell (CD80, CD80, CD86, and MHCII) (11). Ammonia could diminish cell count, phagocytosis, and lymphocyte

stimulation dendritic cell.

The above arguments are also strengthened using the analysis of the relationship between the concentration of the substance (not a log of the concentration of a substance) and the response of the living creatures in the determination of environmental quality standard (32). The determination of environmental quality standard is based on the determination of the NOAEL and LOAEL (Figure 3) with the image of the curve obtained is the curve inverted, whereby at the time when the initial response was still good, then towards the peak response, it finally decreased in the end.

When the immune response decreases, it does give an indication that there has been an effect or impact of ammonia in the body. Similarly, the results of this study when CD8 lymphocytes decreased, it means that there was an effect of ammonia in the body. At the time of the dose of ammonia 0.0103 mg/kg, it was a maximum increase of immune response with an indication of CD8 IRS score decline. Thus, a dose of ammonia 0.0154 mg/kg can be referred to as the highest dose with no effect on the body or on CD8 lymphocyte especially this standard just can be applied in Indonesia workplace. Further research such as determine ammonia NOAEL in human also can be done to elaborate more research on risk assessment in Indonesia.

CONCLUSION

The highest dose of ammonia without effect or NOAEL location is in group 3 with 0.0154 dose mg/kg body weight. The findings of the research revealed a meaningful amount of differential expression of CD8 lymphocyte cells in the lung white mice exposed to ammonia groups and control group.

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