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Determination of Highest Dose of Ammonia without Effect at Work Environment through the Expression of Interleukin-2 Cell in *Rattus Novergicus*

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Abstract

BACKGROUND: For determining the threshold limit value firstly determined the highest dose of ammonia without effect (NOAEL). However, research on the determination of NOAEL ammonia didn't conduct in Indonesia.

AIM: The aim of this study to determine the value of the highest dose of ammonia without effect (No Observed Adverse Effect Level/NOAEL) through interleukin-2 (IL-2) expression on white mice.

METHODS: This study used experimental laboratory research with post-test only control group design using white mice as experimental subjects. The treatment group divided into 6 groups (a group of controls and five groups with different ammonia exposure through inhalation). The trend curve of Remmele Scale Index (IRS) and histopathologic analysis could be used for determining NOAEL.

RESULT: The location of the highest dose of ammonia without any effect (NOAEL) of white mice was in the second group, with 0.0103 mg/kg body weight dose. Analysis of statistical tests Kruskal Wallis stated there was no significant difference in interleukin-2 expression between the control with ammonia exposed group with a significance of p (0.747) > α (0.05).

CONCLUSION: There is no difference between some interleukin-2 expression in the lymphocyte cell lung white mice group exposed to ammonia and control group. The highest dose of ammonia without any effect (NOAEL) on white mice was 0.0103 mg/kg body weight.

Introduction

Indonesia threshold limit value of chemicals was adopted from other countries or institutions in the world, such as the ACGIH and OSHA. Some overseas institutions also had different threshold limit value. OSHA, for example, has an ammonia TLV ppm, NIOSH (50 ppm/5 minutes CEIL), ACGIH (25 ppm). Unfortunately, the threshold limit value of chemicals set by the result of economic and technological considerations [1].

If a country didn't have a chemical threshold limit value, the country could use other research data of the highest dose of ammonia without effect or *No Observed Adverse Effect Level*/NOAEL in the workplace. This also applies to the determination of the highest dose of ammonia gas in Indonesia; the research results can be used as the standard highest dose of ammonia in the working environment. However, until now there has been no research on the standard of the highest dose of ammonia gas in the work environment in Indonesia [2].

The requirement of ammonia in Indonesia is increasing every year. For example, ammonia gas consumption for ZA fertiliser industry averages about

400,000 tonnes per year. Unfortunately, this increase production and distribution of ammonia have an impact on increasing risk accidents for the worker. To assess ammonia standards for workers must be preceded by the experimental animals. During this time, the experimental animals were often used are white mice with Rattus sp. [3].

Effects of ammonia in the mice include anatomical changes observed by hepatologist, but it is also observed by biomolecular immune response aspects including interleukin-2 (IL-2). IL-2 expression of the cells was observed from the number of immunoreactive cell and intensity of colour. It is said to have an effect when there is a decrease in the immune response of lymphocyte cells i.e. IL-2, and it is said to be without effect if there isn't decline in the immune response to cells immunoreactive IL-2. This study never was done in Indonesia.

Therefore, the purpose of this study is to determine the highest dose of ammonia without effect (NOAEL) through the expression of IL-2-2 in white mice.

Material and Methods

This study was experimental laboratory research with post-test only control group design using experimental animals (white mice with Rattus novergicus) as experimental subjects. The treatment in research was giving ammonia with varying doses on white mice. The basic principle as the requirements of experimental studies was two (2) terms. The first requirement was replication or repetition of the conditions given equal treatment with all samples in all treatment groups. The second requirement is a random system or random element in the distribution of sample number 2 (two) groups of the study, the group that is exposed to ammonia through the inhalation system, and the group without being given exposure to ammonia or a control group. The control group was used to increase the validity of research results [4]. Group exposure ammonia divided into five sub-groups. Group 1 with concentration of ammonia 0.0872 mg/m^3, group 2 with concentration 0.1309 mg/m^3, group 3 with concentration 0.1963 mg/m^3, group 4 with concentration 0.2944 mg/m^3, and the last in group 5 with concentration 0.4416 mg/m^3.

Experiment unit

The study used species *Rattus norvegicus* rat or white mice from an animal laboratory of Pharmacy Faculty, University of Airlangga. White mice were selected with male sex, weight 138-142 gram and aged 2-3 months (white mice breeding) [5]. The white mice were kept by following several requirements such as control, environmental and health status monitoring, surveillance on researchers and workers then food and beverages supervision.

Determination of the number of replication follows the formula of determining the replication performed by Federer obtained the number of samples of 24 white mice [6]. This research already approved by the Ethical Committee of Veterinary Faculty, Airlangga University with ethic number 212-KEK in 2012. Tools for experimentation such as 5 sets of Metabolite Kit, measuring cup, 5 pieces of 1 l chemical, Scales, simple Respirometer, 3 ml dropper. For materials was NH_4OH 25%, BJ 0,91 kg/l, aqua shades, *Rattus novergicus*, and KOH powder.

Dose determination of animal toxins in the body

To determine the dose of toxin in the form of gas that enters the living body, including animals used formula of [2]:

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

Note:

α = % Absorbed substances lungs, = 100% when the unknown.

BR = *breathing Rate* (Respiratory rate of experimental animals, the unit m^3 / h

T = *time* (Long working time, hours)

C = c*oncentration* (Concentration of toxin in the air, mg / m^3)

W = w*eight* (Animal weight, kg)

The highest dose of toxin without effect (NOAEL) in the experimental animals

NOAEL determination was performed on a test sub-acute [7], [8], [9], [10]. General protocol sub-acute toxicity test method is tested on 14 days, using 3 doses of test and control. The dose is causing toxic effects but not cause death; smallest dose did not leave a toxic effect, the middle dose is between the two doses of the above and Control = all ingredients used unless the active substances generally are solvent.

This calculation also conducted chemical analyses of blood or urine, and histopathology and others analyse if possible. NOAEL, along with LOAEL was the main indicator of environmental quality criteria. The range between the concentration NOAEL and LOAEL a concentration range of acceptable substances or match (Maximum Acceptable Toxicant Concentration) [1].

Analysis of Statistics

Analysis statistics uses a Kruskal Wallis test with Alfa 0,05 to know the difference in interleukin-2 expression between control and ammonia exposed group (group 1, 2, 3, 4).

Histopathology

IL-2 expression in lymphocyte cells was a semiquantitative scale of IRS IL-2 (Remmele Scale Index) which is the result of multiplication percentage of the immunoreactive cell (A) with colour intensity score on the immunoreactive cell (B) according to the modified Remmele method (IHC staining, 1000 x magnification) [12].

IRS CD8 = (A x 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 the immunoreactive cell was 11-50%, score 3 immunoreactive cell was 51-80% and score 4 if the immunoreactive cell has percentage more than 80%.

B was represented for the colour intensity of the cell. Score 0 for no colour, score 1 for moderate colour intensity, score 2 for moderate colour intensity and score 3 for strong colour intensity [12]. 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.

Results

Observation

Dosage of ammonia in the control group was 0.0000 mg/kg, in the group 1 is 0.0068 mg/kg, group 2 is 0.0103 mg/kg, group 3 is 0.0154 mg/kg, group 4 is 0.0231 mg/kg and group 5 is 0.0346 mg/kg body weight of mice on Table 1.

Table 1: Results of Ammonia Dose in White Mice with Time Exposure 8 hours/day

NH_3 Concentration (mg/m^3)	The percentage of ammonia absorbed (α)	The average rate of respiration (BR) (L/h)	W (average weight) (kg)	NH_3 Dose (Mg / kg)
0.0000	100%	1.3750	0.1405	0.0000
0.0872	100%	1.3755	0.1405	0.0068
0.1309	100%	1.3809	0.1410	0.0103
0.1963	100%	1.3809	0.1410	0.0154
0.2944	100%	1.3657	0.1395	0.0231
0.4416	100%	1.3754	0.1405	0.0346

Source: Primary Data.

The Observation of IL-2 (Interleukin-2) Expression in Lymphocytes

On slide K (control) in Figure 2 is still has a low IL-2 expression by the percentage shown immunoreactive cell number is still less than 10% and moderate colour intensity. In group 1, immunoreactive cells had increased in number between 11-50% and moderate colour intensity. In the group 2 with a dose of 0.0103 mg/kg weight, immunoreactive cell count between 51-80% greater than the number of cells group 1, but the intensity of the colour was same as the intensity of colours in the group 1. In group 3 immunoreactive cell count between 11-50%, but the colour intensity is lower than the intensity of the colour in the group 2. Thus, expression cell score in the group 3 lower than the score of the IRS group 2. In group 4 has immunoreactive cell less than 10% with moderate colour intensity. In group 5 has immunoreactive cell less than 10% and colour intensity was almost the same as group 4 but from expression is still lower than group 4. IRS score tends to fall because of continuous exposure to ammonia.

Table 2: Observations Expression of Interleukin-2 Lymphocyte Cell Lung in White Mice

Group of white mice	NH_3 dose (mg/kg)	Interleukin-2 expression (IRS)
Control	0.0000	2.75
Group I	0.0068	4.00
Group II	0.0103	4.50
Group III	0.0154	4.25
Group IV	0.0231	3.75
Group V	0.0346	3.25

p = 0.747; α = 0.05; Source: Primary Data.

Expression of Interleukin-2 and Color Intensity

From IRS score shown in Table 2, the highest IRS score contained in group 2 (4.50) at a dose of 0.0103. From that number then fell to 3.25 at the highest dose in group 5. From these data, it can be concluded location of the highest dose of ammonia without any effect on the lungs of white mice is in the second group, i.e. 0.0103 mg/kg body weight of white mice.

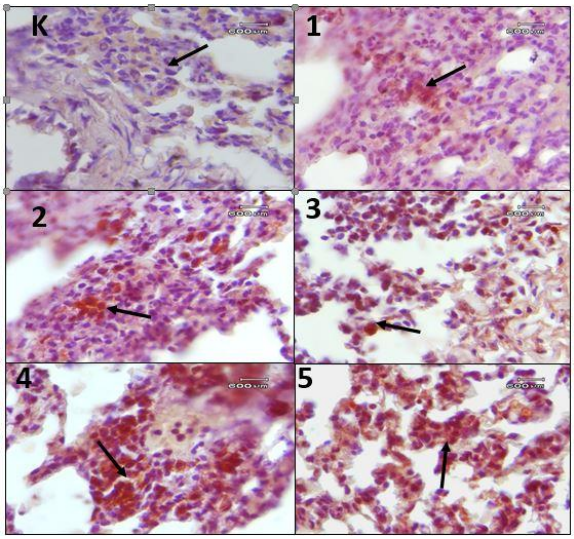

Figure 1: Comparison of Expression of IL-2 on Lung white mice after exposure to ammonia with different doses. K represents a control group with no exposure to ammonia, and 1, 2, 3, 4 and 5 respectively represent the group exposed to ammonia in a row with a dose of ammonia 0.0068; 0.0103; 0.0154; 0.0231; 0.0346 (mg/kg)

From the results of the Kruskal Wallis test, it can be seen that there is no significant difference in the expression of IL-2 (interleukin-2) between the control group and the ammonia exposed group with p significance (0.747) > α (0.05). No significant difference can be seen from the number of IRS score in each group were almost the same as that shown by using immunohistochemical staining (Figure 2).

In Figure 3, the IRS score of IL-2 (interleukin-2) at a dose group 2 is higher than the other, which rose after a dose group 1 and then fell after a dose of group 2. Thus, one can say that the immune response or IRS score of IL-2 (interleukin-2) is group 2 (with a dose of 0.0103 mg/kg) is the highest and could be called as the location of the highest dose without effect or NOAEL.

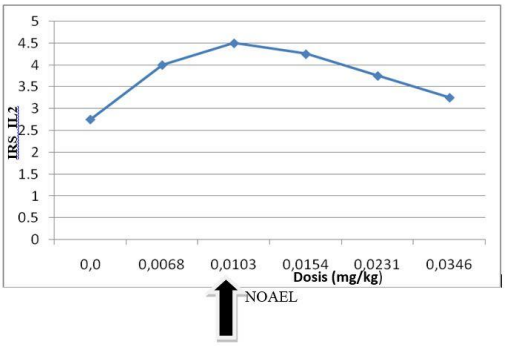

Figure 2: Relationship NH_3 Dose in the White Mice Body Exposed Ammonia Group and Control with IRS score of Interleukin-2 Immune Response

Discussion

Expression of Interleukin-2 in White Mice Exposure to Ammonia and Control Group

Of the four leading causes of death: injuries, infections, degenerative diseases, and cancer - only the first two causes usually cause the patient's death before reproductive age, which means potentially eliminating genes. Therefore, any mechanism that reduces the impact of highly valuable in sustaining life, it can be observed in a sequential process, recovery, and immunity [13].

Ammonia is polar thus can be absorbed into the cell by passive diffusion with facilities/carrier protein. Ammonia as a hapten binds to carrier protein will be immunogenic and entry into T cells, including IL-2 (interleukin-2). With continuous exposure to ammonia at IL-2 (interleukin-2) caused a reduction in lymphocyte cells IL-2 (interleukin-2) immune response.

Recipient T cell or TCR (T Cell Receptor) has the typical domain structure consisting of protein molecules containing amine groups. Thus, structurally T cells that contain amine groups will easily bind with ammonia [13]. As the immune system containing immunoglobulin superfamily, IL-2 (interleukin-2) group containing ammonia. Hapten ammonia has the chemical formula analogous to the amine group on IL-2 (interleukin-2). With the similarity of chemical compounds, IL-2 (interleukin-2) can absorb ammonia [13], [14]. Thus, the number of issued immune response is also almost the same.

IRS score is also increased to 4.50 in group 2 at a dose of 0.0103 mg/kg. This means that the immune response of white mice at group 2 is still good and mice have not shown the effects resulting from exposure to ammonia. This is due to the immune system of white mice were able to function well to recognise and dispose of ammonia hapten [13], [14], [15].

From the description above, it can be concluded a dose of ammonia in group 2 didn't give effect for ammonia caused the white mice body is still able to recognise and dispose of ammonia.

Meanwhile, ammonia groups 3, 4 and 5 affects the body of white mice because the immune response decreases. From these data, it can be concluded that the location of the highest dose of ammonia without effect (NOAEL) in the mice of group 2, at a dose of 0.0103 mg/kg body weight of mice. With ammonia NOAEL found in the mice can be used to determine the highest dose of ammonia without any effect on the worker's body.

Ammonia Highest Dose without Effect (NOAEL) in White Mice

In this histopathologic analysis of the dose in group 2, provides tissue lesion score higher than the first group and the control group, but not statistically significantly different. Thus, the dose group 2 i.e. 0.0103 mg/kg body weight of white mice is the highest dose of ammonia without any effect on mice. This is accordance with the opinion of Vermeire and Leeuwen that there is no significant relationship between dose in the exposed group and the control, and the highest doses of the exposed group were the highest dose without effect called NOAEL [16].

The results of Kruskal Wallis test produces a value above alpha, so it can be seen that there are no significant differences in the expression of IL-2 (interleukin-2) between the control group and the ammonia exposed group 1, 2, 3, 4 and 5 with p (0.747) > α (0.05). However, descriptively, IRS score in dose group 2 is higher than the others which rose after the dose in group 1 and then fell after the dose in group 2, it can be stated that the immune response or IRS IL-2 (interleukin-2) in the group 2 highs and could be called as IRS lies at the highest dose of ammonia without effect or NOAEL in white mice.

According to the EPA, NOAEL is

experimentally determined the dose at which no statistically significant indication of the toxic effect or biological concern. Thus, although Kruskal Wallis test immunoreactive expression of IL-2 (interleukin-2) IRS score did not differ significantly can be used as a reference in determining the NOAEL [17].

Moreover, from the aspect of molecular weight (Mw) were also influential. If compare with another lymphocyte cell such as CD4 and CD8, molecular weight IL-2 (interleukin-2) is 15,000, CD4 has a molecular weight of 60,000 and CD8 was 75,000. Because, CD8 has molecular weight greater doses of the same ammonia smaller mole, so its concentration is too small, and the reaction rate is also smaller. This is by the law of the greater speed of reaction that more greater concentration, it can also increase the reaction rate. Thus, the reaction rate is greater on CD4 and IL-2 (interleukin-2) than the CD8. Therefore, when group 3 began contained ammonia effect on mice. From these findings, IL-2 (interleukin-2) can be used as a marker for determination of ammonia in the highest dose of ammonia without any effect on mice.

From Figure 3 it can be stated the higher ammonia dose, the higher damage include congestion, oedema, infiltration, degeneration, necrosis, fibrosis, and hyperplasia. It is different with the immune response to IL-2 which begins with a rising curve that shows the immune response is still good (have not had a negative effect) but after it decreased immune response that show already negative effect. Thus, the pattern of immune response curve makes it easy to determine the highest dose without effect or NOAEL compared with the curve patterns of tissue damage. This is confirmed by a conception that immune as a gateway to the occurrence of diseases. Meanwhile, the effect occurs because there is a decrease in the immune response [18].

When the immune response decreases, this indicates that there has been an effect or impact of ammonia in the body [19], [20]. Similarly, at the time when the results of this study showed that there would be lymphocyte IL-2 (interleukin-2) decrease, it can be meant there was an impact of ammonia in the body. At the time of the ammonia dose, 0.0103 mg/kg in white mice body is the peak immune response seen from the rise in IRS score of IL-2 (interleukin-2), and after that IRS score, each lymphocyte cells was decreased. Thus, the dose of ammonia 0.0103 mg/kg can be referred to as the highest dose without effect on the body (NOAEL).

The integrity of the immune system is needed to defend themselves against microorganisms and toxic products they produce, and it is said to be a decline in immune response when a T-cell count is low, it can be seen using fluorescence microscopy. From the observation using fluorescence microscopy was found decreased IRS score on the third dose group whereas in dose group II had the highest immune response compared with the first dose group in IL-2 (interleukin-2). Thus, it can be stated that the highest dose of ammonia without any effect on the body lying on a white mouse to the second dose is 0.0103 mg/kg body weight of mice [21].

We can conclude that:

1. The level of Interleukin-2 expression ammonia exposed group did not differ significantly with the number of IL-2 expression on control.

2. The highest dose of ammonia without any effect (NOAEL) was 0.0103 mg/kg body weight of mice.

Recommendation

Based on the findings in this study will also provide a solution for the certification bodies such as the Center for Drug and food (BBPOM) in determining the safety certification of a food or a product. This is due to encountered a product has been certified but later turned out to be harmful to health, such as DDT, sodium benzoate on instant noodles. With the ease of determining the NOAEL will provide facilities that also determine a safe dose for workers.

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Ethical Clearance

Ethical Clearance took from the Faculty of Public Health, Airlangga University, Indonesia Committee.

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