The Effect of Exposure Process of Toluene To The Spermatogonia Cell a Rattus Strain Wistar

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Abstract

***Intoduction**: Acute and chronic exposure to toluene at high doses is known to affect all organs of the body including the spermatogenesis process. In the industrial sector, the use of toluene as a solvent is still widely used, up to 10 million tons per year. The control over health problems that may occur is carried out by applying work exposure threshold values. This research aims to explore the effect of toluene exposure at the threshold value range on spermatogenesis.*
***Method**: This research used laboratory experiment on 30 male Wistar rats which were divided into five groups of different exposure levels, namely 12.5 parts per million (ppm], 25 ppm, 50 ppm, 100 ppm, and no exposure (control). Exposure was given for 4 hours daily over 14 days through a hood with measured release in the glass cage. The toluene exposure markers observed were Malondialdehyde (MDA) in the blood tissue and testicles using the Thiobarbituric Acid Reactive Substances (TBARS) method. The effect on the spermatogenicity process was assessed by counting the spermatogonia A cells of male Wistar rats with Periodic Acid Schiff (PAS) staining and is calculated by the Abercrombie formula. Analysis of the correlation between the level of exposure and its effect on the increase in malondialdehyde, and spermatogenesis was carried out using the Spearman correlation analysis.*
***Result**: There was a moderately positive correlation between levels of toluene exposure and plasma MDA levels ($r = 0.42$; $p = 0.025$). Meanwhile, on [the issue of] the quantity of spermatogonia cells, a high level of negative correlation with exposure levels was obtained ($r = -0.68$; $p = 0.001$).*
***Conclusion**: Toluene exposure in male Wistar rats within the range of threshold values influenced the increase in plasma MDA levels and decreased the Spermatogenia A cells. However, toluene exposure did not affect the testicular MDA levels of male Wistar rats.*

***Keywords**: Toluene, Male Wistar Rat, Spermatogenia A, Malondialdehyde, Abercrombie formula*

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Pengaruh Pajanan Toluena Terhadap Gambaran Sel Spermatogonia A Rattus Strain Wistar

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Abstrak

***Pendahuluan**: Pajanan toluene secara akut maupun kronis pada dosis tinggi diketahui dapat memengaruhi seluruh organ tubuh termasuk proses spermatogenesis. Di bidang industri penggunaan toluena sebagai pelarut masih digunakan luas, bahkan penggunaan di Indonesia mencapai 10 juta ton per tahun. Pengendalian gangguan kesehatan yang mungkin terjadi dilakukan anatara lain dengan penerapan nilai ambang batas pajanan kerja. Penelitian ini bertujuan untuk mengeksplorasi pengaruh pajanan toluene pada kadar kisaran nilai ambang batas terhadap spermatogenesis.*
***Metode**: Desain penelitian ini menggunakan laboratory experimental terhadap 30 tikus Wistar jantan yang dibagi dalam lima kelompok tingkat pajanan yaitu 12,5 part per million [ppm], 25 ppm, 50 ppm, 100 ppm, dan tanpa pajanan (kontrol). Pajanan diberikan 4 jam tiap hari selama 14 hari melalui sungkup dengan pelepasan terukur pada kandang kaca. Marka pajanan toluena yg diperiksa adalah Malondialdehid (MDA) di dalam jaringan darah dan testikel menggunakan metode Thiobarbituric Acid Reactive Substances (TBARS). Efek terhadap proses spermatogenis dinilai dengan menghitung sel spermatogonia A tikus wistar jantan dengan pewarnaan periodic acid Schiff (PAS) dan dihitung dengan rumus Abercrombie. Analisis hubungan antara tingkat pajanan dan pengaruhnya terhadap peningkatan malondialdehid, dan spermatogenesis dilakukan analisis korelasi spearman.*
***Hasil**: Terdapat korelasi positif tingkat sedang antara kadar pajanan toluene dengan kadar MDA plasma (r=0.42; p=0.025). Sedangkan dengan jumlah sel spermatogonia didapatkan korelasi negatif tingkat tinggi dengan kadar pajanan (r= -0.68 ; p=0,001).*
***Kesimpulan**: Pemajanan toluena pada tikus Wistar Jantan dengan kisaran nilai ambang batas memengaruhi peningkatan kadar MDA plasma dan menurunkan sel Spermatogenia A. Akan tetapi, pemajanan toluene tidak memengaruhi kadar MDA testis tikus Wistar Jantan.*

***Kata Kunci**: Toluena, Tikus Wistar Jantan, Spermatogenia A, Malondialdehid, Rumus Abercrombie*

Introduction

Toluene (C6H5CH3, Molecular Weight 92,15), is colorless in room temperature, has a sharp and distinctive smell, volatile, and combustible. Toluene is still widely used in industry today.[1,2] Every year, 4-5 million workers were exposed to toluene. Five to ten million tons of toluene per year were used as industrial raw materials, such as in the industries of paint, plastic, ink, glue, etc.[2,3]
There is no data on the exposure of toluene in Indonesia, but Indonesia's third gross domestic product is contributed by textile and wood industries, which are known to use toluene widely.[4]

According to the Circular of the Minister of Manpower, Transmigration and Cooperatives No. SE-02/MEN/1978, which was later updated in 1997 through the Circular of the Minister of Manpower No. SE-01/MEN/1997, the Threshold Limit Value (TLV) for toluene is 50 ppm.[3]

Toluene enters the body through three mechanisms, namely inhalation, gastrointestinal, or through penetration of the skin.[4] The effects of toluene on the human body can be acute and chronic.[5-7] The acute effect of toluene is determined by the source of the substance's entry into the body. Intake of toluene through acute inhalation or ingestion can cause systemic effects such as euphoria, excitation, hallucinations, dizziness, drowsiness, balance disorders, dysphasia, tremors,

respiratory depression, arrhythmias and convulsions. Comatose and death may occur if exposure happens in large amounts.[6,7] Chronic exposure to toluene can attack almost all human organs, especially the lungs and liver. Other organs such as nerves, kidneys, testes, and bone marrow can also be affected.[8,9]

Research on the effect of toluene exposure on spermatogenesis maturation process is still a controversy.[10] A study by Atsushi, et al. in 1999 on Sprague Dawley rats found that toluene directly affected the epididymis at a dose of 6000 ppm for 5 weeks.[5] Meanwhile, other researches show that toluene exposure for 20 days with a dose of 1500 ppm for 4 hours per day against male Wistar rats did not directly affect the spermatogenesis process.[11]

This research aims to find out the effect of toluene exposure on the early phase of spermatogenesis, namely Spermatogenia A. If any disruption occurs on Spermatogenia A, then the spermatogenesis process will also be affected.

Method

The research was a laboratory experimental research on 30 male Wistar rats conducted at the Department of Community Medicine, Faculty of Medicine, University of Indonesia (FKUI). The selected Wistar rats were 3-months old rats weighing 200-250 grams. Wistar Rats were procured from The Research and Development Agency of Ministry of Health and kept in the Biology Laboratory of FKUI, as well as where its histopathological assessments were conducted.

During the rearing, three rats were placed in a cage made of wire knitting sized 50 x 30 x 18 cm^3 to ensure the comfort and space for the male Wistar Rat. Every day the rats get enough food, about 50 grams of food. The food was obtained from pet stores and consist of macronutrient and micronutrient components that complied with animal laboratory standards in the form of pellets. Drinking water was sufficient, room temperature was between 27-30^oC, and relative humidity is maintained between 50-65%. To eliminate the influence of environmental factors, placement of mice avoided direct sunlight. To assess the appropriate temperature and humidity, a needle thermohigrometer tool was used. Temperature and humidity were maintained by placing [the cage] in an area where temperature and humidity where maintained and measured hourly. For the lighting used, it was in accordance to laboratory conditions of the experimental animals.

Wistar rats were randomly divided into five groups and treated with different doses of toluene exposure (12,5 parts permillion [ppm], 25 ppm, 50 ppm, 100 ppm, and control). The length of toluene exposure given was 4 hours per day for 14 days. To maintain the levels of toluene vapor, The aquarium was made of glass to prevent the reaction between toluene and aquarium materials. Toluene spraying was carried out through an artificial gutter measuring 80x10 cm on one side of the aquarium, which was 10 cm away from the top edge of the aquarium. Through the gutter, the toluene liquid will be flowed using a 5 cc injection syringe according to the respective exposure dose. In order to ensure that the toluene level was evenly distributed, a bubbler and a fan were added in the aquarium. The air flow was made above because the density of toluene vapor is 3,18 times of the air density. The loss of air that comes out of the top of the aquarium was regulated by the bubbler speed, whereby after calculating, the air content that comes out was measured at 5 µl/sec. This situation needs to be corrected by increasing the dose so that the exposure level is stable. In this research, the dose was added every 1 hour.

After fourteen days of exposure, termination was carried out by pulling the tails to trigger the vagal reflex and then decapitation was carried out. Termination measures were carried out using the appropriate method and by paying attention to the safety and health of the officers. After decapitation, and confirmed that the rats were biologically dead, the rats were placed on the styrofoam. Afterwards, to make sure that the blood did not clot, the blood was immediately aspirated from the tail and then kept in a 3 ml EDTA tube for MDA plasma examination. The blood which had been mixed with MDA was placed in an ice flask to avoid auto-oxidation.

The next stage was surgery to take the testicular organs. Surgery was carried out from the base of the testes, then immediately cleaned from the blood using 0.9% NaCl solution and put in a pot containing 10% formalin for 12 hours for periodic acid Schiff (PAS) and tissue MDA examinations. Then the object was placed under a microscope at 400x magnification using emersion oil. Each slide was evaluated as much as 3 fields of view and each field of view was photographed with a Nikon Eclipe E 600W and Opticlab Image Raster 2.1. In each field of view, the quantity of Spermatogonia A cells was assessed according to the Abercrombie formula.[12]

The data processing of the research results was using the SPSS version 16 program[13] Assess-

ment of the data began with a normality test (Kolmogorof Smirnov test or Shapiro-Wilk test). If the data distribution was normal, Anova test would then be carried out, but if the distribution was not normal, the Kruskal Wallis test would be carried out. If the data distribution was normal, the MANOVA test would be continued, whereas if the data distribution was not normal, a linear regression test would be carried out.[14]

Result

Until the end of the exposure, there was no dead male Wistar rat, so the total sample obtained was 30 male Wistar rats. The distribution of characteristics of the five groups obtained can be seen at table 1.

Table 1. Distribution of Research Data

Variable	Group I control	Group II (12.5 ppm)	Group III (25 ppm)	Group IV (50 ppm)	Group V (100 ppm)	*p*
Chamber temperature						
Median	30 °C	30 °C	30 °C	29 °C	29 °C	<0.001kw
Minimum-maximum	29-31 °C	29-32 °C	29-32 °C	27-31 °C	27-31 °C	
Rat's weight						
Mean	240 g	237 g	237 g	239 g	244 g	0.204An
Deviation standard	8 g	6 g	4 g	6 g	4 g	
Chamber humidity (%)						
Median	65%	52%	51%	62%	57%	<0.001kw
Minimum-maximum	50-71 %	31-67 %	40-65 %	54-70 %	52-61 %	

kwKruskal Wallis test and An Anova test

The characteristics of the research showed that the temperature and humidity between groups were significantly different (p <0.001). Lowest Chamber Temperature was 27-32 oC and Chamber Humidity varies from 31-71%. For the average body weight of rats between groups were [recorded at] 237-244 g [which] did not differ significantly among groups (p = 0.204).

Plasma MDA levels, can be seen on table 2, were found to be the highest in the exposure group of 50 ppm (2,667 (0.468-5.413) nmol/l) and the lowest was found in the control group (median 0.893 (0.526-5.959) nmol/l). The lowest MDA level in the testes tissue was in the control group (0.07 (± 0.021) nmol/mg) and the highest was observed at 50 ppm (0.13 (± 0.091) nmol/mg) exposure. The lowest quantity of Spermatogenia A was in the 100 ppm exposure group (41.2 (39-46) cells) and the highest was in the control group (49.5 (44-55)).

Table 2. Average Difference Among Groups on Plasma MDA Levels, Testes MDA Levels, and the Quantity of Spermatogonia A Cell

Variable	Group I control	Group II (12.5 ppm)	Group III (25 ppm)	Group IV (50 ppm)	Group V (100 ppm)	*p*
Plasma MDA level (in nmol/l)						
Median	0.893	0.701	1.164	2.667	1.778	0.118kw
Minimum – maximum	0.526- 5.959	0.475- 0.980	0.614- 2.426	0.468- 5.413	0.875– 3.916	
Testes MDA level (in tissue nmol/mg)						
Average	0.07	0.098	0.098	0.13	0.076	0.680an
Deviation standard	0.021	0.057	0.068	0.091	0.043	
Spermatogonia A Cell quantity						
Median	49.5	49.2	48	45	41.2	0.005kw
Minimum – maximum	44-55	44-52	41-58	41-47	39-46	

kwKruskal Wallis test An Anova test

Table 3. Correlation of Independent Variable Against Dependent Value

Independent Variable	Dependent Variabel	*p*	r^{sp}
Toluene ppm level	Plasma MDA level	0.025	0.416
Toluene ppm level	Testes MDA level	0.985	-0.004
Toluene ppm level	Quantity of Spermatogonia A Cells	0.001	-0.683
Plasma MDA level	Kadar MDA Testis	0.861	-0.045
Plasma MDA level	Quantity of Spermatogonia A Cells	0.606	-0.1
Testes MDA level	Quantity of Spermatogonia A Cells	0.358	0.177

[sp] Spearman test

Table 3. shows that there is a meaningful correlation between toluene exposure levels with plasma MDA levels (p = 0.025) with a moderate level correlation (r = 0.416). There was a meaningful correlation between the rate of toluene exposure with the quantity of spermatogenia A cells (p = 0.001) with a moderate level correlation (r = .0.683). Table 4 shows the linear regression as the base for the linear equation.

Table 4. Linear Regression test between the level of toluene exposure, plasma MDA levels and tissue MDA levels with Spermatogenia A cells

Variable	B	Std. Error	Sig
Constant	51.023	1.240	<0.001
Toulene-Level	-0.087	0.020	<0.001

The linear equation for the quantity of spermatogenia A cells is as follow:

Spermatogenia A cells = -0.870 (ppm toluene) + 51.023…….

Discussion

MDA is a marker that indicates oxidative stress on cells. Toluene that enters the body will damage the integrity of the membrane which will cause oxidative stress which can be assessed from the MDA level. For toluene exposure through inhalation, [stress] levels would be found in lung and hepatocyte cells. These two cells contribute to high blood MDA levels.[15,16] This is in accordance with research conducted, that the greater the toluene exposure, the higher the blood MDA level is. This is evident from the Spearman correlation test with a value of p = 0.025 and r = 0.041 (moderate correlation). Even then, MDA levels in group V (ppm toluene level of100 ppm) had a lower mean value than group IV (ppm toluene level of 75 ppm). This condition can be caused by two factors. The first factor is because researchers cannot accurately ascertain the toluene level at the time of exposure, as there is no means of measuring toluene levels in the air in Indonesia. The second factor is the occurrence of the apoptotic process in cells.[17] The apoptotic process in cells is a programmed cell death so that it does not cause inflammatory/oxidative stress reactions in cells, this may be the factor that cause plasma MDA levels in group V to be not as high as in group IV.

The weakness of the thiobarbituric acid reactive substances (TBARS) method for examining MDA is its low specificity and TBA can react with other molecules, causing bias results. Other supporting examinations can be performed to confirm the value of MDA such as conjugated dienes, lipid hydroperoxide, and exhalation gases such as pentane and isoprotanes which were not carried out in this research.[6,16]

The levels of metabolic toluene (especially hipuric acid) circulating in the blood can affect the testes. However, the exact amount of toluene exposure dose that affects [the result] significantly cannot be observed with certainty. Ono et al in their research said that exposure to 5000 ppm for 2 hours/day, 7 days/week for 5 weeks can directly interfere with the spermatogenesis process.[18] Meanwhile, research by Lemasters et alstates that xylene and toluene exposure of >50 ppm for 30 weeks in painters has influenced the spermatogenesis process.[11]

The results of the research conducted by the authors showed that toluene exposure had influenced MDA levels. Meanwhile, the researches of Lemasters et al and Ono et al with new measures of high doses affected

MDA levels.[11,16,19] Some of the factors that made this possible were because of the deviation between temperature and humidity that should not exist. Other factors was that due to the experimental animal that might experience excessive stress during the research, such as [due to] the limited space for movement or the condition of the chamber that was made of glass. Another factor was the exposure in group V was carried out at a different time than the exposure in the other group due to the limited tools for exposure. This situation also made the difference in MDA scores in group V possible.

Testicular MDA levels in the research will be smaller than plasma MDA levels. This condition is possible because of the presence of Blood Testis Barrier (BTB), which is a protective barrier for the testis from harmful substances.[5] However, there was a pattern of increasing testicular MDA levels that is similar to plasma MDA levels.

The three variables, namely Blood MDA Levels, Testicular MDA Levels, and Spermatogonia A Cell Counts, if the correlations among groups is made, it is found that the only significant results are the quantity of Spermatogonia A cells. Several hypotheses, according to the authors, are that due to the unavailability of a toluene measuring device in the air, the tests were carried out in vivo where the response of each individual test could be different on the different toluene exposure of each group.[15,19]

In this research, the calculation of the number of Spermatogonia A cells was using the Abercrombie technique. This technique is recommended in experimental animal research to observe spermatogenesis.[12] The assessment was carried out by including the diameter of the seminiferous tubules to the spermatogenesis process. However, this research only assessed the diameter of seminiferous tubule and spermatogonia A. This is because other spermatogenesis processes such as spermatids or spermatozoa were difficult to quantify on the slide. Difficulty which occured was caused by overlapping cells, many cells are missing or unclear. According to the authors, spermatogonia A was chosen because it the cells are easy to identify and it is also the initial cell in the spermatogenesis process. Therefore, if there is any disturbance in spermatogonia A, the next sequence can be assumed to be interfered.

Conclusion

Toluene exposure in male Wistar rats increased the plasma MDA levels significantly and had moderate correlation. In addition, toluene exposure in male Wistar rats significantly decreased Spermatogenia A cells and had moderate correlation. However, toluene exposure was not significantly correlated and had no correlation with Wistar rat testicular MDA.

Conflict of Interest

All contributing authors declare no conflict of interest.

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