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Subchronic Accumulation of Arsenic (Arsenic Acid) on Sprague Dawley Rats

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Abstract: Arsenic is one of toxic metals can cause circulatory system and internal organ disturbance. The aim of these studies was to detect accumulation of *arsenic acid* after subchronic exposure, to describe histopathology alteration of the liver and brain and to evaluate blood profile for liver function in *Sprague Dawley* rats. Sixteen male rats were divided for 4 goups. *Arsenic acid* was orally and daily administrated with doses 0 mg/kg Body Weight (control); 2,5 mg/kg BW; 5,0 mg/kg BW and 10,0 mg/kg BW for 28 days. The blood samples were collected for analyzing the *aspartate amino transferase* (AST) and *alanine amino transferase* (ALT) enzymes. The samples of liver and brain were collected and analized using an *inductively coupled plasma-mass spectrometry* (ICP-MS) method. The results showed that evaluation of the liver enzymes demonstrated the significant elevation of AST and ALT levels of treated groups of rats as compare to controls. High values of inorganic arsenic were occurred in livers but lower concentration in brains with clearly histopathological lesions. Histopathological lesion of the liver indicated a mild to moderate infiltration of mononuclear cells in portal areas accompanied with degeneration and necrosis of hepatocytes. Subchronic exposure of inorganic arsenic in rats was also made inflammatory cell infiltration in hippocampus with necrosis in area.

Key words: Arsenic Acid · Rat · ICP-MS · Liver · Brain · Histopathology

INTRODUCTION

Arsenic toxicity is a global health problem affecting many millions of people. Contamination is caused by arsenic from natural geological sources leaching into aquifers, contaminating drinking water and may also occur from mining and other industrial processes. Arsenic is known to cause arsenicosis owing to its manifestation in drinking water, the most common species being arsenate [$HasO_{2-4}$; As (V)] and arsenite [H_3AsO_3; As (III)]. As III is 60 time more toxic than As V. Organic arsenic is non-toxic whereas inorganic arsenic is toxic [1]. Organic arsenicals in the pentavalent oxidation state are much less toxic than inorganic arsenicals because, unlike inorganic arsenic, these ingested organic arsenicals are not readily taken up into cells and undergo limited metabolism [2].

Experimental studies have indicate that the liver is an important site of arsenic methylation, especially following ingestion, when the absorbed arsenic initially passes through the liver [3]. Exposure of mice to arsenic in drinking water causes elevation of liver enzymes in plasma [4] and capitalization of liver sinusoidal endothelium. Liver is the major site of arsenic metabolism and hence arsenic exposure causes liver disease in both humans and animals. Arsenic has been shown to cross the placenta and studies have also shown that in utero exposure may occur. Once arsenic gains access to the neonate, however, it may cross the blood brain barrier (BBB) and directly affect the central nervous system (CNS) [5]. All forms of arsenic, including inorganic and methylated arsenicals, accumulate in many parts of the brain, with the highest accumulation in the pituitary [6].

Several methods have been widely used to detect the presence of arsenic compounds, one of which is by using inductively coupled plasma-mass spectrometry (ICP-MS). This method has a high sensitivity with a concentration of up to part-per-trillion (ppt), capable of detecting sample liquid and liquid extracts of biological and environmental samples [7].

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The number of negative effects caused by exposure to arsenic requires the government to be a regulator to set a threshold of arsenic contamination in drinking water. According to the Minister of Health regulation number 492/Menkes/Per/IV/2010 April 29, 2010 states that the maximum level of arsenic allowed in drinking water is 10 ug/L (ppb). The government regulations are applicable to a total of arsenic only, while element of inorganic arsenic is an element that is more toxic than organic arsenic. Related policy restriction at permissible levels of inorganic arsenic in drinking water is essential to ensure the safety and public health as set out in several other countries.

There have been no report of concentration of arsenic acid after sub chronic accumulation on rats animal models in Indonesia so that this study was the first study carried out. Based on the background shown above, the purposes of this research are to know how large the concentrated inorganic arsenic is after sub chronic exposure, to study the pathology changes that occurred on the internal organ and blood profile towards the liver function as a place of biotransformation on the *Sprague Dawley* experimental rat. Results of this study are expected to provide benefits such as adding information on the toxicity of inorganic arsenic that can be used by governments as a database and a reference in setting policies relating to restrictions permissible levels of inorganic arsenic in beverages consumed by people in Indonesia.

MATERIAL AND METHODS

The experiments were carried out in National Quality Control Laboratory of Drug and Food, Jakarta and Laboratory of Pathology Department of Veterinary Clinic Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, from February to April 2015.

Chemicals: Arsenic acid (H_3AsO_4) supplied by Merck Ltd. was used for the study. All other chemicals used were of analytical grade.

Animals: Male Sprague Dawley rats, aged 8-10 weeks and weighing 180±20 g were procured from the animal facility of National Quality Control Laboratory of Drug and Food, Jakarta. They were housed individually in cages under standard laboratory conditions and normal photoperiod (12 hr dark and light). Each animal was offered food pellets and tap water *ad libitum*. The animals were allowed to acclimatize to the laboratory environment for 7 days prior to the study. All animal treatments and protocols employed in this study received prior approval of the Institutional Ethical Committee of Bogor Agricultural University No. 05/RSH IPB/2015.

Experimental Design: Sixteen male rats were divided for 4 groups. Route of administration selected for the study was oral by gavage. Group I (control) animals were fed only distilled water while groups II, III and IV animals were fed arsenic acid (1 mg/ml solution from Merck Ltd.) at dose 2.5, 5.0, 10.0 mg/kg body for 28 days. Rats were anesthetized by CO_2 after 28 days.

Sample Collection: After completion of the treatment period, blood was collected through cardiac puncture before sacrifice for blood biochemical test. Liver and brain samples were removed. Small representative slices were fixed in 10% formalin for routine histopathology. A portion of the liver and brain (250–300 mg) was used for estimation of inorganic arsenic concentration.

Blood Analysis: Blood sampling carried out immediately after the animals are anesthetized. Blood was collected through cardiac puncture to the blood biochemical test including the levels of the enzyme *aspartate amino transferase* (AST) and *alanine amino transferase* (ALT). AST and ALT measurements were taken using enzymatic reaction kinetics according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Inorganic Arsenic Analysis by ICP-MS Method

Making the Test Solution: Each organ sample was finely cut and crushed then weighed quantitatively. Organ samples were put in a centrifuge and then homogenized for furthermore and incubated at 90°C for 20 minutes. After incubation, the sample solution allowed to stand at room temperature prior to further centrifuged 4000 rpm for 10 minutes. Of 0.5 mL of the supernatant was taken and then put into the column *solid phase extraction*(SPE) which had previously undergone preconditions with 3.0 mL of methanol and 3.0 mL of water of 18.2 MΩ. The next analytes were eluted using 3.0 mL of 0.5 N HCl and 2.0 mL of 18.2 MΩ water with a flow rate of 1 drop per second (as Solution A).

Raw Solution: It consists of the Arsenic acid stock solution with a concentration of 1000 mg/L. Raw solution I, it contains a number of 1.0 mL of solution that was pipetted of the Arsenic acid stock solution and put in a 100 mL volumetric flask and then diluted with 0.5 N HCl up to the mark of 100. Raw solution II, it contains a 1.0 mL solution of Raw solution I pipette and put in a 100 mL

volumetric flask and then diluted with 0.5 N HCl up to the mark of 100. Working raw solution with a concentration of each 0; 2; 4; 6; 8; 10; 12; 14 ng/L was prepared by pipette solution of raw solution II each 0; 1; 2; 3; 4; 5; 6; 7 mL, put in a 50 mL volumetric flask and then diluted with 0.5 N HCl up to the mark of 50 (as Solution B).

Blank Solution: Made similar to the test solution without organ samples (as Solution C).

Determination Procedure: Solution C, A and B were measured using ICP - MS. Levels of inorganic arsenic in the sample were calculated using the following formula:

$$\text{Level of Arsenic (ng/g)} = \frac{\text{Csp}}{\text{w}} \times V \times F$$

Csp is the level of inorganic arsenic that is obtained from the calculation using the calibration curve (ng/mL), V is the volume of the test sample solution (mL) and F is the dilution factor.

Histopathological Study: Collected morbid tissues were preserved in 10% buffered formalin. About 4-5ìm thick sections were cut and processed for histopathological examination using the standard method of dehydration in ascending grades of ethanol, clearing in xylene and stained with hematoxylin and eosin [8].

Data Analysis: The analysis of the data used in this study is the experimental design used to form a completely randomized design (CRD) to determine the effect of the internal organs of rats and dosage on the accumulation of inorganic arsenic and inorganic arsenic accumulation effect on levels of the enzyme. If the analysis indicates a significant influence, then the test is continued with a Duncan test. Data were analyzed using SPSS 22. Descriptive analysis was also carried out on pathological changes in the liver and brain.

RESULT AND DISCUSSION

Blood Analysis Results: Analysis of the *aspartate amino transferase* (AST) and *alanine amino transferase* (ALT) enzymes were used as one of the parameters of the damage to the liver. The effect of arsenic acid with different doses of the AST enzyme levels in the liver showed significant differences between the treatment groups with the control. In the treatment group, the increased AST enzyme levels are higher than the control group. The same thing happened on the measurement

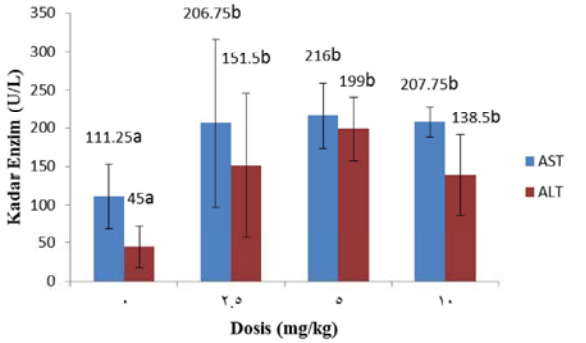

Fig. 1: The effect of arsenic acid dosage on the enzyme levels of AST and ALT.

results of the ALT enzyme levels and it was seen that there were significant differences among treatment groups with the control. Treated rats showed levels of arsenic acid treatment in which ALT enzymes were higher when compared with the control group. Results of the analysis of the enzymes AST and ALT levels in this study are presented in Figure 1.

From these results, it can be concluded that arsenic acid is capable of causing an increase in the levels of both AST and ALT enzymes in the liver which indicates the occurrence of disturbances in liver function.The increment of the activities of AST and ALT in plasma may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream [9], which gives an indication on the hepatotoxiceffect of arsenic.

The AST and ALT are two important enzymes working as an important link between carbohydrates and protein metabolism. They provide much needed keto acids for the functioning of Krebs's cycle. The activities of these aminotransferases were shown to be altered in tissues under several pathological conditions [10, 11]. Treatment of animals with toxic agents is known to produce pathological lesions being associated with increased proteolysis[12].

Organic Arsenic Analysis Results using the ICP-MS Method: In this study, Sprague Dawley rats' internal organs that were taken for measurement of the concentration of arsenic acid using inductively coupled plasma-mass spectrometry (ICP-MS) is the liver and brain. The statistical analysis showed that the liver and the brain influenced the accumulation of inorganic arsenic. Rats given the treatment showed arsenic acid accumulation in the brain, but the accumulation of bigger heavy metals were found in the liver (Figure2A). This is in line with the results of studies in rats that were given an acute inorganic arsenic[13] and in mice with chronic administration [14].

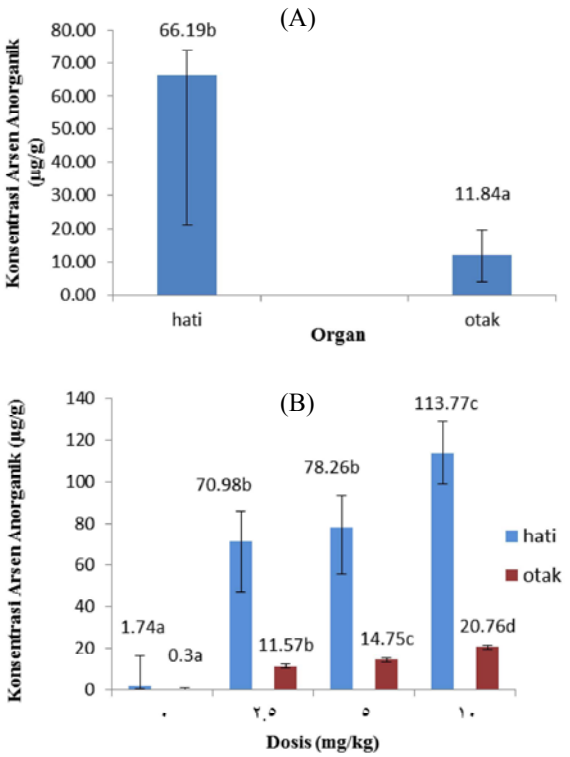

Fig. 2: Concentration of organic arsenic in the heart and brain (A), The impact of organ and dosage towards the concentrated organic arsenic (B).

Data in Figure 2B show the average concentration of arsenic acid in the highest liver at doses of 10 mg/kg of body weight. The dose of 2.5 and 5 mg/kg for 28 days did not differ significantly, but the third dose of the treatment when compared with the control group showed significant difference. These results indicated that the administration of arsenic acid for 28 days, dose of 2.5 to 10 mg/kg body weight in rats responded in the increasing accumulation in the liver.

The liver is an important organ in a variety of metabolic processes and the effect of the inclusion of chemicals and xenobiotics into the body will be detected first in the liver. The process of arsenic metabolism occurs in the liver which is the main place for methylation of arsenic in the body [15]. A study of inorganic arsenic with a single administration [16]and advanced research that is repeated administration sub chronic of inorganic arsenic in drinking water at a dose of 0.014 to 1.4 mg/L in mice[17], suggesting that the capacity of the arsenic methylation and excretion did not differ significantly.

Statistical analysis showed brain accumulation of arsenic acid that were significantly different at a dose of 2.5 to 10 mg/kg of body weight when compared with the control group. The mean dose of the highest dosage of 10; 5; 2.5 and lowest at doses of 0 mg/kg body weight respectively (Figure 2B). This study shows that arsenic acid is able to penetrate the *blood brain barrier* and accumulates in the brains of rats. Lipophilic action of the organic metal easily penetrates the blood brain barrier, as well as inorganic metal will be able to reach the brain tissue. Toxic effects of inorganic arsenic, both acute and chronic involve multiple organ systems including the central nervous system [18].

Research found a marked reduction in cognitive function with decreased intelligence, verbal coefficient and learning and memory disorders associated with the occurrence of chronic exposure to heavy metals such as arsenic [19]. Cognitive disorders and nervous system depend on the amount of concentration, time and duration of exposure to arsenic [20]. The heavy metals arsenic has potential to be a teratogen that can penetrate the placenta during the development phase with high concentrations, which causes impaired growth and nervous system defects [21].

Histopathological Lesions: Description of lesions was found in the liver with HE staining which included control groups that were generally hepatocytes with no noticeable changes, there was little congestion that commonly occurred during the process of euthanasia. A dose of 2.5 mg/kg showed a change began to occur in the portal area with infiltration of inflammatory cells and some cells degeneration. A dose of 5 mg/kg noticeable changed almost equal to a dose of 2.5 mg/kg, but the area that was experiencing degeneration occurring was characterized by the expansion of the nucleus that is smaller and lost, in the portal area of ??edema accompanied by infiltration of inflammatory cells. At a dose of 10 mg/kg of apparent damage to their widespread, degeneration and necrosis with infiltration of inflammatory cells are more and more (Figure 3).

Hepatic necrosis may be due to oxystress induced by arsenic that further involved in the cellular protein degradation. The sinusoidal space were expanded due to shrinkage and necrosis of hepatic cells because arsenic increases the permeability from which infiltration of cellular material take place [22]. Mononuclear infiltration in the portal area in association with biliary hyperplasia could be due to production of IL-33, IL-1α and production of intra and extracellular damage associated molecules from the necrotic cells in the liver those are recognized by the macrophages and generated the cytokines for the recruitment and removal of the necrotic cells from the liver [23].

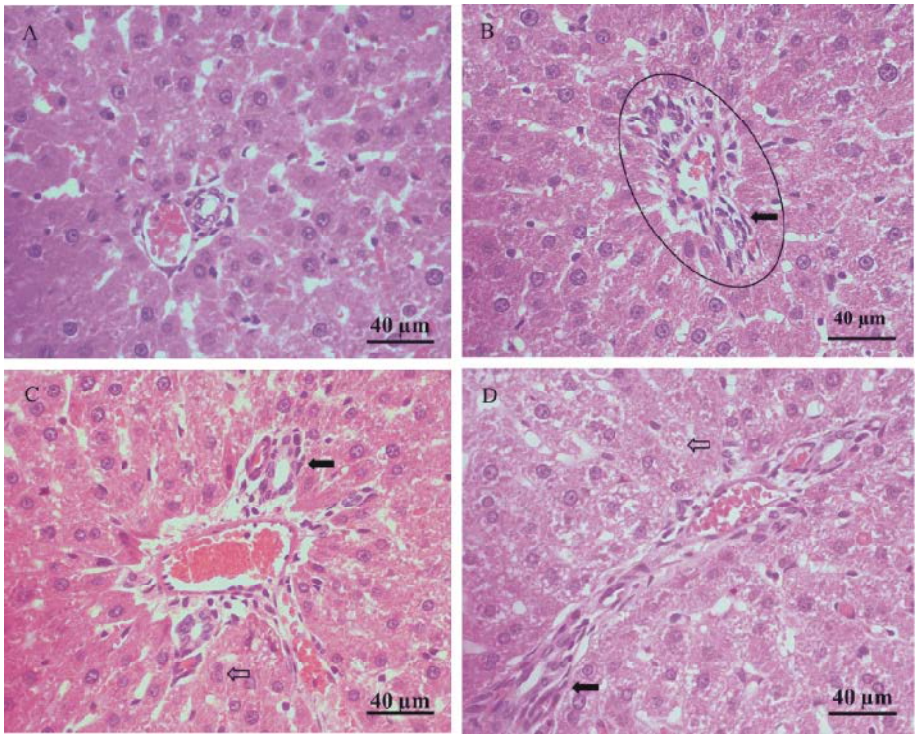

Fig. 3: Histopathological liver lesions. (A) control; (B) dose of 2,5 mg/kg body weight; (C) dose of 5 mg/kg body weight; (D) dose of 10 mg/kg body weight. Visible presence of inflammatory cell infiltration (⬅) in the portal area and hepatocytes cells undergo necrosis (⇦). HE staining.

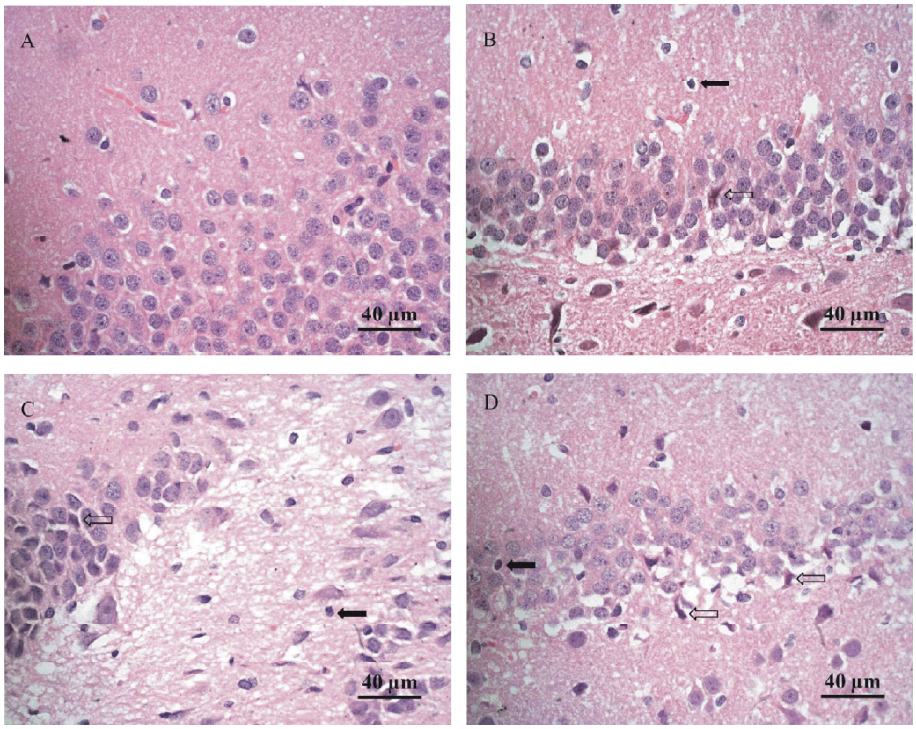

Fig. 4: Histopathological brain lesions. (A) control; (B) dose of 2,5 mg/kg body weight; (C) dose of 5 mg/kg body weight; (D) dose of 10 mg/kg body weight. Visible presence of inflammatory cell infiltration (⬅) and neuron cells undergo necrosis (⇦). HE staining.

In the brain, the area that was evaluated was in the hippocampus because in this area, it is very important in the process of receiving information, a new memory storage and disclosure of long memory [24]. Histopathological lesions that occur in the brain is presented in Figure 4. The changes seen among others; for the control group in general, there were no changes. At a dose of 2.5 mg/kg, the infiltration of inflammatory cells was seen. Doses of 5 and 10 mg/kg were an apparent necrosis of neuron cells that were quite a lot, nearly 40 percent of visible death of neurons, allegedly as a result of arsenic acid that can penetrate the blood brain barrier and accumulate in the brain. Tissue damages were seen in the HE staining, which in line with the results of the analysis using ICP - MS which showed that the average concentration of inorganic arsenic were higher with increasing dose.

Once arsenic gains access to the neonate, however, it may cross the blood brain barrier (BBB) and directly affect the central nervous system (CNS). The BBB is a structure composed of tight junctions between capillary endothelial cells in the brain and epithelial cells in the choroid plexus specialized to prevent proteins and smaller molecules from mixing with the cerebrospinal fluid[25]. Reports on developmental exposures to heavy metal mixtures suggest that combined exposure is associated with greater risk for cognitive dysfunction, including behavior and impaired neurological (CNS) development[26]. Arsenic exposure in rats resulted in individual shrunken cells with condensed cytoplasm and nucleus in the hippocampus; mitochondrial changes were noted, edema was seen around capillaries and there were decreased synaptic vesicles in the synapses. All of these effects were dose-dependent [27].

CONCLUSION

Exposure to inorganic arsenic (arsenic acid) in rats for 28 in the study is able to reduce the function of the liver characterized by an increase in the value of the enzyme aspartate amino transferase (AST) and alanine amino transferase (ALT). Inorganic arsenic accumulation in the liver is higher than in the brain. Histopathological lesions in the liver show infiltration of mononuclear cells in the portal area with degeneration and necrosis of hepatocytes. Changes in the hippocampus is characterized by infiltration of inflammatory cells and necrosis of neuronal cells.

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