In Vitro Diabetogenic Effect of Cadmium on Liver

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ABSTRACT
The objectives of this study were to determine the effect of cadmium (Cd) on glucose metabolism disruption in liver cells homogenate in vitro. The glucose metabolism disruption was analyzed by measuring the level of liver glucose, glycogen and methylglyoxal (MG), and the activity of glucokinase activity. In this experiment, a liver sample was taken from male rats (Rattus norvegicus). Samples then homogenized and divided into four groups with; C served as control which contains liver homogenate only; T1 which contains liver homogenate + 0.03 mg/l of cadmium sulphate (CdSO₄); T2 which contains liver homogenate + 0.3 mg/l of CdSO₄; and T3 which contains liver homogenate + 3 mg/l of CdSO₄. After treatment, liver glucose, glycogen, and MG levels, and glucokinase activity were estimated. The activity of liver glucokinase was estimated by measuring the Michaelis-Menten constant (KM) value. The results revealed that Cd exposure could significantly increase glucose and MG levels, the KM value of glucokinase, and decreased the glycogen level in liver cells (P<0.05). These results indicated that Cd exposure induced the disruption of glucose metabolism in the liver.

Keywords: Cadmium, Glucose, Glucose Metabolism, Liver.

INTRODUCTION
Cadmium (Cd) is a widely but sparsely distributed element found in the earth’s crust and is primarily association with zinc ore. Cd is a common product of processing zinc-bearing ore¹-². It has long been recognized as one of the most toxic elements because its tend to have a slow elimination rate and its half-life in the human body ranges between 10–30 years³-⁵. For most people, diet is a primary exposure source. For a large portion of the general population, tobacco smoke is a secondary source. Cd from dietary and smoking exposures can accumulate in various organs and tissues, but the most extensive accumulation occurs in the kidney for chronic exposure and liver for acute exposure⁶-⁷.

Recently, there is growing evidence that Cd exposure may be related to the increasing risk of diabetes mellitus and its complications. Diabetogenic effects of Cd have been demonstrated in experimental studies. A growing body of evidence from population-based studies suggests an association between body burden of Cd and type 2 diabetes⁸. Some experimental studies indicated that the relationship of Cd and diabetes may be caused the effect of Cd to several organs that was involved in glucose metabolism, including liver⁹.

The liver is an important organ performing vital functions including biotransformation, migration of lipids, glycogen storage and release of glucose into the blood¹⁰. The liver may be exposed to large concentrations of exogenous substances and their metabolites. One of the exogenous substances that are harmful to the liver is heavy metal, such as Cd¹¹. Several previous studies in experimental animals and in vitro models have shown that Cd have a toxic effect on liver cells¹²-¹⁴. This might be one of the reasons why Cd could alter the glucose metabolism. Considering liver is one of the central organs in glucose metabolism, study of the Cd effect on several glucose metabolism parameters in this organ is important. Therefore, the present the study has been designed to investigate the effect of Cd on several glucose metabolism parameters in the liver in vitro.

MATERIAL AND METHODS
Samples collection
The liver samples were collected from 24 old male rats (Rattus norvegicus) with 2-3-month-old and weighing 200-250 g. The rats were purchased from the Abadi Jaya farm at Yogyakarta, Indonesia, in healthy condition. Animals were the acclimatized to the laboratory conditions before samples collection. The rats were caged in a quite temperature controlled room and had free access to water and standard rat diet. After the acclimatization period, the liver samples were taken by surgical procedure with ether as an anesthesia. Then the liver fixed in phosphate buffer at pH 7.0. The liver was ground to form a liquid. Subsequently, the solution was taken and centrifuged at 3500 rpm for 10 min and the top layer was taken and stored until it uses. All animals used and care was in compliance with the Ethics Commission of the Faculty of Medicine,

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**Experimental models**

Homogenate samples were divided into 4 groups (1 control group and 3 treatment groups). Control (T0) group: liver homogenate only; Treatment 1 (T1) group: liver homogenate + 0.03 mg/l of Cd-sulphate (CdSO₄); Treatment 2 (T2) group: liver homogenate + 0.3 mg/l of CdSO₄; Treatment 3 (T3) group: liver homogenate + 3 mg/l of CdSO₄. Each solution then incubated at 37°C for 1 hour. After incubation, liver glucose, glycogen, and methylglyoxal (MG) levels, and glucokinase activity were estimated. In addition, all experimental models and measurement were done in Medical Chemical/Biochemical Laboratory, Faculty of Medicine, Lambung Mangkurat University, Banjarbaru, South Kalimantan, Indonesia.

**Estimation of liver glucokinase activity**

Liver tissues were homogenized in 50% Trichloroacetic Acid (TCA), keeping the proportion of 100 mg per 1.0 ml of TCA. After centrifuging for 5 min at 5000 rpm, the contents of glucose were determined in the supernatant. Homogenate samples were submitted to the same procedure, keeping the same proportions (100 μl of homogenate/1.0 ml TCA). Glucose was determined by Dubois hydrolytic method. It consists of a suitable aliquot of glucose into a final volume of 0.5 ml added of 0.7 ml of 3% phenol. After shaking, 2 ml of 0.5 ml of concentrated sulfuric acid (H₂SO₄) was added into one stroke developing strong heat of reaction. The product was determined at 540 nm in a single colorimeter.

**Estimation of liver methylglyoxal concentration**

This assay was performed as described by Bidinatto et al. Samples of liver were quickly separated from freeze tissues and transferred to tubes containing 1.0 ml of 6 mol/l potassium hydroxide (KOH). The tubes were transferred to a boiling water bath and left along 3-5 min for complete dissolution. Aliquots of the resultant solution (250 μl) were added to 3 ml of 95% ethanol-water and after mixing, 100 μl of 10% potassium sulfate (K₂SO₄) was appended. A cloudy white precipitate was formed and the supernatant was discharged after centrifuging at 3000 rpm for 3 min. It was added 2.5 ml of distilled water to the precipitate, which was promptly dissolved. Suitable aliquots of such solution were employed to Dubois reaction. Glycogen concentration is expressed in μmol of glucosil-glucose per g of wet tissue.

**Estimation of liver glucokinase activity**

Glucokinase activity was measured using a method which previously described by Bustos and Iglesias. Glucose with several concentrations (100 mM, 200 mM, 300 mM, 400 mM and 500 mM) were taken 3 ml and added 3 ml of phosphate buffer pH 7 in each solution. Furthermore, mixed the solutions until homogeneous. A total of 1 ml homogenate are added to each solution, and then measured the levels of glucose [G₀]. After 20 minutes, each mixture of glucose is measured again [G₁] by the method of hydrolytic Duboie's. The rate of oxidation of glucose by glucokinase (v) is expressed in changes in the concentration of glucose per minute.

Glucokinase activity is expressed by measuring Michaelis-Menten constant (Kₘ) which calculated by creating a linear graph between 1/[G] with 1/v. From the linear graphs, a straight line equation with Kₘ/V_max as slope and 1/V_max as an intercept was obtained. The estimation of liver methylglyoxal concentration

MG compounds are measured using modified Dinitro-Phenyl hydrazine (DNPH) method. From each test solution, 0.5 ml solution was taken, and then each solution was divided into 2 tubes with 0.25 ml volume in each tube. The first tube was the sample (A) and the second tube was a blank (B) solution. Then 1 ml DNPH were added into each A tube and 1 ml HCl 2.5 mol/l into each B tube. The tubes were incubated for 45 min at room temperature and protected from light, and then tubes were shaken with a vortex for 15 min. The next step is added 1 ml of TCA 20% into each tube (A and B), then the tubes were incubated for 5 min. Tubes were centrifuged for 5 min at 1400 rpm of speed to separate the supernatant. The pellets are centrifuged and washed three times with the addition of 1 ml ethanol-ethyl acetate. The last step was added 1 ml of urea 9 mol/l and incubates the solution for 10 min in 37°C while it was shaken. The solution was centrifuged again for 5 min at 1400 rpm of speed. Then the absorbance of tube A and B were measured at λ = 390 nm (ΔA).

Furthermore, a total of 25 μl of the homogenate was added to 350 μl DNPH (0.1% DNPH in 2 mol/l HCl) and then 2.125 ml distilled water was added. It is incubated for 15 min at 37°C, then 1.5 ml NaOH 10% was added. Absorbance was measured at λ = 576 nm (A₁).

MG level was calculated following to equation: MG level (%) = (A₁ - ΔA) × 100%

**Statistical evaluation**

The results were expressed as mean ±SE for three replicates. The significance of mean differences of glucose, glycogen, and MG levels between treatment and control groups were statistically compared using one-way Analysis of Variance (ANOVA) or Kruskal-Wallis test and followed by a post hoc Tukey’s Honestly Significant Difference (HSD) or Mann-Whitney test for multiple range test. Significance was set at P<0.05. For the glucokinase activity, the comparison was made by the descriptive statistics. The software used for the data analysis were the Statistical Package for the Social Sciences (SPSS) version 16.0 and Microsoft Excell 2010 for Windows Vista.

**RESULTS**

In this present study, the level of liver glucose with the presence of Cd in different concentrations was investigated. The result shows in figure 1. Cd exposure caused the distortion of the liver glucose levels. A liver concentrations of glucose were significantly higher in all group of treatments compare to control (ANOVA test, p<0.05). Post-Hoc Tukey HSD test results show that there are significant differences between all group of treatments (table 1).
The liver glycogen level that received different doses of Cd and the controls are presented in the Figures 2. After the administration Cd, the liver glycogen level seems to be decreased compared to the controls (Figure 2). There was a dose-dependent decrease in glycogen level in the liver tissues. The statistical analysis test results show that the decreasing of MPO activity was statistically significant (ANOVA test, P<0.05). Post-Hoc Tukey HSD test results show that there are significant differences between all group of treatments (table 1).

The effect of different dose of Cd on liver MG level is presented in figure 3. It was observed that the MG level is increased in all Cd treatment groups as compared to control group. The increase in MG level was in a dose-dependent manner. ANOVA test results show that there is a significance difference in liver MG level between the group of treatments (p<0.05). Post-Hoc Tukey HSD test shows that there are significant differences between group of treatments except between T1 and T3 (table 1).

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and a latter one due to inflammation and oxidative stress\textsuperscript{22-23}. Thus, will disrupt the level of liver glucose and glycogen.

The result of this present study also indicated that the disruption of liver glucose and glycogen level may be caused by the effect of Cd on interference the enzyme that has a role in glucose metabolism in liver such as glucokinase. It can be seen from the result of this study that the Km value of glucokinase was increased by the increasing of Cd concentration in liver cells homogenate. From this point of view, it can be said that Cd exposure could increase the affinity between glucose-glucokinase complex\textsuperscript{24}. It means the binding between glucose and glucokinase might be weak by the presence of Cd. This condition will increase the concentration of glucose as a substrate and reduce the activity of glucokinase through a negative feedback mechanism. This condition can also lead to the breaking of glycogen to glucose that will decrease glycogen levels in the liver.

The interference effect of Cd on glucokinase is caused by covalent bond to -SH group of glucokinase. Glucokinase is an intracellular enzyme that plays a role in the conversion of glucose to glucose-6-phosphate\textsuperscript{25}. Glucokinase known to have -SH group on the amino acid cysteine 213, 220, 230, 233, 364, 371, and 382-385. Cd could be expected to bind to the -SH group of those amino acid cysteine in glucokinase\textsuperscript{28}.

The result of this present study also clearly demonstrates that Cd exposure could increase the MG level. The increasing of liver MG level may be caused by the increasing of glucose level in liver cells homogenate. It is well known that High MG levels are thought to be due to excess blood sugar\textsuperscript{29}. MGO is a highly reactive dicarbonyl and a precursor to free radicals and advanced glycation end products (AGEs). It is formed from the spontaneous dephosphorylation of triose phosphates during glycolysis, the spontaneous fragmentation of a Schiff base during the
Maillard reaction, and from ketone and threonine metabolism\textsuperscript{26}. Interesting results are seen from the comparison of MG levels between Cd treatment with dose 0.03 µg/dl and 3 µg/l. The most likely reason why MG levels are not significant in this comparison is caused by the degree of liver cells damaged. In greater concentration (3 µg/l of CdSO\textsubscript{4}) the degree of liver cell damage will also be getting greater. Liver cells damaged might be inhibited the glycolysis, and will decrease the MG level. This result is supported by Suhartono et al.\textsuperscript{11} results. The result of these study indicated that 3 mg/l of CdSO\textsubscript{4} could significantly decrease MG level in liver cells homogenate compared to control.

In conclusion, the present study demonstrated that Cd-induced glucose metabolism disruption as can be seen from the level of liver glucose, glycogen, and MG, and glucokinase activity.

**CONFLICT OF INTEREST**
We declare that we have no conflict of interest.

**REFERENCES**

Table 1: Comparison and Statistical analysis test results of liver glucose\textsuperscript{a}, glycogen\textsuperscript{a}, and MG\textsuperscript{b} levels in the different group of treatments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group of Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.944\textsuperscript{c,d,e}</td>
</tr>
<tr>
<td>Glycogen</td>
<td>136.222\textsuperscript{c,d,e}</td>
</tr>
<tr>
<td>MG</td>
<td>16.195\textsuperscript{c,d,e}</td>
</tr>
</tbody>
</table>

MG: methylglyoxal; C: control group; T1: treatment with 0.03 µg/dl; T2: treatment with 300 µg/dl; T3: treatment with 3000 µg/dl.

\textsuperscript{a} Indicates p-value when compared between C group.

\textsuperscript{b} Indicates p-value when compared between T1 group.

\textsuperscript{c} Indicates p-value when compared between T1 and T2 group.

\textsuperscript{d} Indicates p-value when compared between T2 group.

\textsuperscript{e} Indicates p-value when compared between T3 group.

\textsuperscript{p}-Values were calculated using the One Way ANOVA or Kruskal–Wallis test and followed by Post Hoc Tukey HSD or Mann-Whitney test; \textsuperscript{p} < 0.05 was considered statistically significant.


