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Changes in the Concentration and Localization of Accumulated Mercury in Kidney, Liver, and Spleen of Mice over Time

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This study investigated the localization and changes in the concentration of injected mercury in the kidney, liver, and spleen of mice. To evaluate changes in the concentration of mercury over time, the mice were euthanized 10, 150, and 300 days post-treatment. Localization of accumulated mercury was identified by the autometallography method. Mercury was densely located in the supranuclear cytoplasm of epithelial cells of proximal tubules of the kidney but was not detected in the glomerulus 10 days post-treatment. In the liver, mercury was mainly found in hepatocytes around the portal vein and in sinusoidal Kupffer cells 10 days post-treatment. Mercury was scattered throughout both white and red pulp of the spleen 10 days post-treatment. In terms of changes in the concentration of mercury, the levels were lower in the renal cortex and medulla 150 and 300 days post-treatment as compared with those 10 days post-treatment. Mercury was found at low concentrations in liver hepatocytes 150 and 300 days post-treatment. The mercury concentration was also low in both the white and red pulp of the spleen 150 and 300 days post-treatment. Therefore, the concentrations of accumulated mercury in the kidney, liver, and spleen 150 and 300 days post-treatment were lower than those 10 days post-treatment. We identified the localization of mercury in cells and tissues of several organs and observed that accumulated mercury in organs decreased naturally over time.

Key words : Autometallography, kidney, liver, mercury, spleen

Introduction

Mercury is a well-known toxic metal that tends to be released into the environment via various industrial activities. Reports have suggested that people can be exposed to mercury through whitening creams applied to the skin [3], amalgam fillings [5, 33] used as dental materials, and by the ingestion of certain types of fish [23, 37]. In humans, the main route of exposure to methylmercury is through ingestion of certain types of seafood and freshwater fish [12].

The major organs affected by mercury toxicity in mammals are the kidneys and the nervous system [15]. Mercury is also an environmental contaminant that has toxic effects on the immune system and the lungs [26]. Mercury can induce various metabolic changes, particularly in the nervous system, kidneys, and liver [28]. It has been reported that high concentrations of mercury can accumulate in the liver, kidney, and brain [1]. Moreover, the concentration of mercury in the kidney has been reported to be up to five times higher than that in the liver [6]. It has also been observed that administration of mercury can bring about changes in the epithelial cells constituting the tubules in the kidneys in mice [32], along with changes in the hepatocytes in the liver [26]. In rats, various changes caused by mercury were observed in the kidneys [27, 32, 36] and liver [36]. In the spleen, although it was seen that the accumulation of mercury differs according to the mouse strain, the concentration of mercury accumulated in the spleen increased in proportion from the time of mercury administration [24]. As a result, the lymphocytes of the spleen have been reported to decrease in response to the accumulation of mercury [30].

In this study, we administered compounds containing mercury into pubertal mice once a week for a total of 3 weeks, after which the administration was stopped and the levels and the localization of mercury in the kidney, liver, and spleen were measured at various time points by autometallography [16, 17].

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Materials and Methods

Experimental animals, mercury administration and perfusion

Six-week-old pubertal female mice (n=36) belonging to the ICR (Damul Sciences) strain were used for this experiment. Adolescent mice were established according to the criteria reported by Emanuele et al [20]. The average weight of the mice was 24 g. During the experimental period, solid feed and distilled water were supplied ad libitum. In addition, the environment of the feeding room was maintained at a temperature of 22±2℃, with a relative humidity of 50±5%, and an alternating light-dark cycle of 12 hr each. The experiment was conducted with the approval of the Ethical Committee of Animal Experimentation at Sunchon National University (Approval No. SCNU IACUC-2014-05).

For the administration of mercury into the mice, 0.01 mg of methylmercuric chloride (CH_3HgCl) was dissolved in 0.1 ml of physiological saline solution, then subcutaneously injected into the back of the neck of the mice for 3 weeks at a dose of 0.01 mg of mercury per week. For the control group, 0.1 ml of physiological saline was injected subcutaneously in the same manner.

The mice in the control and the mercury-treated groups were randomly divided into three groups after receiving the mercury doses for 3 weeks and were killed at 10, 150, and 300 days post treatment. After the euthanasia, 12 mice were used in the control (n=6) and the mercury treated (n=6) groups. Before perfusion, heparin was administered into each mouse intraperitoneally, followed by inhalation anesthesia with diethyl ether. The anesthetized mice were then weighed and perfused. For perfusion, the needle was inserted into the left ventricle, and the animals were perfused with a peristaltic pump (Spectra/Chrom MP-1) for 2 min using Ringer's solution. This was subsequently replaced with 4% glutaraldehyde (pH 7.4) solution and perfused for 20 min.

Tissue preparation and mercury staining

The kidneys, livers, and spleens were excised from the perfused mouse, placed on filter paper to remove the water, and then weighed and averaged. A p value of <0.05 was considered statistically significant in terms of organ weight. Each organ was weighed and subsequently stored in 4% glutaraldehyde (pH 7.4) at 4℃ until further use. The tissues of these organs were subsequently trimmed to the appropriate size, rinsed with 0.1 M phosphate buffer (pH 7.4), dehydrated with 70%(twice), 80%(once), 95%(twice) and 100%(thrice) ethanol at 1 hr intervals, followed by infiltration solution treatment, and then embedded in JB-4 solution (Polysciences, Inc.).

After the embedding process, the tissues were sectioned at a thickness of 2.5 μm using a microtome (RMC Rotary Microtome, MT 990) and stained using an autometallography-based method [16, 17]. Briefly, tissue sections were treated with distilled water for 10 min and developed for 2 hr at 26℃. The samples were then processed for 55 min in distilled water, 12 min in 5% sodium thiosulfate, 10 min in distilled water, and 5 s in 0.5% toluidine blue at room temperature. Next, the sections were washed with distilled water for 10 min, dried in a slide warmer at 35℃ for about 1 hr, sealed with permount (Fisher Chemical), and observed under an optical microscope.

Measurement of mercury concentration

A SPOT camera (Model No. 11.2 Color Mosaic) attached to an optical microscope (Olympus BX 50) was used to examine the localization of the accumulated mercury and the changes in the mercury concentration in the kidney, liver, and spleen. To measure the concentration ratio of the accumulated mercury, the color range was adjusted so that the area with the accumulated mercury could be seen clearly using the color range designation module of the Adobe Photoshop CS 5 program, after which the pixel value of the area with accumulated mercury was obtained. For calculating the ratio, the pixel value was divided by the total pixel value of the photograph, then multiplied by 100 to obtain the ratio of the accumulated mercury in the tissue.

Statistical analysis

Statistical differences between the control and the mercury treated groups were analyzed via one-way ANOVA using Microsoft Office Excel, and a differences with a p value of <0.05 were considered to be statistically significant.

Results

Localization of mercury compound in organs

Compared to the control group, the mean weights of the kidneys, livers, and spleens in mice that were euthanized 10~150 days post treatment, were found to be similar or increased in the mercury-treated group. When the mice were euthanized at 300 days, the weight of each organ was found to be lower in all the mercury-treated mice than in the control mice (Table 1). Microscopic observations of the renal tissue showed that the mercury accumulation was in the form

Table 1. The average organ weight of the control and mercury-treated mice taken each day post treatment

Organ	Group	Day post treatment		
		10	150	300
Kidney (g)	Control	0.25±0.01	0.27±0.05	0.32±0.03
	Mercury	0.29±0.02*	0.28±0.04	0.25±0.01*
Liver (g)	Control	1.82±0.18	2.33±0.73	2.61±0.66
	Mercury	2.58±0.23*	2.54±0.62	2.01±0.33
Spleen (g)	Control	0.14±0.02	0.15±0.05	0.18±0.06
	Mercury	0.22±0.05*	0.15±0.06	0.12±0.02

Values represent the means ± SD.
For obtaining the organ weights each day post treatment, 12 mice were used as control (n=6) and as mercury treated (n=6) specimens.
*p<0.05 compared with the control group of the same day post treatment.

of granules, mainly in the epithelial cells constituting the proximal convoluted tubule in the cortical area at 10 days post treatment (Fig. 1). In the epithelial cells, mercury was located in the cytoplasm near the nucleus or in the supra-nuclear cytoplasm, rather than in the basal lamina (Fig. 1B). Mercury was also accumulated in the epithelial cells of the distal convoluted tubule albeit in relatively small amounts as compared to that in the proximal convoluted tubule. We did not find any mercury accumulation in the glomerulus.

When the mice were euthanized at 150 days post treatment, the glomeruli were found to be atrophied. Additionally, we observed that the mercury granules were mainly accumulated in the free surface of proximal tubular epithelium (Fig. 1C). We also found mercury granules in the cytoplasm of the distal tubule epithelial cells, as well as in the epithelial cells constituting the collecting ducts and Henle's loop tubules in the medulla region (Fig. 2). When the mice were euthanized after 300 days of treatment, a small amount of mercury was found to be accumulated in the epithelial cells

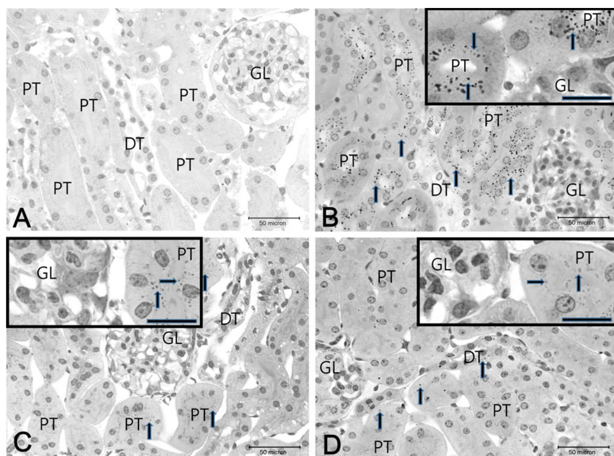

Fig. 1. Microscopic photographs of the renal cortex region before and after mercury treatment. Images of the renal cortex of the control mice at (A) 10 days, and mercury-treated mice at 10 (B), 150 (C) and 300 (D) days post treatment. Mercury granules (indicated by arrows) were found to be accumulated in the epithelial cells of the proximal tubule. The accumulated mercury (indicated by arrows) was mainly distributed around the nucleus of the proximal tubule epithelium, or from the nucleus to the free surface of the cell (B, inset). As shown in the enlarged magnification, mercury was distributed on the free surfaces of proximal tubular epithelial cells, which was mainly in contact the lumen (C and D, inset). DT, distal tubule; GL, glomerulus; PT, proximal tubule. Bars = 50 µm in A-D and 20 µm in B-D insets.

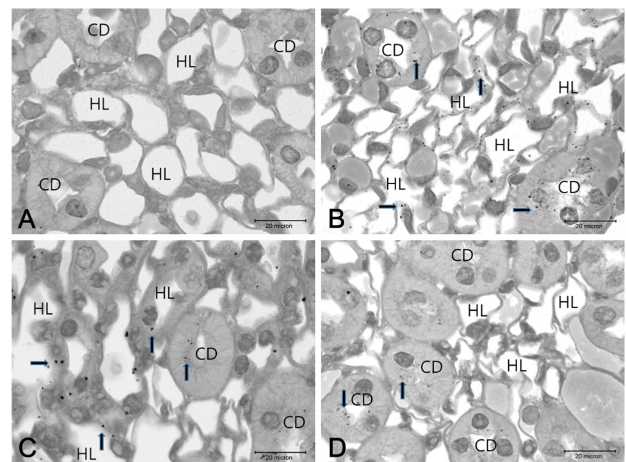

Fig. 2. Microscopic photographs of the renal medulla region before and after mercury treatment. Images showing the renal medulla in the control mice at (A) 10 days post treatment, and mercury treated groups at (B) 10, (C) 150 and (D) 300 days post treatment. The accumulated mercury (indicated by the arrows) was seen in granular form in the collecting duct and Henle's loop epithelial cells. The amount of mercury was decreased at 150 days, and there was very little or no mercury observed at 300 days post treatment. CD, collecting duct; HL, Henle's loop. Bars = 20 µm in A-D.

of the proximal and distal convoluted tubular portion of the kidney cortex (Fig. 1D). In case of the renal medulla (Fig. 2), although we found accumulated mercury in the epithelium constituting the Henle's loop and collecting duct, the amount was much lesser than that observed in the proximal convoluted tubule (Fig. 2B). The epithelial cells of the collecting ducts and Henle's loop tubules in the medulla region contained very little or no mercury at 150 and 300 days post treatment (Fig. 2C, Fig. 2D).

In the liver, mercury was mainly distributed in the form of granules in the hepatocytes around the portal vein at 10 days post treatment (Fig. 3). In addition, mercury was accumulated in the hepatocytes along the hepatocyte plate from the portal vein to the central vein and the mercury deposition was observed to be linear (Fig. 3B). The mercury accumulation in the hepatocytes decreased toward the central vein as the hepatocytes surrounding the central vein showed little or no mercury accumulation. Mercury was also found to be accumulated in the Kupffer cells in the sinusoid. When mice were euthanized at 150 days post treatment, there was either no accumulation of mercury or very low concentrations of mercury were observed in the hepatocytes.

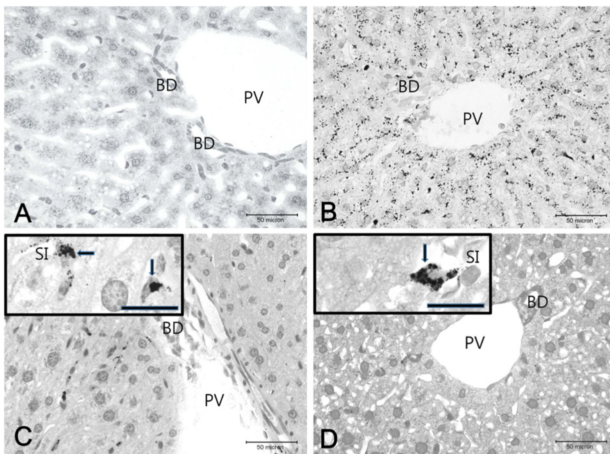

Fig. 3. Microscopic photographs of the liver before and after mercury treatment. Images of the livers in the control mice at (A) 10 days, and mercury treated groups at (B) 10, (C) 150 and (D) 300 days post treatment. The mercury was distributed in the form of granules in hepatocytes around the portal vein and bile ductile at 10 days post treatment. A small amount of mercury was found to be accumulated in the hepatocytes, but many mercury granules (arrows) were still distributed in Kupffer cells in the sinusoid at 150 and 300 days post treatment (C and D, inset). BD, bile ductile; PV, portal vein; SI, sinusoid. Bars = 50 μm in A-D, and 20 μm in C and D, inset.

However, Kupffer cells in the sinusoid contained mercury granules (Fig. 3C). When the mice were euthanized at 300 days post treatment it was revealed that though mercury accumulation did not appear in the hepatocytes, but it was still observed in Kupffer cells in sinusoid (Fig. 3D). In addition, vacuoles were observed in the hepatocytes.

In the spleens of (Fig. 4) mice that were euthanized at 10 days post treatment, there was relatively higher amount of accumulated mercury in the white pulp than in the red pulp (Fig. 4B). Compared to the granular form of mercury observed in the liver and kidney, the mercury granules in the spleen were observed in the form of aggregations. When the mice were euthanized at 150 days post treatment, the distribution of black color in the white and red pulp portions of the spleen clearly indicated the aggregation of mercury granules (Fig. 4C). The bulk of the mercury was found to be mainly accumulated in the red pulp of the spleen at 300 days post treatment (Fig. 4D).

The concentration of accumulated mercury in the organs

The amount of mercury accumulated in the tubular epithelial cells of renal cortex gradually decreased at 150 and

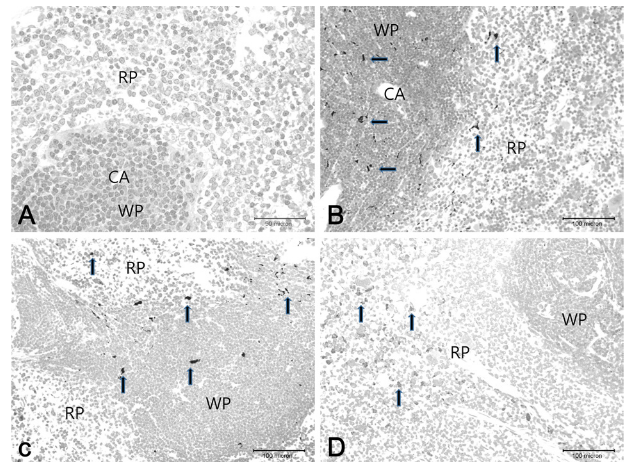

Fig. 4. Microscopic photographs of the spleen before and after mercury treatment. Images showing the spleen in control mice at (A) 10 days, and mercury treated groups at (B) 10, (C) 150 and (D) 300 days post treatment. The mercury grains (arrows) as in the form of agglomerations were mainly distributed in the white pulp around the central arteriole and red pulp at 10 days post treatment. The amount of mercury accumulated in white pulp and red pulp was decreased at 150 and 300 days post treatment. CA, central arteriole; RP, red pulp; WP, white pulp. Bars = 50 μm in A and 100 μm in B - D.

300 days post treatment compared to the levels observed at 10 days post treatment. The concentration of mercury in the renal medulla region was decreased at 150 days, and there was very little or no mercury observed at 300 days post treatment. The ratio of the accumulated mercury concentration in the kidneys was 1.06% when the mice were killed at 10 days post treatment and it went down to 0.25% and 0.06% at 150 days and 300 days post treatment, respectively (Table 2).

The hepatocytes of the liver showed lower concentrations of mercury at 150 and 300 days post treatment compared to those at 10 days post treatment. The mercury concentration of the Kupffer cells in the sinusoid at 150 and 300 days post treatment was lower than that observed in the livers of mice that were euthanized at 10 days. The mercury concentration ratio in the liver decreased gradually over time from 1.38% on day 10 to 0.62% on day 150, and 0.22% on day 300 post treatment (Table 2).

A small amount of mercury was observed in the white and red pulps at 150 and 300 days post treatment compared to that 10 days post treatment. The red pulps showed a decrease in mass of mercury at 300 days post treatment compared to 150 days. The ratio of the concentration of accumulated mercury in the spleen gradually decreased over time from 2.37% at day 10 after the mice were killed to 1.61% at day 150 and 0.69% at day 300 (Table 2).

Discussion

In this study, we investigated the distribution of mercury in various organ and tissues and the changes in the concentrations of accumulated mercury over time. At 6 weeks of age, female mice were treated with a compound containing mercury (methylmercuric chloride) and then killed at 10, 150

Table 2. Mercury concentration rates (%) of kidney, liver and spleen in the mercury-treated group at each day post treatment

Organ	Day post treatment		
	10	150	300
Kidney	1.06±0.354	0.25±0.051	0.06±0.003
Liver	1.38±0.346	0.62±0.156	0.22±0.074
Spleen	2.37±0.395	1.61±0.404	0.69±0.232

Values represent the mean ± SD.
For obtaining the mercury concentration rates each day post treatment, 12 mice were used as control (n=6) and as mercury treated (n=6) specimens.

and 300 days post treatment. We observed that the mean weights of the kidney, liver, and spleen in the group treated with mercury were either similar or higher at 10 days and 150 days post treatment compared to the control group, while they were lower at 300 days. In a previous study, methylmercury was orally administered into adult male mice daily for 35 days, after which the weight of their livers was found to be significantly lower (0.99 g) in the mercury-treated group compared to the control group (1.90 g) [18]. However, another study found that oral administration of mercuric chloride into male mice for 14 days led to a significant increase in the kidney and spleen weights in the mercury-treated group compared to the control group [30]. In the present study, the weights of the kidney, liver, and spleen of the mice in the mercury-treated group decreased significantly, especially at 300 days post treatment, which is considered to be the result of accumulation of mercury for a long period of time.

Based on several reports, it has been observed that more mercury accumulates in the kidney than in the liver [1, 3, 41, 42]. However, a lethal dose of mercury compounds killed mice within 2-4 min of intraperitoneal injection and the concentration of mercury accumulated in the liver was found to be 10 times that of the mercury concentration in the kidney and 50 times that of the mercury concentration in the spleen [14]. In this case, the mercury accumulated in a very short period of time, which is extremely unusual.

In the present study, the kidneys of mice that were euthanized 10 days post treatment were found to have mercury accumulation in the epithelial cells constituting the proximal and distal convoluted tubules of the cortex, as well as in the collecting ducts and the Henle's loop of the medulla. These results are consistent with reports that have shown that mercury accumulates in the proximal tubular epithelial cells of the kidneys in mice [10] and rats [21]. In the kidneys of the mice that were euthanized 10 days post treatment, mercury was mainly distributed around the nuclei of the epithelial cells in the proximal convoluted tubules, or from the nucleus to the free surface of the cell. In comparison, mercury was mainly located on the free surface side of epithelial cells in the proximal convoluted tubules of mice killed at 150 or 300 days post treatment. This phenomenon might be attributed to the fact that the mercury accumulated in the cytoplasm of the epithelial cells might migrate towards the free surface of the cells and eventually appear in the filtrate of the proximal convoluted tubule lumen. It has been

reported that cytoplasmic vacuole formation [32, 34], pyknotic nucleus, and cell degeneration occurred in the epithelial cells of the proximal convoluted tubules following the accumulation of mercury [35]. In the present study, glomerular atrophy and vacuole formation was noted in response to mercury treatment, which is consistent with the results of previous studies [35, 36].

The effects of mercury also depends on the sex and the strain of the animal models. In the A.SW mouse strain, the accumulation of mercury is 2-5 times higher in the kidneys of the males than in those of females. The accumulation of mercury was also reported to be higher in the kidneys of the mice belonging to the A.SW strain than in the B10.S strain [19]. These findings indicate that the concentration of mercury that accumulates in the kidney differs from strain to strain, which means that the accumulation of mercury in the body is controlled or released depending on the genetic background of the animal. Higher accumulation of mercury was found in the A.SW, DBA/2, and BALB/C strains than in the B10.S strain, which might indicate that *Pprc1* is an important regulator of mercury excretion in the kidney [2]. In this experiment, although the mercury concentrations in the kidney, liver, and spleen were not measured directly, the relative concentration of mercury was analyzed by comparing the degree of mercury accumulation in the tissues of each organ under a microscope.

Generally, the blood in the hepatic portal vein flows towards the central vein. In this study, mercury was mainly found to be distributed in the hepatocytes around the portal vein after the initial contact with mercury in the blood and Kupffer cells in the sinusoid capillary. However, this was not the case in the hepatocytes around the central vein at 10 days post treatment. This suggests that mercury distribution might be related to the flow of blood. These results were confirmed by the autometallography method using sections frozen and embedded in Epon [10]. Mercuric chloride was also found in the Kupffer cells of the sinusoid capillary [11]. In the livers of mice killed on 150 and 300 days post treatment, although the amount of mercury accumulated in the hepatocytes was reduced to near zero, the mercury accumulated in the Kupffer cells still remained. Therefore, the effects of mercury on the hepatocytes is expected to decrease naturally over time. When the mercuric chloride was subcutaneously administered into adult male mice or rats, degeneration of hepatocytes and destruction of normal liver tissue structure was observed [26, 27]. The mechanism of action behind the damage caused by mercury to living tissue is due to its ability to increase the concentration of reactive oxygen species in cells such as superoxide and hydrogen peroxide, which leads to oxidative stress and subsequent tissue damage [22, 38]. In this experiment, vacuoles were still observed in hepatocytes of the mice killed at 150 and 300 days post treatment, which may be related to the damage to the hepatocytes.

According to previous reports, mercuric chloride was injected intraperitoneally into mice and autometallography confirmed that the mercury was localized in the macrophages of the spleen, lymph nodes, and thymus [11]. The results of studies conducted on various types of fish collected from eight national parks in the United States suggest that the aggregation of macrophages in the spleen increases in response to mercury, which implies tissue damage to the spleen [40]. In the present study, the mercury that accumulated in the kidney tubular epithelial cells or hepatocytes appeared in the form of small granules, whereas mercury that accumulated in the spleen appeared in the form of agglomerated clusters and was distinctly different from that seen in the kidneys and liver. This may be explained by the fact that mercury is predominantly found in the macrophages that are concentrated in the splenic tissues and appear to be in the form of lumps. Previous reports have shown that the concentration of mercury accumulating in the C57BL/6 strain mouse spleen increased based on the duration of the subcutaneous administration of mercury into the female mice. However, the concentration of mercury in the spleen of the DBA/2 mouse did not show a significant increase with time [24]. Similar to the kidneys, these findings indicate that mercury accumulation in the spleen differs depending on the mouse strain.

In this study, the ratio of the concentration of mercury in the organs of the mercury-treated mice and the control mice decreased over time, which is consistent with the results of a previous study [25]. Although mercury levels were seen to decline naturally over time, the pathways regulating the emission of mercury are not yet fully understood [9, 43]. One mechanism that might account for mercury excretion is via the binding of mercury to glutathione (GSH), which is present in almost all cells, to form GSH-Hg complexes [29, 39, 43]. This complex, which is the initial form of mercury that can be transported out of the cell [13], might be transported through the organic anion transporter to the proximal tubular cells, which are eventually transported into

the urine by multidrug resistant proteins (MRPs) [8]. These GSHs are induced by metallothionein (MT), which is a cysteine-rich protein present in various animal tissues; it has four isomers (MT-I, II, III, and IV). Metallothionein also has the ability to remove heavy metals [42].

A widely recommended method of detoxifying mercury is the conversion of mercury to a chelate complex [31]. In animals and humans, chelating compounds such as 2,3-dimercapto-1-propanesulfonic acid are known to be effective at removing mercury [4]. However, 2,3-dimercapto-1-propanesulfonic acid compounds have recently been reported to induce toxicity in the kidneys of mice treated with mercury [7]. The GSH-Hg complexes are transported into the urine by MRPs, *m*eso-2,3-dimercaptosuccinic acid (DMSA), and 2,3-dimercaptopropane-1-sulfonic acid (DMPS) [4, 8]. The MRPs play a functional role in the DMSA- and DMPS-mediated extraction of mercuric ions from proximal tubular epithelial cells of kidney and resulting in the release of mercury through the urine.

Methylmercuric chloride was subcutaneously injected weekly into pubescent female mice for 3 weeks in the present study. Localization of accumulated mercury was identified by autometallography. When mice were euthanized at 10 days post treatment, mercury was found to be distributed in the renal tubules and collecting ducts, especially in the proximal convoluted tubules. In the liver, mercury accumulated in the hepatocytes around the portal vein and Kupffer cells in the sinusoid capillary. In addition, vacuoles with accumulated mercury were observed in hepatocytes. In the spleen, an unusual mass of lumpy mercury was distributed in the white and red pulp. When animals were euthanized at 150 and 300 days post treatment, the relative concentrations of mercury accumulated in the kidney, liver, and spleen reduced naturally.

In conclusion, the present findings suggest that the endogenous glutathione protein can form a complex with mercury, and this complex can be excreted out via urine. As a result, the concentration of mercury accumulated in cells and tissues naturally reduces over time.

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초록 : 생쥐 신장, 간, 비장 내 시간에 따른 수은 농도 변화와 수은 화합물의 위치

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본 연구에서는 생쥐 신장, 간, 비장 내 축적된 수은의 위치와 아울러서 시간에 따른 수은 농도 변화를 조사하였다. 수은 투여 종료 후 10일, 150일, 300일에 생쥐를 희생하여 수은 농도변화를 분석하였다. 10일에 희생시킨 생쥐 신장의 경우, 근위세뇨관 상피세포의 핵 위쪽 세포질에 수은이 다량으로 분포하였으나, 사구체에는 분포하지 않았다. 간의 경우, 수은이 주로 간문맥 주위에 있는 간세포와 굴모세혈관에 있는 Kupffer 세포에 분포하였다. 10일에 희생시킨 비장의 경우, 백색 수질과 적색 수질에 수은이 흩어져 분포하였다. 수은의 농도 변화에 있어서, 150일과 300일에 희생시킨 신장의 피질과 수질에 축적되어 있던 수은이 낮은 농도로 나타났다. 역시 간세포에 축적되어 있던 수은도 150일과 300일의 경우, 낮은 농도로 나타났다. 비장의 경우, 백수와 적수 조직에 있던 수은 농도가 감소되었다. 이런 결과를 통해 세포나 조직에 축적되어 있던 수은의 위치가 확인되었으며, 또한 이 결과는 기관에 축적되어 있던 수은 농도가 시간이 지남에 따라 자연스럽게 감소된다는 사실을 확인해 주고 있다.