Enzyme Histochemical Study of Germanium Dioxide-Induced Mitochondrial Myopathy in Rats

Shin Young Yim¹, Il Yung Lee¹, and Tai Seung Kim²

Abstract

The purposes of this study were 1) to determine the earliest pathological changes of germanium dioxide (GeO₂)-induced myopathy; 2) to determine the pathomechanism of GeO₂-induced myopathy; and 3) to determine the minimal dose of GeO₂ to induce myopathy in rats. One hundred and twenty five male and female Sprague-Dawley rats, each weighing about 150 gm, were divided into seven groups according to daily doses of GeO₂. Within each group, histopathological studies were done at 4, 8, 16, and 24 weeks of GeO₂ administration. Characteristic mitochondrial myopathy was induced in the groups treated daily with 10 mg/kg of GeO₂ or more. In conclusion, the results were as follows: 1) The earliest pathological change on electron microscope was the abnormalities of mitochondrial shape, size and increased number of mitochondria; 2) The earliest pathological change on light microscope was the presence of ragged red fibers which showed enhanced subsarcolemmal succinate dehydrogenase and cyttochrome c oxidase reactivity; 3) GeO₂ seemed to affect the mitochondrial oxidative metabolism of muscle fibers; 4) GeO₂ could induce mitochondrial myopathy with 10 mg/kg of GeO₂ for 4 weeks or less duration in rats.

Key Words: Cytochrome c oxidase, germanium dioxide, mitochondrial myopathy, oxidative metabolism, ragged red fibers

INTRODUCTION

Germanium (Ge) is a grayish-white crystalline metal with atomic number 32, atomic weight 72.60, and specific gravity 5.33 g/cm³. In 1948, it came to be used for its semiconductor properties by U.S. Bell Laboratories, and played a major role in the development of the semiconductor electronics industry.² It has been discovered that germanium is present in almost all biomaterials and the average daily human intake ranges from 0.9 – 3.2 mg. After oral administration, Ge is rapidly absorbed in the gastrointestinal tract and is mainly excreted via urine and feces, with the kidney being the main excretory organ.³⁻⁴

Ge compounds are divided into two groups: inorganic compounds such as germanium dioxide (GeO₂) and germanium tetrachloride; and organic compounds such as spirgermanium, germanium lactate citrate, and carboxyethylgermanium sesquioxide, some of which have antitumor and immunomodulative effects.⁵⁻¹²

Many Japanese reportedly ingest a Ge preparation as an elixir to maintain or restore their health. In Europe, Ge supplements have been increasingly recommended. Ge is also self-administered by patients with cancer diseases and immune deficiencies such as AIDS.¹³⁻¹⁵ In Korea, Ge-containing drinking water or Ge-containing hot springs have become popular as a cure-all on a large scale through publicity, but actually on scientific grounds its effect on health is unclear.

More than 20 cases of Ge intoxication were reported in Japan by 1992, and the mortality rate was high; 6 out of 20 patients died.¹⁵ The most common clinical symptoms of germanium intoxication were renal failure, anemia and muscle weakness.¹⁶⁻¹⁸

It is well known that experimental myopathy is induced by the administration of GeO₂ in rats. Although it has been known that mitochondrial dysfunction may be the most important factor in the genesis of experimental myopathy,¹⁶⁻¹⁹,²⁰ the precise pathomechanism of GeO₂-induced myopathy is not known. A few studies reported that the earliest pathological changes in GeO₂-induced myopathy were decrease in cytochrome c oxidase activity (CCO) and accumulations of electron-dense materials in mitochondria.¹⁹,²⁰ But the determination of the minimal dosage of GeO₂ to induce myopathy is the essential prerequisite to the determination of the earliest pathological changes in GeO₂-induced myopathy. According to our literature review, the minimal dose of GeO₂

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to induce myopathy has not been proved and these previous studies were carried out without knowledge of the minimal dosage of GeO₂ to induce myopathy.

The purposes of this study were: 1) to determine the earliest pathological changes of GeO₂-induced myopathy; 2) to determine the pathomechanism of GeO₂-induced myopathy; and 3) to determine the minimal dose of GeO₂ to induce myopathy in rats.

MATERIALS AND METHODS

Animals and Method of GeO₂ administration

One hundred and twenty five male and female Sprague-Dawley rats, weighing about 150 gm each at the beginning of the experiment, were divided into seven groups after adjusting rats to the experimental environment for one week: group 1 treated daily with 150 mg/kg GeO₂ dissolved in drinking water via orogastric tube; group 2 given 100 mg/kg GeO₂; group 3 given 50 mg/kg GeO₂; group 4 given 20 mg/kg GeO₂; group 5 given 10 mg/kg GeO₂; group 6 given 5 mg/kg GeO₂; and a control group not given GeO₂ (Table 1). Each dosage group was divided again into four subgroups according to duration of GeO₂ treatment: 4, 8, 16, and 24 weeks. Forty-five rats died during the experiment. These rats showed marked weight loss and severe autolysis of muscle fibers. We excluded them from histopathological studies. The total numbers of rats used in the enzyme histochemical studies are shown in Table 1.

Enzyme histochemical studies

Rats in each group were sacrificed at 4, 8, 16, and 24 weeks of the experiment, respectively. The soleus muscles were obtained for histopathological studies. Part of the muscle specimens was immediately frozen in isopentane cooled to −160°C with liquid nitrogen, and then prepared for histochemical examination. Serial frozen sections, 7 µm in thickness, were stained with hematoxylin-eosin (H-E), modified Gomori trichrome (mGt), nicotinamide adenine dinucleotide, reduced-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome c oxidase (CCO), adenosine triphosphatase staining pH 9.4, and pH 4.6 (ATPase pH 9.4, pH 4.6), respectively.

Electron microscopic examination

1 mm³ sized muscle specimens were fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide for 1 hour, and embedded in Epon mixture. Ultrathin sections were stained with both uranyl acetate and lead nitrate, and examined with a Hitachi H500 electron microscope.

Quantification of reactivity of SDH and CCO staining according to fiber types of skeletal muscles of rats

To determine the pathomechanism of GeO₂-induced myopathy, we conducted quantification of reactivity of SDH and CCO staining according to the fiber types of skeletal muscles of rats. Photomicrographs of muscles stained with SDH and CCO in each group were scanned by Polaroid Sprint Scan 35 (Polaroid Co., Cambridge, Mass., U.S.A.). The color photo computer images were transformed into gray-scale images using a 256-scale, where 0 represented solid black and 255 represented pure white. Reactivities of SDH and CCO staining were measured in 20 muscle fibers for each muscle fiber type.

Table 1. Daily Dose and Duration of Germanium Dioxide and Number of Rats Used in Enzyme Histochemical Studies

<table>
<thead>
<tr>
<th>Group</th>
<th>Daily dose of GeO₂ (mg/kg)</th>
<th>Number of rats used in experiment</th>
<th>Number of rats used in enzyme histochemistry according to duration of experiment</th>
<th>Number of rats which died during experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4wk</td>
<td>8wk</td>
</tr>
<tr>
<td>Group 1</td>
<td>150</td>
<td>23</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Group 2</td>
<td>100</td>
<td>30</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Group 3</td>
<td>50</td>
<td>24</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Group 4</td>
<td>20</td>
<td>14</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Group 5</td>
<td>10</td>
<td>13</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Group 6</td>
<td>5</td>
<td>13</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>125</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

Statistical analysis

One-way ANOVA and multiple-comparison test using the Tukey method were used for statistical evaluation of reactivity of SDH and CCO staining according to the fiber types of skeletal muscles of rats.

RESULTS

Enzyme histochemical studies

Groups 1, 2, 3, and 4 showed ragged red fibers (RRF) on mGt staining. RRFs could be classified by the degree of staining reactivity on mGt staining. That is, thin subsarcolemmal RRFs were fibers showing thin, subsarcolemmal, increased reactivity on mGt staining; thick subsarcolemmal RRFs were fibers showing thick, subsarcolemmal, increased reactivity; diffuse RRFs were fibers showing diffuse, sarcolemmal increased reactivity (Fig. 1). Group 4 showed only thin subsarcolemmal RRFs in all durations of experiment. Groups 1, 2, and 3 showed thin subsarcolemmal RRFs in 4 and 8 weeks of experiment. Thick subsarcolemmal RRFs and diffuse RRFs were observed in groups 1, 2, and 3 of 16 and 24 weeks of experiment. Necrosis and degeneration of muscle fibers, and the infiltration of macrophages and inflammatory cells were observed in groups 1, 2, and 3 of 16 and 24 weeks of the experiment.

Thin and thick subsarcolemmal RRFs showed increased subsarcolemmal reactivity while diffuse RRFs showed diffuse reactivity on NADH-TR and SDH staining (Fig. 2).

Thin subsarcolemmal RRFs showed enhanced subsarcolemmal CCO reactivity on CCO staining (Fig. 3). However, thick subsarcolemmal RRFs and diffuse RRFs proved to be CCO-deficient fibers on CCO staining (Fig. 3).

Enzyme histochemical studies using NADH-TR, SDH, CCO, ATPase pH 9.4 and ATPase pH 4.6 respectively, showed that most RRFs were type 1 fibers, some of them were type 2A fibers, but none of them were type 2B fibers (Fig. 1).

Groups 5, 6, and the control group did not induce any pathological changes of muscles in all durations of experiment on enzyme histochemical studies.

Electron microscopic findings

Group 5 at all durations showed the abnormalities of mitochondrial shape, size and increased number of

![Fig. 1. Mirror images of soleus muscles at 8 weeks in group 2. Enzyme histochemistry using ATPase pH 9.4, CCO, and NADH-TR proved that fibers with increased subsarcolemmal basophilic reactivity in H-E staining, and thin RRFs on mGt staining (arrowhead) were type 1 and 2A fibers. Open arrowhead: thin RRFs of type 1 fibers. Dark arrowhead: thin RRFs of type 2A fibers. HE, H-E. TRC, mGt. ATP, ATPase pH 9.4. 1, type 1 fiber. 2A, type 2A fiber. 2B, type 2B fiber.](image-url)
mitochondria (pleocornia). Group 5 at all durations did not show electron-dense materials in mitochondria. Groups 1, 2, 3 and 4 at all durations of experiment showed pleocornia, occasionally containing electron-dense materials and enlarged mitochondria (megacornia) with proliferated cristae in subsarcolemmal and intermyofibrillar areas (Fig. 4). There were no accumulations of lipid droplets in any of the experimental groups. Group 6 and the control group did not show any abnormalities on electron microscopic studies in all durations of experiment.
Fig. 4. Electron microscopic findings of soleus muscles at 8 weeks in group 1. (A) Subsarclemmal accumulation of enlarged mitochondria with proliferated cristae. (B) Note many enlarged mitochondria containing electron-dense materials (arrowhead).

Table 2. Reactivity* of SDH † Staining According to Fiber Types of Skeletal Muscles of Rats in Group 2 at 16 Weeks of Experiment

<table>
<thead>
<tr>
<th>Fiber type</th>
<th>Number of fibers</th>
<th>Reactivity of SDH</th>
<th>p †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal type 1</td>
<td>20</td>
<td>87.76 ± 8.41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal type 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2A</td>
<td>20</td>
<td>102.56 ± 10.90</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Type 2B</td>
<td>20</td>
<td>108.59 ± 2.46</td>
<td></td>
</tr>
<tr>
<td>Fibers with increased subsarcolemmal reactivity</td>
<td>20</td>
<td>27.50 ± 8.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fibers with diffuse reactivity</td>
<td>20</td>
<td>36.36 ± 8.20</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Reactivity is expressed using a 256 gray scale, where 0 represents black and 255 represents white. Values are given as mean ± standard deviation.
† Succinate dehydrogenase.
†† One-way ANOVA and multiple-comparison test (Tukey) show significant (p<0.0001) differences among four kinds of fiber types. There is no significant difference between normal type 2A and 2B fibers.

Quantitation of reactivity of SDH and CCO staining according to muscle fiber types

Mean reactivities of SDH staining according to muscle fiber types in group 2 at 16 weeks of experiment were as follows (Table 2): normal type 2B fibers at 108.6; normal type 2A fibers at 102.6; normal type 1 fibers at 87.8; fibers with diffuse reactivity 36.4; and fibers with increased subsarcolemmal reactivity (thin RRF) at 27.5. Therefore, normal type 2B fibers showed the lightest color and fibers with increased subsarcolemmal reactivity showed the darkest color. Other groups also showed that normal type 2B fibers showed the lightest color and fibers with increased subsarcolemmal reactivity showed the darkest color. One-way ANOVA and multiple-comparison test (Tukey) showed significant (p<0.0001) differences among four kinds of fiber types such as type 1 fibers, type 2 fibers, fibers with increased subsarcolemmal reactivity, and fibers with diffuse reactivity. There was no significant difference between normal type 2A and 2B fibers.

Mean reactivities of CCO staining according to muscle fiber types in group 2 at 16 weeks of
Table 3. Reactivity* of CCO † Staining According to Fiber Types of Skeletal Muscles of Rats in Group 2 at 16 Weeks of Experiment

<table>
<thead>
<tr>
<th>Fiber type</th>
<th>Number of fibers</th>
<th>Reactivity of CCO</th>
<th>p †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal type 1</td>
<td>20</td>
<td>112.01±9.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal type 2A</td>
<td>20</td>
<td>148.80±6.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal type 2B</td>
<td>20</td>
<td>169.91±9.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fibers with increased</td>
<td>20</td>
<td>42.55±12.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>subsarcolemmal reactivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCO deficient fibers</td>
<td>20</td>
<td>197.50±8.20</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Reactivity is expressed using a 256 gray scale, where 0 represents black and 255 represents white. Values are given as mean±standard deviation.
† Cytochrome c oxidase.
† One-way ANOVA and multiple-comparison test (Tukey) show significant (p<0.0001) differences among five kinds of fiber types.

Experiment were as follows (Table 3): CCO-deficient fibers at 197.5; normal type 2B fibers at 169.9; normal type 2A fibers at 148.8; normal type 1 fibers at 112.0; and fibers with increased subsarcolemmal reactivity at 42.6. Therefore, CCO-deficient fibers showed the lightest color and fibers with increased subsarcolemmal reactivity showed the darkest color. One-way ANOVA and multiple-comparison test (Tukey) showed significant (p=0.0001) differences among five kinds of fiber types such as normal type 1 fibers, type 2A fibers, type 2B fibers, fibers with increased subsarcolemmal reactivity, and CCO-deficient fibers.

**DISCUSSION**

Characteristic mitochondrial myopathy was induced in rats by the daily administration of 10 mg/kg of GeO<sub>2</sub> for 4 weeks. Therefore, germanium dioxide can induce mitochondrial myopathy with 10 mg/kg of GeO<sub>2</sub> for 4 weeks or less duration in rats. Further study is required to determine the minimal duration of GeO<sub>2</sub> to induce mitochondrial myopathy in rats. Studies on GeO<sub>2</sub>-induced mitochondrial myopathy reported up to now have administered GeO<sub>2</sub> mixed with animal feed to the experimental animals, so it was impossible to obtain accurate dosage measurements of GeO<sub>2</sub> actually ingested by the animals. Therefore, in our study, we utilized an orogastric tube for accurate daily dosage of GeO<sub>2</sub>. The severity of mitochondrial myopathy increased approximately in proportion to the dosage and duration of GeO<sub>2</sub> administration. Therefore, dose-dependency in GeO<sub>2</sub>-induced myopathy was observed in our study similar to GeO<sub>2</sub>-induced nephrotoxicity that is reported to show dose dependency. Higuchi et al. reported that the earliest pathological changes in GeO<sub>2</sub>-induced myopathy in rats were a decrease in CCO activity and accumulation of high electron-dense materials in mitochondria with 100 mg/kg of GeO<sub>2</sub> for 4 months of experiment. In our experiment, increased CCO reactivity was the earliest finding and this change was induced with 10 mg/kg of GeO<sub>2</sub> for 4 weeks. One of the reasons for the contrary results might be that the experimental duration used by Higuchi et al. was too long to observe thin RRFs which showed enhanced subsarcolemmal CCO reactivity. Therefore, the earliest pathological change on electron microscope is the abnormalities of mitochondrial shape, size and increased number of mitochondria and the earliest pathological change on light microscope is the presence of ragged red fibers which showed enhanced subsarcolemmal SDH and CCO reactivity. These findings suggest that at the early stage of GeO<sub>2</sub>-induced myopathy, muscle fibers show increased reactivity to CCO staining via a compensatory mechanism, but as the myopathy progresses, the depletion of CCO is so severe that this compensatory mechanism no longer works.

The administration of GeO<sub>2</sub> caused severe pathological changes in mitochondria-rich type 1 and type 2A muscle fibers, but did not cause any pathological changes in type 2B fibers. In conclusion, GeO<sub>2</sub> seems to affect the mitochondrial oxidative metabolism of muscle fibers.

Most human mitochondrial myopathy showed the accumulation of lipid droplets, but GeO<sub>2</sub>-induced mitochondria myopathy did not show any lipid.
droplets. Therefore, GeO₂-induced mitochondrial myopathy is a new form of mitochondrial myopathy.

The conclusions are as follows: 1) The earliest pathological change on electron microscope was the abnormalities of mitochondrial shape, size and increased number of mitochondria; 2) The earliest pathological change on light microscope was the presence of ragged red fibers which showed enhanced subsarcolemmal SDH and CCO reactivity; 3) GeO₂ seemed to affect the mitochondrial oxidative metabolism of muscle fibers; 4) GeO₂ can induce mitochondrial myopathy with 10 mg/kg of GeO₂ for 4 weeks or less duration in rats.

Further biochemical and molecular biological studies are necessary to elucidate the pathomechanism of GeO₂-induced mitochondrial myopathy.

REFERENCES