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

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Abstract

The purposes of this study were 1) to determine the earliest pathological changes of germanium dioxide (GeO_2)-induced myopathy; 2) to determine the pathomechanism of GeO_2-induced myopathy; and 3) to determine the minimal dose of GeO_2 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 GeO_2. Within each group, histopathological studies were done at 4, 8, 16, and 24 weeks of GeO_2 administration. Characteristic mitochondrial myopathy was induced in the groups treated daily with 10 mg/kg of GeO_2 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 cytochrome *c* oxidase reactivity; 3) GeO_2 seemed to affect the mitochondrial oxidative metabolism of muscle fibers; 4) GeO_2 could induce mitochondrial myopathy with 10 mg/kg of GeO_2 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/cm.[1] 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.[2,3] 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.[1,3]

Ge compounds are divided into two groups: inorganic compounds such as germanium dioxide (GeO_2) and germanium tetrachloride; and organic compounds such as spirogermanium, germanium lactate citrate, and carboxyethylgermanium sesquioxide, some of which have antitumor and immunomodulative effects.[1,4-11]

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-administrated by patients with cancer diseases and immune deficiencies such as AIDS.[12-14] 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 GeO_2 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 GeO_2-induced myopathy is not known. A few studies reported that the earliest pathological changes in GeO_2-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 GeO_2 to induce myopathy is the essential prerequisite to the determination of the earliest pathological changes in GeO_2-induced myopathy. According to our literature review, the minimal dose of GeO_2

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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 GeO_2 to induce myopathy.

The purposes of this study were: 1) to determine the earliest pathological changes of GeO_2-induced myopathy; 2) to determine the pathomechanism of GeO_2-induced myopathy; and 3) to determine the minimal dose of GeO_2 to induce myopathy in rats.

MATERIALS AND METHODS

Animals and Method of GeO_2 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 GeO_2 dissolved in drinking water via orogastric tube; group 2 given 100 mg/kg GeO_2; group 3 given 50 mg/kg GeO_2; group 4 given 20 mg/kg GeO_2; group 5 given 10 mg/kg GeO_2; group 6 given 5 mg/kg GeO_2; and a control group not given GeO_2 (Table 1). Each dosage group was divided again into four subgroups according to duration of GeO_2 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 $-160^{\circ}C$ 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 mm^3 - sized muscle specimens were fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetradioxide 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 GeO_2-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

Group	Daily dose of GeO_2 (mg/kg)	Number of rats used in experiment	Number of rats used in enzyme histochemistry according to duration of experiment				Number of rats which died during experiment
			4wk	8wk	16wk	24wk	
Group 1	150	23	3	3	3	3	11
Group 2	100	30	3	3	3	3	18
Group 3	50	24	3	3	3	3	12
Group 4	20	14	3	3	3	3	2
Group 5	10	13	3	3	3	3	1
Group 6	5	13	3	3	3	3	1
Control	0	8	2	2	2	2	0
Total		125	20	20	20	20	45

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

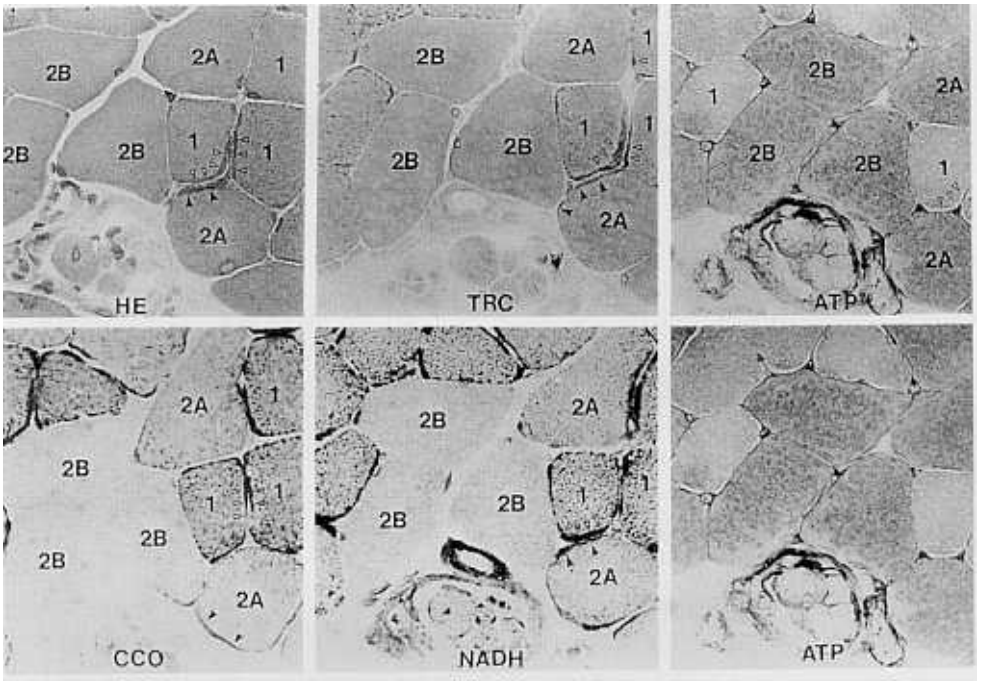

Fig. 1. Mirror images of soleus muscles at 8 weeks in group 2. Enzyme histochemistry using ATPase pH 9.4, CCO, and NADH-TR proved that fibers with increased subsarcolemmal basophilic reactivity in H-E staining, and thin RRFs on mGt staining (arrowhead) were type 1 and 2A fibers. Open arrowhead: thin 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.

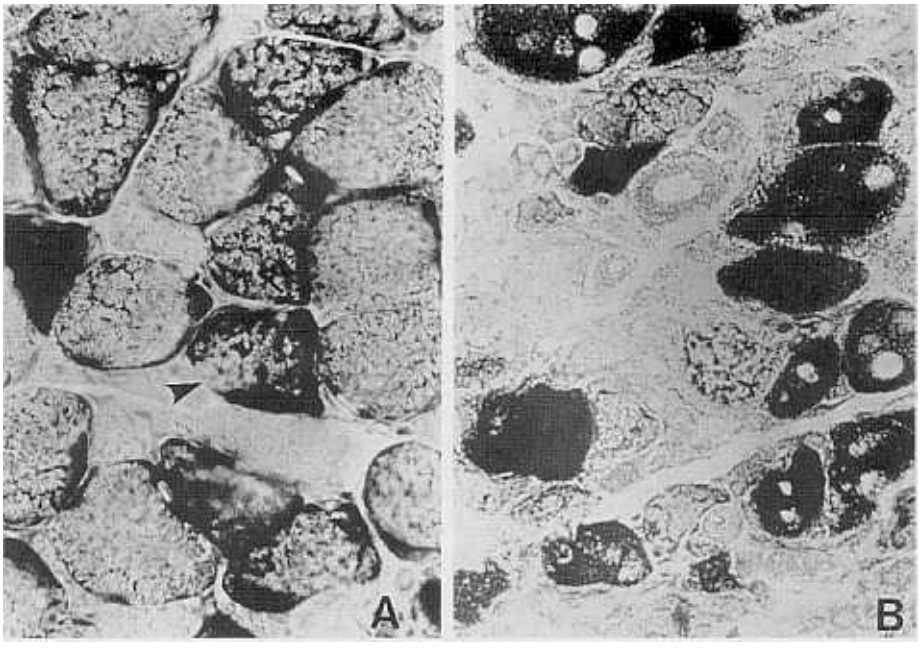

Fig. 2. Succinate dehydrogenase staining of soleus muscles at 24 weeks in group 1. (A) Note fibers with increased subsarcolemmal reactivity (arrowhead) and a fiber with diffusely increased reactivity. (B) Note fibers with diffuse reactivity.

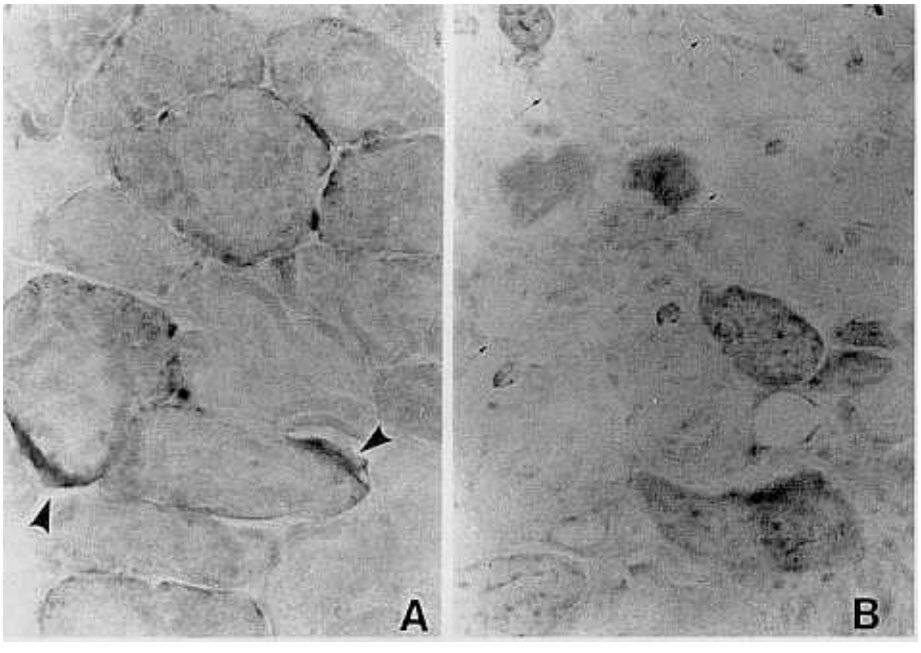

Fig. 3. Cytochrome c oxidase staining of soleus muscles at 8 weeks in group 1. (A) Note fibers with enhanced subsarcolemmal cytochrome c oxidase reactivity (arrowhead). (B) Note many cytochrome c oxidase-deficient fibers (arrows).

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.

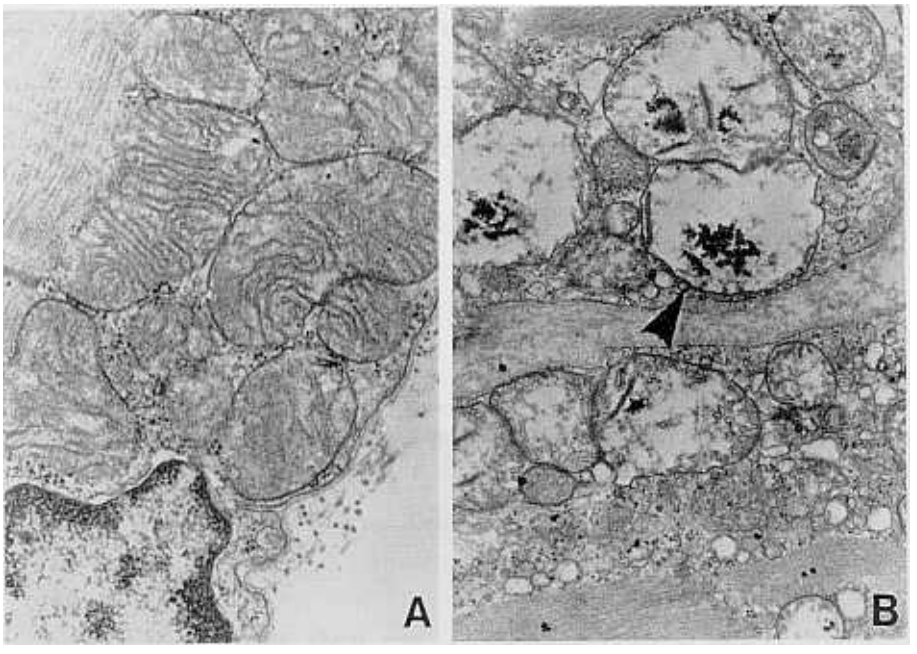

Fig. 4. Electron microscopic findings of soleus muscles at 8 weeks in group 1. (A) Subsarcolemmal accumulation of enlarged mitochondria with proliferated cristae. (B) Note many enlarged mitochondria containing electron-dense materials (arrowhead).

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

Fiber type		Number of fibers	Reactivity of SDH	p‡
Normal type 1		20	87.76±8.41	<0.0001
Normal type 2	Type 2A	20	102.56±10.90	<0.0001
	Type 2B	20	108.59±2.46	
Fibers with increased subsarcolemmal reactivity		20	27.50±8.12	<0.0001
Fibers with diffuse reactivity		20	36.36±8.20	<0.0001

* 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

Fiber type	Number of fibers	Reactivity of CCO	p‡
Normal type 1	20	112.01±9.67	<0.0001
Normal type 2A	20	148.80±6.25	<0.0001
Normal type 2B	20	169.91±9.67	<0.0001
Fibers with increased subsarcolemmal reactivity	20	42.55±12.91	<0.0001
CCO deficient fibers	20	197.50±8.20	<0.0001

* 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_2 for 4 weeks. Therefore, germanium dioxide can induce mitochondrial myopathy with 10 mg/kg of GeO_2 for 4 weeks or less duration in rats. Further study is required to determine the minimal duration of GeO_2 to induce mitochondrial myopathy in rats. Studies on GeO_2-induced mitochondrial myopathy reported up to now have administered GeO_2 mixed with animal feed to the experimental animals, so it was impossible to obtain accurate dosage measurements of GeO_2 actually ingested by the animals.[16,19,20] Therefore in our study, we utilized an orogastric tube for accurate daily dosage of GeO_2.

The severity of mitochondrial myopathy increased approximately in proportion to the dosage and duration of GeO_2 administration. Therefore, dose-dependency in GeO_2-induced myopathy was observed in our study similar to GeO_2-induced nephrotoxicity that is reported to show dose dependency.[10]

Higuchi et al. reported that the earliest pathological changes in GeO_2-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_2 for 4 months of experiment.[19] In our experiment, increased CCO reactivity was the earliest finding and this change was induced with 10 mg/kg of GeO_2 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_2-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_2 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_2 seems to affect the mitochondrial oxidative metabolism of muscle fibers.

Most human mitochondrial myopathy showed the accumulation of lipid droplets, but GeO_2-induced mitochondria myopathy did not show any lipid

droplets.[16] Therefore, GeO_2-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) GeO_2 seemed to affect the mitochondrial oxidative metabolism of muscle fibers; 4) GeO_2 can induce mitochondrial myopathy with 10 mg/kg of GeO_2 for 4 weeks or less duration in rats.

Further biochemical and molecular biological studies are necessary to elucidate the pathomechanism of GeO_2-induced mitochondrial myopathy.

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