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# Microscopic Study of Decomposition-Inhibition in Stabilized $ClO_2$ Gas in Skeletal Muscle of Rat

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# 흰쥐 골격근에서 안정화 이산화염소(Stabilized $ClO_2$)의 부패억제에 관한 현미경적 연구

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## ABSTRACT

This study was conducted to determine the antiseptic effect of stabilized chlorine dioxide (S-$ClO_2$) on muscle tissue of rats. Skeletal muscle of 8-week old Sprague-Dawley rats was used. Light and transmission electron microscopic findings were observed in the control group, which was not treated with stabilized chlorine dioxide, and in the experimental group, which was treated with a stabilized chlorine dioxide powder in aqueous solution.

According to the LM and TEM observations, the day 1 control group showed the initiation of endomysium collapse resulting in an unclear boundary of muscle fibers, and partial collapse of the mitochondrial membranes. All endomysium had collapsed, and bacteria were observed among muscle fibers in the day 2 and later groups. Shapes of muscles were not distinguishable in day 3 or later groups. In contrast, the day 1 and 3 experimental groups revealed detailed structure of typical muscles, but partial collapse of the mitochondrial membranes was observed in the day 3 and later groups. Subsequently, connective tissues collapsed and structures in the shape of concentric circles were observed.

In summary, the day 1 control group showed the initial collapse of tissues, and shapes were not distinguishable in the day 3 and later groups because most of the tissues had collapsed. In contrast, the day 3 experimental group showed partial collapse, but the overall shapes of muscles were maintained as time went on, confirming the antiseptic effect of stabilized chlorine dioxide on muscles.

**Keywords :** Endomysium, Skeletal muscle, Stabilized chlorine dioxide

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## INTRODUCTION

Microbicidal and preservative agents, which are very cheap alcohol group chemicals with moderate disinfective effects, and chloride group chemicals, which have effective antiseptic substances but are harmful to the human body have been used recently. The safety of these chemical compounds is still controversial, particularly in terms of toxicity, which limits the amount used. Many studies have been conducted to identify effective, nontoxic, non-invasive, and environmentally friendly preservatives and have focused on stabilized chlorine dioxide which has strong deodorization and disinfective effects on viruses, bacteria, and fungi (Harakeh et al., 1988; Han et al., 2001).

The deodorization effect of stabilized chlorine dioxide is excellent, because it reacts with amino acids containing S-radicals or aromatic rings in a natural state. Its microbicide effect is generated by protein cellular membrane denaturation (Tan et al., 1987). Kastner et al. (2003) reported that stabilized chlorine dioxide shows excellent microbicide effects under acidic conditions and at a low temperature. Additionally, chlorine dioxide is tasteless and odorless (Prokop, 1991; White, 1992; Edwards & Amirtharajah, 1993).

Unlike other chlorine compounds that show microbicide effect due to chlorination, stabilized chlorine dioxide is an ideal agent with microbicide and deodorizing effects based on strong oxidation; thus, it does not cause immune or homeostatic problems (Fukayama et al., 1986; Chang et al., 2000). Thus, stabilized chlorine dioxide has been applied to the agricultural, fisheries, and livestock, industries (Chang et al., 2000; Han et al., 2001; Kastner et al., 2003). Studies on stabilized chlorine dioxide have focused on its disinfection and deodorizing effects on food and the water supply (Fukayama et al., 1986; Chang et al., 2000; Świetlik et al., 2002, 2003).

However, research about the decomposition-inhibition effect and mechanism of stabilized chlorine dioxide is rare. The present study was performed to investigate the decomposition-inhibition effect of stabilized chlorine dioxide in rat muscle tissues using light and transmission electron microscopy (TEM).

## MATERIALS AND METHODS

### 1. Laboratory animals

Male Spargue-Dawley rats were purchased from Samtaco Co. (Gyeonggi-do, Korea). Healthy 8-week old Sprague-Dawley rats weighing 230 ~ 250 g were used for the experiments. They were divided into control group and an experimental group (stabilized chlorine dioxide (S-$ClO_2$) powder in aqueous solution). Each group was subcategorized into a day zero group, day 1 group, day 2 group, day 3 group, day 4 group, and day 5 group. Each subgroup contained five rats. All animals were anesthetized before excising the right thigh muscle. Experiment was conducted on a 9 cm-diameter Petri-dish with gauze on it.

### 2. Process of stabilized chlorine dioxide (S-$ClO_2$)

The control and experimental groups were treated in an incubator at 37˚C and 80 ± 5% humidity. A nonwoven fabric bag of powdered stabilized chlorine dioxide was added to the experimental group from the beginning to end of the experiment, and an aqueous solution of stabilized chlorine dioxide was sprayed once per day for 5 days from the beginning of the experiment.

### 3. Light microscopy and TEM

Samples were pre-fixed with a 2.5% paraformaldehyde-glutaraldehyde phosphate buffer (4˚C, pH 7.4) for 1 hour, washed twice for 15 minutes each with 0.1 M phosphate buffer (4˚C, pH 7.4), and then post-fixed in 1% osmium tetroxide in the same buffer for 1 hour at 4˚C. The samples were dehydrated in an ethanol series for 10 minutes in each concentration, and twice in 100% propylene oxide for 20 minutes. Samples were embedded in Epon 812 mixture according to the protocol and polymerized in a vacuum drying oven (Yamato, Japan) at 60˚C for 2 days. The embedded tissues were prepared as 1 μm semithin sections using an ultramicrotome (LKB-2088), stained with 1% toluidine blue, and observed through a light microscope (Olympus BX51, Japan) under 200× magnification. At the same location, 60 ~ 80 nm of ultra-thin sections were prepared, mounted on a 200 mesh copper grid, and stained with 2% uranyl acetate for 20 minutes and lead citrate for 5 minutes. Photo microscopy was performed using a Hitachi H-7600 TEM at 80 kV.

## RESULTS

### 1. Light microscopic findings

#### 1) Control groups

Samples in the day zero control group were darkly stained

with toluidine blue, and the shape of the muscle fibers was clear with horizontally severed multinucleated cells surrounded by endomysium. Nuclei were oval in shape, and found mostly at the lower area of the cells. The connective tissue among the muscle fibers was dense (Fig. 1A). The endomysium surrounding the muscle fiber was separated in the day 1 control group, and connective tissue among the muscle fibers was collapsed, resulting in partly spaced muscle fibers among them. Nuclei below the cell membrane were mostly collapsed and not observed (Fig. 1B). The endomysium surrounding the muscle fibers in the day 2 control group was collapsed, barely showing its overall shape, and muscle fiber boundaries were vaguely observed in some cases. Myofibrils inside the muscle fibers had collapsed showing light staining (Fig. 1C). The boundaries among muscle fibers in the day 3 control group were hardly distinguishable as endomysium, and the connective tissue among the muscle fibers had collapsed (Fig. 1D).

2) Experimental groups

Samples in the day 1 experimental group were densely stained with toluidine blue. The shapes of the muscle fibers and endomysium were clearly observed with dense muscle fibers. Nuclei below the cell membrane were also densely stained (Fig. 1E). Boundaries among muscle fibers in the day 2 group were clear, and myofibrils in the muscle fibers and nuclei below the cell membrane were also clearly observed (Fig. 1F). Collapse of connective tissues among muscle fibers had increased slightly in the day 3 group compared with that in the day 2 group, but the shapes of myofibrils were similar to those in the day 2 control group (Fig. 1G). The day 4 experimental group showed a faint toluidine blue stain but the overall shape was similar to that of the day 3 group (Fig. 1H). The day 5 experimental group

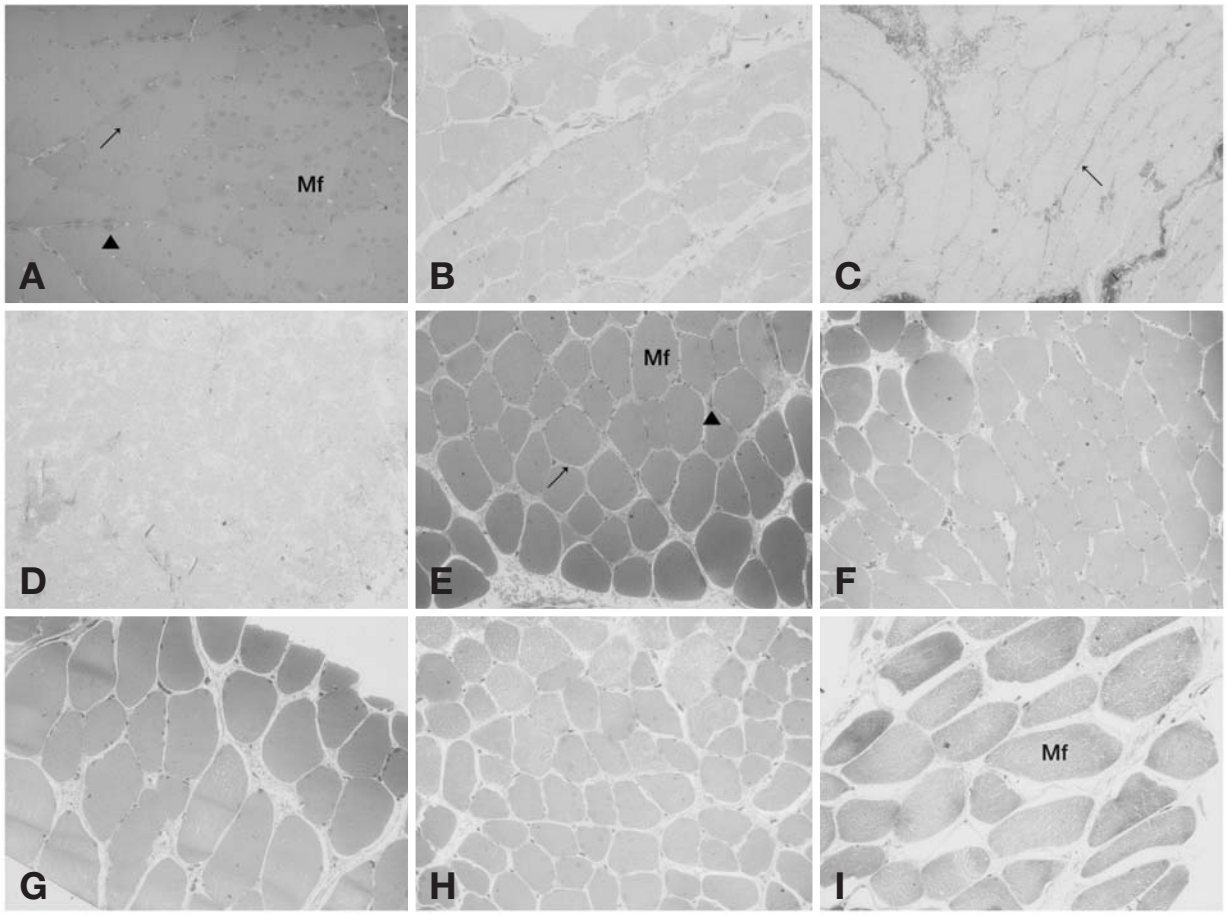


**Fig. 1.** Light micrographs of the control and experimental groups with passage of time Muscle fibers (Mf), endomysium (arrow) and nuclei (head of arrow) were clearly observed in the day zero control and day 1 experimental groups. However, only the endomysium outline (arrow) was present in the day 2 control group. Connective tissues had collapsed in the day 5 experimental group, but the overall shape of muscle fiber (Mf) was maintained. All photos were taken under 200× magnification (control: A=0 day, B=1 day, C=2 days, D=3 days; experiment: E=1 day, F=2 days, G=3 days, H=4 days, I=5 days).

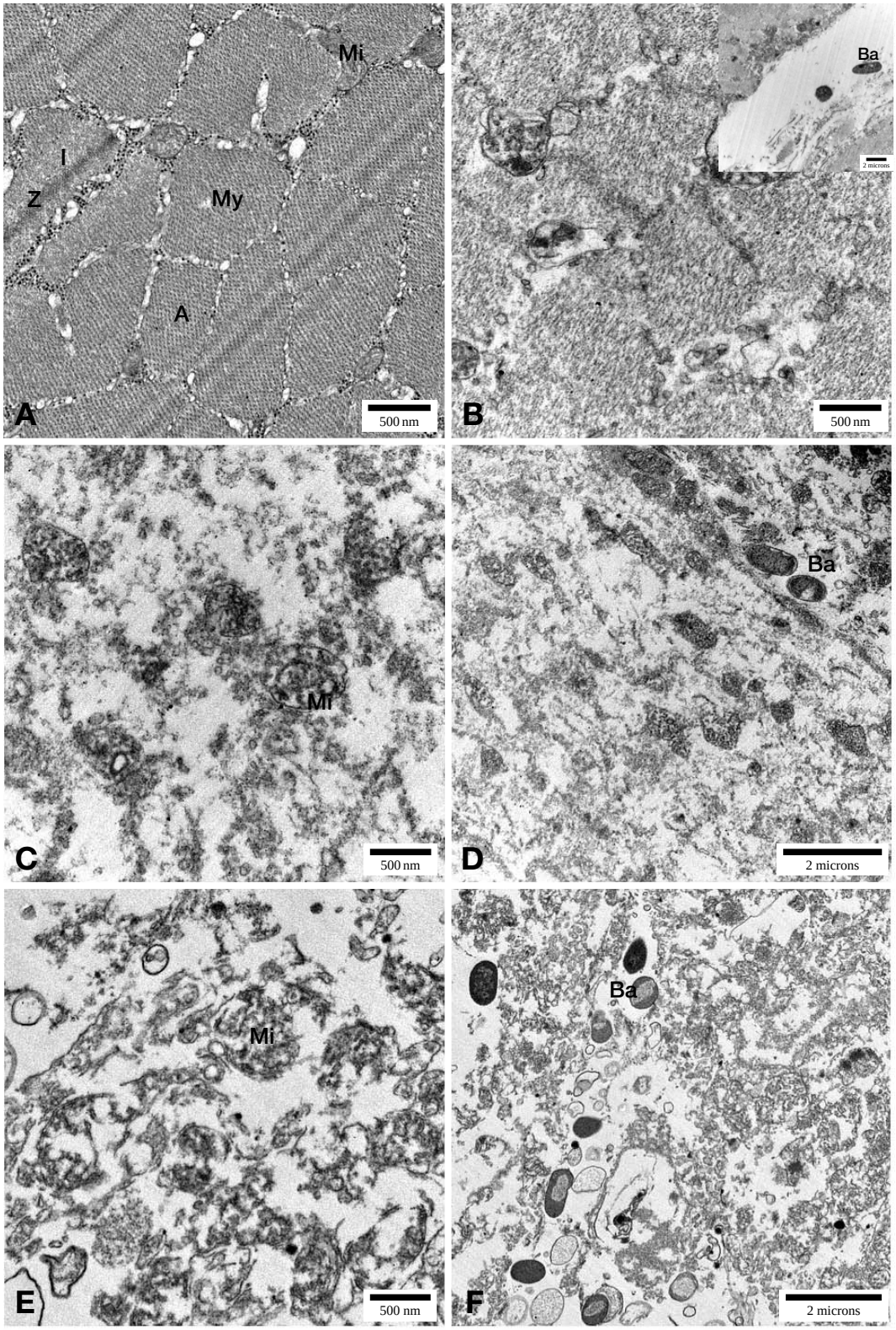


**Fig. 2.** Electron micrographs of the control groups with passage of time Myofibrils (My) were clearly found, and light band (I band: I), dark band (A band: A), and the Z line (Z) comprising muscle fibers as well as mitochondria (Mi) were observed in the zero group. The double membrane of mitochondria was destroyed in the day 1 group and accordingly, cristae and matrix were congregated. Bacteria (small photo: Ba) were observed. Shapes of muscles were not noticeable in the day 3 group, because the endomysium was destroyed. Many septic bacteria were observed (A=0 day, B=1 day, C, D=2 days, E, F=3 days).

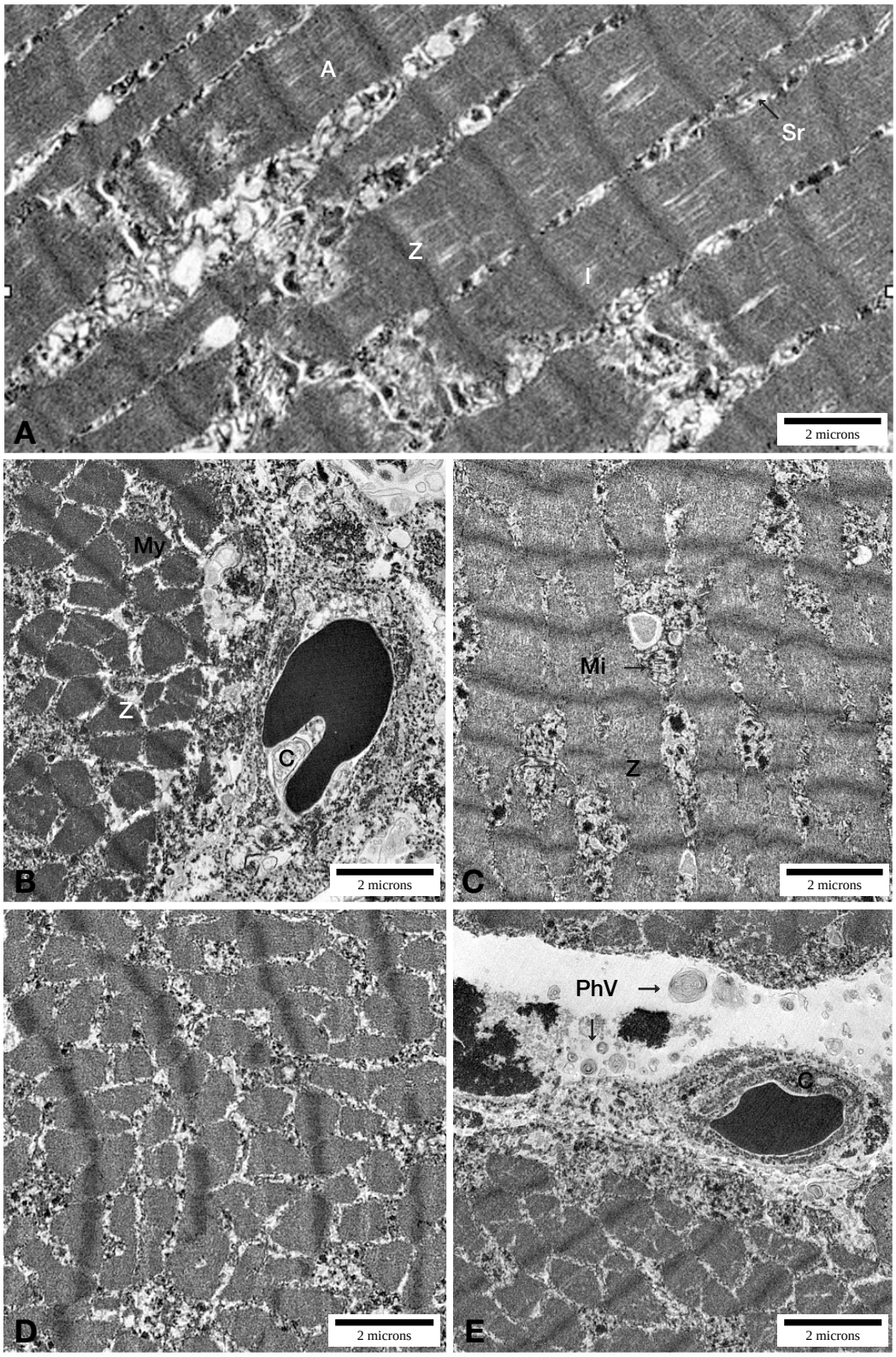


**Fig. 3.** Electron micrographs of the experimental groups with passage of time The light band (I band: I), dark band (A band: A), Z line (Z), and sarcoplasmic reticulum (Sr) comprising muscle fibers were clearly observed in the day 1 group. Similar shapes to those of the day 1 group and capillaries were observed in the day 2 group. Partial destruction of mitochondria was observed in the day 3 group. In the day 5 group, phagocytic vacuoles (PhV) in the shape of concentric circles were observed due to collapsed connective tissue (A=1 day, B=2 days, C=3 days, D=4 days, E=5 days).

showed wide spaces among muscle fibers due to collapsed connective tissue. However, no morphological changes occurred in the endomysium surrounding the muscle fibers. Other than the wide space among muscle fibers, the day 5 experimental group was similar to the day 4 group overall (Fig. 1I).

### 2. TEM findings

#### 1) Control groups

The horizontally sectioned day zero control group showed clear myofibrils in muscle fibers with thin and thick filaments, which are proteins composing muscle fibers. Additionally, the A band overlapped with thin and thick filaments, and the I band and Z line, composed of thin filaments, were clearly observed. Mitochondria were also found among muscle fibers and showed their typical shape for skeletal muscle (Fig. 2A). Boundaries of muscle fibers were unclear in the day 1 group due to a collapsed endomysium, and some of the myofibrils were scattered. Due to the partly destroyed double membrane of mitochondria among the muscle fibers, the cristae and matrixes were congregated, and bacteria were observed (Fig. 2B). All of the endomysium was destroyed in the day 2 control group, and no boundaries remained among muscle fibers. Myofibrils were mixed up, and bacteria were observed among some muscle fibers (Fig. 2C, D). All myofibrils bundles had collapsed in the day 3 group, and mitochondria as well as sarcoplasmic reticulum were completely destroyed and scattered, and the shape of muscles was not detectable due to collapsed muscle fibers and endomysium. Moreover, the unit mitochondrial membrane was mostly destroyed, and many bacteria were observed among the myofibrils (Fig. 2E, F).

#### 2) Experimental groups

Typical muscle fibers were observed on the longitudinal section with regularly arranged thin and thick filaments in the day 1 experimental group. Additionally, the A, H, and I bands as well as the Z line were clearly observed in the myofibrils. Sarcoplasmic reticulum was also found among the filaments (Fig. 3A). The shape of the muscle fibers was almost the same in the day 2 experimental group as that in the day 1 experimental group. Capillaries containing red blood cells and Z lines were also observed among muscle fibers (Fig. 3B). Under high magnification of the horizontal section, the day 3 experimental group clearly showed thick and thin filaments and Z lines, but only part of mitochondrial membranes were observed among muscle fibers (Fig. 3C). Collapse of connective tissues among muscle fibers occurred in the day 4 group, and there were expanded spaces among connective tissue compared with those of the day 2 and 3 groups, but the overall shape of the muscle fibers was well maintained (Fig. 3D). The shapes and spaces of the muscle fibers were similar in the day 5 experimental group than those in the day 3 group. Capillaries containing red blood cells were found in the connective tissue among muscle fibers, and part of the structures with the shape of concentric circles were observed due to collapse of the connective tissue. However, no bacteria were observed in the muscle fibers (Fig. 3E).

## DISCUSSION

Stabilized chlorine dioxide ($ClO_2$) has been used as an effective disinfectant mainly in the food industry due to its strong oxidation and disinfective effects. As the chemical bond of chlorine dioxide is unstable, it can cause an explosion if mixed with air at a concentration of 10% or more, and it is easily dissolved at high temperature and under strong light. Stabilized chlorine dioxide is based on chlorine dioxide by integrating chlorine dioxide into the powder or particles of calcium silicate or zeolite and then reverse diffusing it into air little by little (Jinli et al., 2001).

Harakeh et al. (1988) reported that "stabilized chlorine dioxide" has microbicidal effects based on its strong oxidation effects, and is an ideal agent which does not develop problem of immunity or tolerance, but has deodorization effects. Prokop (1991) and Kastner et al. (2003) reported that stabilized chlorine dioxide has effective microbicidal effects at acidic condition, well functions at low temperature, has no taste or odor, and especially has deodorization effects. Smith & Willhite (1990) compared stabilized chlorine dioxide with other chlorine group compounds. Also, stabilized chlorine dioxide does not form trihalomathane (THM) which is a carcinogenic substance generated from the reaction between free chlorine ions of chlorine group compounds and organic matters in water. The reason is that the reaction is not a chlorination reaction which is generally seen in chlorine group compounds but an oxidation reaction (Smith & Willhite, 1990; Świetlik et al., 2002, 2003).

Harakeh et al. (1988) and Kastner et al. (2003) reported that the disulfide bond in the three-dimensional structure of a protein is a major deodorization mechanism, and that the effects of antiseptic and microbicides are accomplished by the disulfide bond through denaturing functions of enzymes. Dorn & Hopkins (1998) reported that stabilized chlorine dioxide has antiseptic effects in tissues by virtue of the disulfide bond,

whereas preservatives and disinfection agents with aldehyde groups show their antiseptic and disinfective effects by combining the aldehyde with a nitrogen group, which is mainly present in amino acids.

According to the light microscopic observation of the present study, the control group showed expanded space among muscle fibers in accordance with separation of the endomysium which surrounds muscle fibers, and destruction of connective tissue. As time went on, nuclei below the cell membrane and endomysium were destroyed, and most of the connective tissue among the muscle fibers was also necrotized, resulting in difficulty distinguishing muscle fibers. This is due to enhanced activity of decomposition caused by air and water contacts (Sundberg & Jönsson, 2008). According to the TEM observations, the endomysium was destroyed, mixed myofibrils were found, and decomposed bacteria were found among the muscle fibers. As time went on, muscle shape could not be distinguished, as muscle fibers, endomysium, and connective tissue were destroyed. Mitochondria among the muscle fibers were also destroyed leaving only part of the outer membrane. These results are similar to the light microscopic results and coincide with those of a chronological antiseptic study conducted on kidney tissues of rats (Hwang et al., 2008).

The day 2 experimental group showed a clear boundary among muscle fibers, myofibrils inside the muscle fibers, and nuclei below the cell membrane on light microscopy. These results were similar to those of the day zero control group. The day 3 group showed necrosis in the connective tissue among muscle fibers, but the shape of the myofibrils was similar to that of the day zero control group. In contrast, the day 4 and 5 groups did not show morphological changes in muscle fibers and overall, the results were similar to those of the day 3 group. These results were closely related with those of an antiseptic study on the disulfide bond conducted by Tan et al. (1987), and those of Han et al. (2001) who reported a microbicide effect, which was realized by nonactivated $ClO_2$ gas reacting with the *E.coli* cell surface. Additionally, chlorine dioxide was considered to inhibit decomposition through oxidation with peptide and amino groups resulting in a slow decomposition process.

The day 1 and 3 experimental groups showed shapes of typical muscle fibers with regular arrangements of thin and thick filaments under TEM. Additionally, sarcoplasmic reticulum was observed among filaments. Thin and thick filaments and the Z line were clearly found in the day 3 group, but part of the mithchodrial membranes among muscle fibers were destroyed. The day 4 group showed slightly expanded spaces due to destroyed connective tissue among muscle fibers, but the shape of the muscle fibers was still maintained. The day 5 experimental group showed concentric circle structures due to destruction of connective tissue among the muscle fibers, but no bacteria were found. This result coincided with that of Kastner et al. (2003) who reported that acidic excrement activates stabilized chlorine dioxide resulting in an enhanced microbicide effect. In other words, as acidity increase, the activity of stabilized chlorine dioxide increase to inhibit acidification caused by decomposition.

In summary, the day 1 control group showed the beginning of tissue collapse and shapes were not distinguishable after day 3 due to almost completely collapsed tissues. By comparison, the experimental group showed partial collapse on day 3 and later groups, but the overall shape of the muscles was maintained as time went on confirming the antiseptic effect of stabilized chlorine dioxide on skeletal muscle.

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## ＜국문초록＞

강한 산화력과 소독효과로 인해 광범위하게 사용되고 있는 안정화 이산화염소(S-$ClO_2$)의 근육조직에 대한 부패억제 효과를 알아보고자 본 연구를 수행하였다. 본 실험을 위해 8주령 SD계 흰쥐 골격근(skeletal muscle)을 사용하였고, 안정화 이산화염소를 처리하지 않은 대조군과, 안정화 이산화염소의 분말과 수용액을 처리한 실험군으로 분류하여 광학 및 투과전자현미경으로 관찰하였다.

광학현미경 관찰 결과, 대조군 1일부터 근섬유막이 분리되기 시작하였고 핵의 붕괴가 관찰되었다. 2일군에서는 근섬유막이 붕괴되어 근섬유의 형태만 남아 있었고, 근원섬유도 붕괴되는 양상을 나타내었다. 3일군 이후 대조군의 경우는 대부분의 근섬유막과 결합조직의 붕괴로 근섬유의 구분이 불분명하였다. 실험군의 경우, 3일군에서 근섬유 사이 결합조직의 부분적인 붕괴가 관찰되었다. 그러나 4, 5일군에서도 3일군과 유사한 양상을 나타내었다.

한편, 전자현미경적 소견은 대조군의 경우 1일군부터 근섬유막의 붕괴로 근섬유의 경계가 불분명하였고, 미토콘드리아막도 부분적인 파괴가 관찰되었다. 2일째부터 근섬유막이 모두 붕괴되었고 근섬유 사이에서 세균이 관찰되었다. 3일군 이후는 근육의 형태를 구별할 수 없었다. 그러나 실험군의 경우, 1일과 2일군은 전형적인 근육의 미세구조를 관찰할 수 있었고, 3일군부터 부분적인 미토콘드리아막의 붕괴가 관찰되었다. 이후 시간경과에 따라 결합조직이 붕괴되어 동심원상의 구조물이 일부 관찰되었다.

따라서 본 연구결과, 대조군은 1일군부터 조직의 붕괴가 시작되어 3일군 이후는 대부분 붕괴되어 형태를 구분할 수 없었으나, 실험군의 경우는 3일군부터 부분적인 붕괴가 시작되었고 이후 시간이 경과되어도 전체적인 근육의 형태가 유지되어 안정화 이산화염소가 근육에 부패억제 효과가 있는 것으로 확인되었다.