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Differential Effect of Denervation on Free Radical Scavenging
Enzymes in Slow and Fast Muscle of Rat

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Footnote:

Abbreviation used;

CuZnSOD; cuprozinc superoxide dismutase

MnSOD ; manganosuperoxide dismutase

EDL ; extensor digitorum longus muscle

Abstract

To determine the effect of denervation on the free radical scavenging systems in relation to the mitochondrial oxidative metabolism in the slow twitch soleus and fast twitch extensor digitorum longus (EDL) muscles, the sciatic nerve of the rat was crushed in the mid thigh region and the muscle tissue levels of 5 enzymes were studied 2 and 5 weeks following crush. Recently developed radioimmunoassays were utilized for the selective measurement of cuprozinc (cytosolic) and manganano (mitochondrial) superoxide dismutases. Total tissue content of cuprozinc superoxide dismutase showed a mild decrease after denervation in slow but not in fast muscle. Manganosuperoxide dismutase and fumarase decreased markedly at 2 weeks and returned toward control levels by 5 weeks, the changes appearing to be greater in slow than in fast muscle. At 2 weeks, cytochrome c oxidase decreased significantly in slow, but not in fast muscle. GSH-peroxidase at baseline was 10 fold higher in slow than in fast muscle, markedly decreased at 2 weeks in slow muscle and returned toward control levels at 5 weeks, while the total enzyme activity in fast muscle did not change through 5 weeks. These data represent the first systematic report of free radical scavenging systems in slow and fast muscles in response to denervation. Selective modification of cuprozinc and manganosuperoxide dismutases and differential regulation of GSH-peroxidase was demonstrated in slow and fast muscle.

Key words: cuprozinc superoxide dismutase; manganosuperoxide dismutase; glutathione peroxidase; denervation; soleus (slow); extensor digitorum longus muscle (fast).

Running title: Denervation; free radical scavenging enzymes.

In aerobic cells oxygen-containing free radicals, i.e. superoxide, hydrogen peroxide etc., are generated as by-products of oxidative metabolism. These toxic species are primarily scavenged by certain enzymes. Superoxide dismutases (SOD), which catalyze the conversion of superoxide to hydrogen peroxide, are found in two forms in mammalian cells. One contains copper and zinc and is found predominantly in cytosol, the other contains manganese and is found predominantly in mitochondria (Asada et al., 1980). GSH-peroxidase catalyzes the reduction of hydrogen peroxide and certain lipid peroxides. This enzyme is considered to play a major role as the scavenger of hydrogen peroxide under low endogenous production rates of hydrogen peroxide pertaining under physiological conditions (Jones et al., 1981).

Metabolic alterations leading to the depressed energy production by both aerobic (Nemeth, 1980; Dubois and Max, 1983) and anaerobic metabolism (Shakelford and Leberherz, 1981) in denervated muscles have been shown. However, the changes in free radical scavenging systems after denervation have not been studied systemically. There is an increasing interest in the possible involvement of these systems in the pathophysiology of muscle disease (Kar and Pearson, 1979; Matkovics et al., 1982; Mizuno, 1983). The present study was designed to determine if the depression of energy metabolism after denervation would produce selective modification in free radical scavenging enzymes (CuZn and MnSOD and GSH-peroxidase) in the slow twitch soleus or in fast twitch extensor digitorum longus (EDL) muscle. In rat the fast twitch EDL has a predominance of type II glycolytic fibers, while the slow twitch soleus is composed mainly of type I oxidative fibers. Recently developed radioimmunoassays for rat CuZn and MnSOD (Asayama and Burr, 1985) enable accurate, specific measures of SODs in small specimens. In order to define

the specificity of any change in MnSOD, two mitochondrial marker enzymes were also measured: cytochrome c oxidase as a marker of membrane associated enzymes and fumarase as a marker of matrix associated enzymes.

Materials and Methods

Surgical Procedures

Male, Harlan Sprague-Dawley rats (160 to 180g) were anesthetized with ether. The left sciatic nerve was crushed at the sciatic notch over a 3 mm segment for 30 s with a serrated hemostat, and the efficacy of functional denervation and subsequent reinnervation were assessed as described previously (Dettbarn, 1981). After nerve crush, hindlimb muscles showed a progressive denervation throughout the initial 2 weeks. Beginning of reinnervation was observed between the 10th and 12th day following nerve crush. Full functional reinnervation, as established by toe spreading and response to indirect stimulation, occurred around the 5th week. Rats were sacrificed by decapitation at 2 and 5 weeks after nerve crush and the left soleus and EDL muscles were removed after the response to electric indirect stimulation was observed. To eliminate the variables introduced by possible functional compensation induced by the contralateral surgery (Luttges et al., 1976; Theiler et al., 1978), muscles from age-matched nonoperated animals were used as controls rather than the right hindlimb muscles of the operated animals.

Muscles were homogenized with a 10 volume (w/v) of 10mM sodium phosphate buffer (pH 7.6) by a Potter-Elvehjem homogenizer. The homogenate was sonicated on ice for 1 min (15 sx 4 times) and then centrifuged at 13,000xg. The supernatant was stored frozen at -70°C and diluted with the appropriate assay buffer just prior to each enzyme assay.

Biochemical Analyses

The radioimmunoassays for rat CuZn and MnSOD have been described previously (Asayama and Burr, 1985). GSH-peroxidase activity was assayed by the enzyme-coupled method of Beutler et al. (1977) with modifications. The reaction mixture consisted of sample, 2 mM GSH, 0.2 mM NADPH, 1 Unit of yeast glutathione reductase (E.C. 1.6.4.2., Sigma Chemical Co., St Louis, MO) and 0.35 mM t-butyl hydroperoxide in 1 ml of 100 mM Tris/HCl/0.5 mM EDTA buffer (pH 7.6). The assay was performed at 25°C. A Unit of activity was defined as μmol NADPH oxidized/min. Cytochrome c oxidase activity was assayed spectrophotometrically by the method of Wharton and Tzagoloff (1967). The activity was defined in terms of the first-order velocity constant (s^{-1}). Fumarase activity was assayed by the method described by Stitt (1984) using L-malate as the substrate. A Unit was defined as μmol fumarate produced/min. Protein was measured by the method of Lowry et al. (1951). The data for the enzyme assays are expressed as total muscle content for SOD or activity for the other enzymes (i.e. μg or Unit/muscle). For the calculation of specific tissue concentration (or activity), the data were also normalized according to protein content.

Statistical significance was assessed utilizing the Student's t-test.

Results

Muscle Weights

Table 1 summarizes the changes in muscle weight following nerve crush. There was a significant loss of muscle weight in both soleus and EDL muscles. After 2 weeks of denervation, the mean muscle weight had decreased to 46% and 59% in soleus and EDL, respectively, of age-matched controls. Five weeks

after operation, muscle weights had returned toward but had not reached those values obtained in control animals.

Superoxide Dismutase Levels

As shown in Fig. 1., the CuZnSOD content in soleus muscle (slow twitch) after 2 weeks of denervation decreased to 51% of the controls, while the specific enzyme concentration (i.e. per mg protein) remained at the control level (Table 3a). After 5 weeks of nerve crush, CuZnSOD increased to 69% of the 5 weeks controls. The MnSOD content in soleus had decreased to 14% of the controls after 2 weeks and returned to the control levels by 5 weeks (Fig. 1.).

Fig. 2. shows that the CuZnSOD content in EDL muscle (fast twitch) while failing to decrease during the first 2 weeks after denervation, remained lower than that of the 5 week controls. The MnSOD levels in EDL showed similar qualitative changes to that in the soleus, whereas the effect was quantitatively less marked- 30% of the control level being retained after 2 weeks of denervation.

GSH-peroxidase Levels

The GSH-peroxidase activities in both soleus (slow) and EDL (fast) muscle are summarized in Fig. 3. When the control values both at 2 and 5 weeks were compared, soleus had a 10 times higher specific activity than EDL muscle. After 2 weeks of denervation, the total muscle activity in soleus decreased to 10% of the control level and then returned to 47% of the controls at 5 weeks. On the other hand, the total activity in EDL muscle did not change significantly through 5 weeks of the study period, while the specific activity in the 2 weeks operated group was 2.5 fold higher than that of the 2 weeks controls (Table 3a).

Cytochrome c Oxidase and Fumarase Levels

Table 2 summarizes the total muscle activities of the two mitochondrial marker enzymes in both soleus and EDL muscles. The cytochrome c oxidase level in soleus decreased to 15% of the controls after 2 weeks of denervation and then returned to 37% of the controls by 5 weeks. Conversely, the activity in EDL showed a smaller (statistically insignificant) change and the specific activity did not change through the 5 weeks of study (Table 3a and 3b).

The fumarase levels after 2 weeks of denervation decreased to 10% and 29% of the 2 weeks controls in soleus and EDL, respectively, and returned to 63% and 103% of the controls after 5 weeks in soleus and EDL, respectively, i.e. the changes were quantitatively less in EDL than in soleus.

Discussion

The present studies have demonstrated significant effects of denervation and subsequent reinnervation on the free radical scavenging enzymes and other mitochondrial enzymes in slow (soleus) and fast twitch (EDL) muscles of the rat. MnSOD decreased markedly after denervation and returned toward normal levels during the period of reinnervation. Fumarase activity decreased in a quantitatively similar extent to MnSOD in both slow and fast muscles after denervation. However, the decrease of cytochrome c oxidase after denervation was statistically significant in slow but not in fast muscle.

In contrast to MnSOD, CuZnSOD showed a mild decrease in total muscle content and no decrease in specific tissue content after denervation in both slow and fast muscles. GSH-peroxidase activity was 10 fold higher in slow than in fast muscle under basal conditions, and decreased markedly in slow, but not in fast after denervation.

Denervation had a similar but more profound effect on slow than on fast muscle in terms of muscle weight and changes in MnSOD, fumarase and cytochrome c oxidase levels. The greater loss of weight in soleus than in EDL muscle has been previously observed (Margreth et al., 1972; Dettbarn, 1981). It has also been shown that denervation leads to a decrease in the levels of enzymes involved in oxidative metabolism such as cytochrome c oxidase (Nemeth et al., 1980) and tricarboxylic acid-cycle-associated dehydrogenases (Turner and Manchester, 1972).

Numerous publications have described the relationship between indices of mitochondrial function and/or enzyme levels and muscular activity. Denervation or disuse of muscle results in a loss of various mitochondrial functions (Joffe et al., 1981; 1983) as well as decrease in several mitochondrial enzyme activities (Turner and Manchester, 1972; Booth and Kelso, 1972; Rifenberick et al., 1973; Nemeth et al., 1980). Conversely, endurance training induces a biochemical adaptation of muscle mitochondria, leading to an increase in oxidative capacity and specific activity of several mitochondrial enzymes (Davies et al., 1981). Electrical stimulation has been shown to prevent the denervation induced decreases in oxidative enzyme levels (Nemeth, 1982). In the present study, the enzymes in the matrix space of the mitochondria (i.e. MnSOD and fumarase) decreased to a greater extent than the membrane bound enzyme, cytochrome c oxidase. This data is consistent with the observation of Rifenberick et al. (1973) who observed a greater decrease in a matrix enzyme (malate dehydrogenase) than in membrane bound enzymes (cytochrome c oxidase and monoamine oxidase) in mitochondria isolated from atrophic muscle.

The present study has demonstrated that denervation results in a marked

decrease in MnSOD levels in both the slow and the fast muscle, but only a marginal change in CuZnSOD levels. To our knowledge, this is the first report indicating a selective modification of CuZn and MnSOD levels in muscle in response to depression of cell function, including oxidative metabolism.

The decrease in MnSOD following denervation was associated with the anticipated decrease in fumarase activity indicative of a decrease in mitochondrial activity. It is known that a major contributor to free radical production within mitochondria is the ubiquinone-semiubiquinone system (Koren et al., 1983), the activity of which reflects general mitochondrial metabolic activity. Further, free radical production in muscle mitochondria is increased by muscular contraction (Davies et al., 1982; Koren et al., 1983). These data collectively suggest that MnSOD concentration reflects mitochondrial free radical production and that this is in turn a function of mitochondrial metabolic activity. This conclusion is supported by a previous study in which a selective induction of mitochondrial MnSOD was observed during functional stimulation of monocytes, a change associated with enhanced oxidative metabolism (Asayama et al., *Am. J. Physiol.* In press, 1985).

With respect to GSH-peroxidase, there was a significant difference between slow and fast muscle in terms of both baseline specific activity and response to denervation. Type I fibers of the soleus are more susceptible to atrophy and the proportion of type II fibers is increased in the atrophic soleus muscle (Templeton et al., 1984). Further, type I (slow) fibers can be converted to type II (fast) fibers during the course of disuse induced muscle atrophy (Corley et al., 1984). Thus, the decrease of the specific activity of GSH-peroxidase in slow muscle to a level comparable to that of fast muscle might be influenced by the metabolic alteration related to the conversion of type I to type II fibers.

The 10 fold higher GSH-peroxidase activity in slow muscle when compared with that of the fast muscle may have important practical implications. Histochemical classification of muscle fiber types is based on differences in activity of enzymes distributed in these muscles. Since GSH-peroxidase activity is higher in slow muscle, the histochemical identification of this enzyme may be a useful tool in the studies of muscle fiber type identification.

A common enzymic pattern in dystrophic muscle is a decrease in NAD-linked dehydrogenases and an increase in the NADP-linked enzymes (McCaman, 1963). Indeed, increases in the specific activities of glucose 6-phosphate dehydrogenase (Max et al., 1981) and glutathione reductase (Turner and Manchester, 1972) have been observed in denervated fast twitch muscle. GSH-peroxidase is coupled with glutathione reductase and both enzymes catalyze the oxidation-reduction cycle of glutathione. The increase in specific activity of GSH-peroxidase observed in our experiments in fast muscle after 2 weeks of denervation is in keeping with these observations.

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Figure Legends

Fig 1. Effect of nerve crush on immunoreactive superoxide dismutase levels in rat soleus muscle. The animals were studied at 2 and 5 weeks after surgery. The data are expressed as total muscle content ($\mu\text{g}/\text{muscle}$). The bars indicate SEM ($n=6$). P values were calculated by Student's t-test. CuZnSOD; cuprozinc superoxide dismutase, MnSOD; manganosuperoxide dismutase, C; unoperated controls, O; operated. * $p<0.001$ v controls; CuZnSOD 2wC v 5wC $p<0.005$, 2wO v 5wO $p<0.001$; MnSOD 2wO v 5wO $p<0.001$.

Fig 2. Effect of nerve crush on immunoreactive superoxide dismutase levels in rat extensor digitorum longus muscle. The bars indicate SEM ($n=6$); Student's t-test. The abbreviations in the figure are defined in the legend of figure 1. * $p<0.01$ ** $p<0.001$ v controls; CuZnSOD 2wC v 5wC $p<0.05$; MnSOD 2wO v 5wO $p<0.001$.

Fig 3. Effect of nerve crush on the activity of GSH-peroxidase ($\mu\text{mol}/\text{min}/\text{muscle}$) in rat soleus and extensor digitorum longus (EDL) muscle. The bars indicate SEM ($n=6$), Student's t-test. The abbreviations (C and O) are defined in the the legend of figure 1. * $p<0.02$, ** $p<0.001$ v controls; Soleus 2wO v 5wO $p<0.001$.

Table 1. Muscle weights after nerve crush.

Weeks	Soleus		EDL	
	Operated	Controls	Operated	Controls
2	57 \pm 4 ^a	125 \pm 4	80 \pm 5 ^c	136 \pm 2
5	112 \pm 6 ^{b,e}	130 \pm 1	116 \pm 4 ^{d,f}	131 \pm 1

Values are in mg, mean \pm SEM (n=6).

a. $p < 0.001$, b. $p < 0.02$, c. $p < 0.001$, d. $p < 0.005$ versus controls.

e. $p < 0.001$, f. $p < 0.001$ versus 2 week operated. Student's t-test.

EDL; extensor digitorum longus muscle.

Table 2. Activities of Cytochrome c oxidase and fumarase
in rat muscle sonicate.

	Soleus		EDL	
	Cytochrome c Oxidase	Fumarase	Cytochrome c Oxidase	Fumarase
2 weeks Controls	0.312 \pm 0.082	4.19 \pm 0.61	0.210 \pm 0.031	3.30 \pm 0.53
Operated	0.047 \pm 0.005 ^a	0.42 \pm 0.25 ^d	0.125 \pm 0.032	0.95 \pm 0.70 ^f
5 weeks Controls	0.326 \pm 0.063	3.82 \pm 0.52	0.227 \pm 0.036	4.04 \pm 0.39
Operated	0.122 \pm 0.015 ^{b,c}	2.42 \pm 0.36 ^e	0.160 \pm 0.022	3.41 \pm 0.45 ^g

Values are mean \pm SEM, in Unit $k(s^{-1})$ muscle for cytochrome c oxidase and $\mu\text{mol}/\text{min}/\text{muscle}$ for fumarase.

a. $p < 0.005$, b. $p < 0.02$ versus controls. c. $p < 0.001$ versus 2 weeks operated.

d. $p < 0.001$ versus controls. e. $p < 0.005$ versus 2 weeks operated.

f. $p < 0.01$ versus controls. g. $p < 0.005$ versus 2 weeks operated.

Student's t-test ($n=4-6$).

EDL; extensor digitorum longus muscle.

Table 3a. Specific enzyme concentrations in muscle at 2 weeks after nerve crush

Enzymes	Soleus		EDL	
	Controls	Operated (%)	Controls	Operated (%)
CuZn superoxide dismutase	0.664 \pm 0.070	0.689 \pm 0.010 (104)	0.403 \pm 0.032	0.519 \pm 0.052 (129)
Mn superoxide dismutase	0.570 \pm 0.050	0.160 \pm 0.024 ^a (28)	0.378 \pm 0.027	0.184 \pm 0.021 ^a (49)
GSH peroxidase	311 \pm 35	68 \pm 34 ^a (22)	29 \pm 5	75 \pm 18 ^c (257)
Cytochrome c oxidase	40.2 \pm 12.1	15.3 \pm 1.4 ^c (38)	24.8 \pm 3.4	27.4 \pm 7.7 (110)
Fumarase	530 \pm 97	113 \pm 52 ^b (21)	404 \pm 51	205 \pm 76 (51)

Values are mean \pm in $\mu\text{g}/\text{mg}$ protein for superoxide dismutases, mU/mg protein for GSH peroxidase and fumarase, and Unit $\text{k}(\text{s}^{-1})/\text{g}$ protein for cytochrome c oxidase. The relation of the concentration of the operated tissue as a percentage of the controls is indicated by the figures in parantheses. Student's t-test ($n=4-6$). EDL; extensor digitorum longus muscle.

a. $p<0.001$, b. $p<0.005$, c. $p<0.05$ (v controls).

Table 3b. Specific enzyme concentrations in muscle at 5 weeks after nerve crush

Enzymes	Soleus		EDL	
	Controls	Operated (%)	Controls	Operated (%)
CuZn superoxide dismutase	0.838 \pm 0.065	0.532 \pm 0.042 ^a (63)	0.736 \pm 0.088	0.469 \pm 0.032 ^c (64)
Mn superoxide dismutase	0.542 \pm 0.025	0.411 \pm 0.026 ^b (76)	0.520 \pm 0.040	0.426 \pm 0.036 (92)
GSH peroxidase	381 \pm 64	271 \pm 38 (71)	39 \pm 4	45 \pm 12 (114)
Cytochrome c oxidase	34.9 \pm 4.5	21.6 \pm 4.0 (62)	26.1 \pm 4.3	26.4 \pm 3.6 (101)
Fumarase	423 \pm 54	434 \pm 81 (103)	346 \pm 46	579 \pm 48 (133)

Values are mean \pm in ug/mg protein for superoxide dismutases, mU/mg protein for GSH peroxidase and fumarase, and Unit k(s⁻¹)/g protein for cytochrome c oxidase. The relation of the concentration of the operated tissue as a percentage of the controls is indicated by the figures in parantheses. Student's t-test (n=5-6). EDL; extensor digitorum longus muscle.

a. p<0.005, b. p<0.01, c. p<0.05 (v controls).

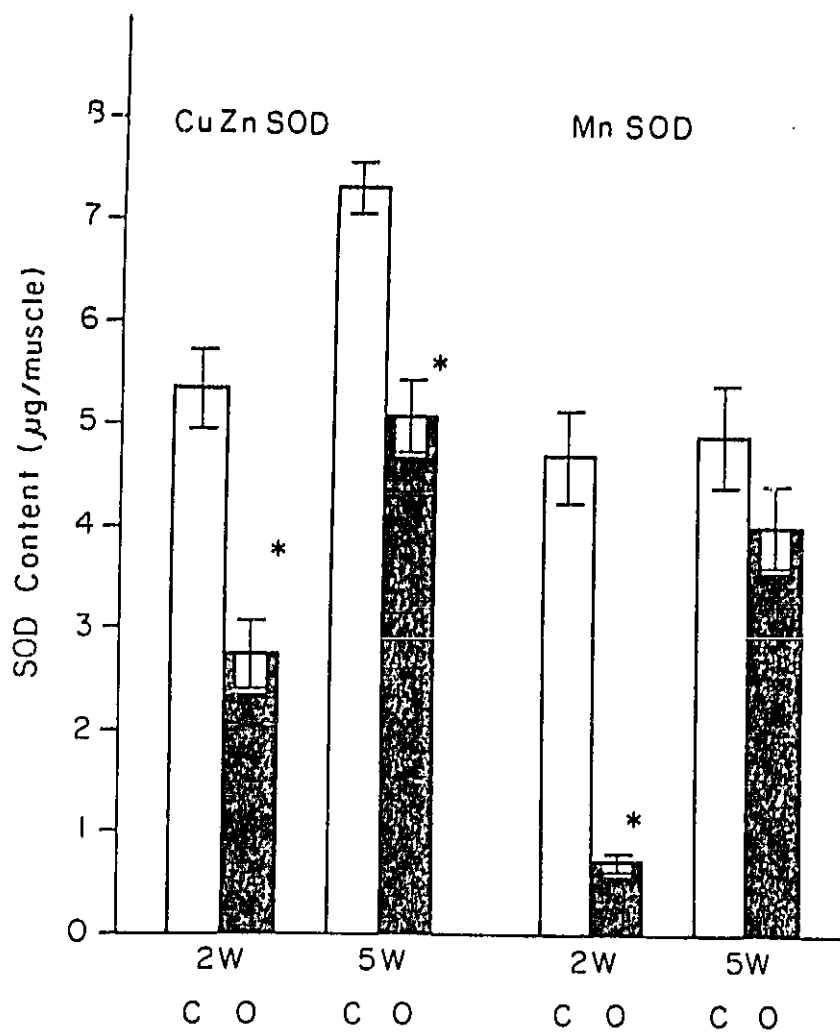


Fig. 1.

CHRONIC EFFECTS
OF POOR QUALITY

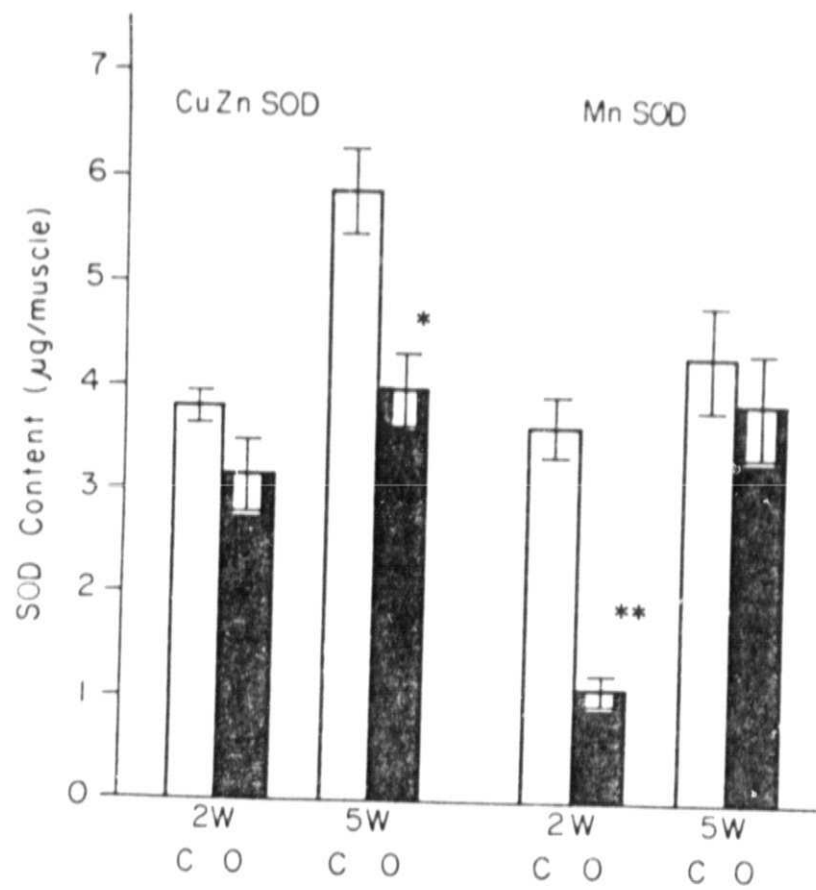


Fig. 2.

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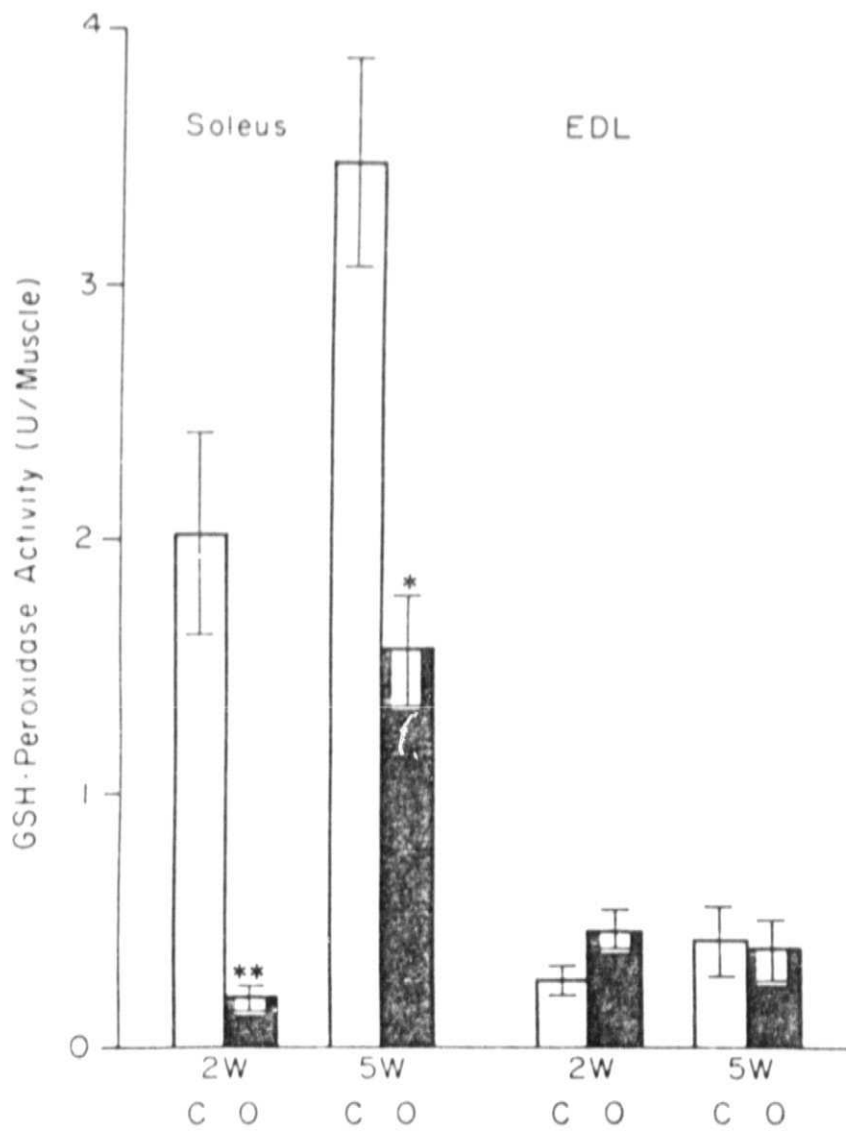


Fig. 3.