Effect of zinc deficiency on muscle fibre type frequencies in the post-weanling rat

BY C. A. MALTIN, L. DUNCAN, A. B. WILSON AND J. E. HESKETH
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

(Received 9 February 1983 – Accepted 23 May 1983)

1. Male weanling rats were maintained on diets either deficient or adequate in zinc for a period of 4 weeks. The rats on the deficient diet showed a reduction in food intakes and growth.

2. After 4 weeks both soleus muscles and the lateral portion of the diaphragm were studied histochemically to examine the relative frequencies of the fibre types.

3. The soleus muscles of the deficient animals showed a significant change in the proportion of slow and fast fibres. The diaphragm muscles of the deficient animals had a significant increase in the proportion of fast-twitch oxidative glycolytic fibres and a significant decrease in fast-twitch glycolytic fibres compared with the controls.

4. Stainable lipid increased in the diaphragm muscle of the deficient animals with respect to their pair-fed controls.

Zinc is an essential micronutrient which is necessary for normal growth in animals and man (Underwood, 1977). Zn deficiency is characterized by growth retardation, reduced intake and impaired utilization of food, alopecia, keratosis, infertility, malabsorption and delayed wound healing (Chesters, 1978). Despite the fact that Zn is a known component of many metalloenzymes, such as lactate dehydrogenase (EC 1.1.1.27) and carbonic anhydrase (EC 4.2.1.1), the physiological and biochemical consequences of Zn deficiency are not well understood; thus at present it is not possible to explain all the known manifestations of Zn deficiency.

Zn appears to play an important role in nucleic acid metabolism, lipid metabolism and protein synthesis: it may even be a structural component of enzymes involved in these processes (Scrutton et al. 1971). An impairment of DNA synthesis (Swenerton et al. 1969) and a decrease in RNA polymerase (EC 2.7.7.6) activity (Terhune & Sandstead, 1972) have been reported in Zn deficiency. Recent studies suggest that Zn may play a particular role in regulating the metabolism of essential fatty acids (Bettger et al. 1979).

Since muscle is the largest single tissue in mammals, it is likely that the growth retardation associated with Zn deficiency has a major impact on this tissue. Large amounts of Zn are found in muscle; slow-twitch fibres are reported to contain three times as much Zn as fast-twitch fibres (Cassens et al. 1967) and Zn ions have been shown to be involved in both muscular contraction (Edman, 1958, 1960; Issacson & Sandow, 1963) and neuromuscular transmission (Sandow & Bien, 1962).

However, deficiency per se has been shown to have little effect on muscle Zn content (Jackson et al. 1982). Furthermore, O'Leary et al. (1979) using weight-restricted controls, have stated that, with the possible exception of soleus muscle, deficiency per se has little effect on the number and size of the muscle fibres. On the other hand, Huber & Gershoff (1973) showed a depression in the activity of a Zn metalloenzyme, lactate dehydrogenase, in heart and gastrocnemius muscle from Zn-deficient rats thereby implying some metabolic effect in the deficiency.

In order to investigate further the role of Zn in muscle, a study has been made of the effects of nutritional Zn deficiency on muscle in the young, growing, male rat. Two muscles have been studied. Soleus muscle was chosen because, from the work of O'Leary et al. (1979),
it appears to be a 'target' muscle. Diaphragm was selected since it is constantly active from birth and has a metabolism which relies heavily on fat as an energy source. Furthermore, diaphragm is often described as having characteristics similar to both cardiac and skeletal muscle, and might therefore reflect the deficiency (Huber & Gershoff, 1973).

**MATERIALS AND METHODS**

Male Hooded Lister weanling rats from the Rowett Research Institute strain, weighing 46–55 g were randomly divided into three groups: one Zn-deficient and two control groups. The Zn-deficient (n 15) and ad lib. control (n 4) groups received their feed ad lib. The second control group (n 13) was pair-fed with the Zn-deficient animals. All animals were individually housed and fed for a 4-week period on a semi-synthetic diet based on egg-albumin, sucrose and arachis oil supplemented with vitamins and minerals (Williams & Mills, 1970). The deficient diet contained less than 1·1 mg Zn/kg, while the control diet was supplemented to give a Zn content of 40 mg/kg. Body-weights and food intakes were recorded.

The animals were anaesthetized with anaesthetic diethyl ether and exsanguinated by cardiac puncture. The blood was collected in heparinized acid-washed tubes for atomic absorption spectrometric determination of the Zn concentration in the trichloroacetic acid soluble fraction of plasma.

The soleus and diaphragm muscles were removed. Soleus was dissected from tendon to tendon and the mid-region was selected for examination. The lateral region of diaphragm midway between the ventral midline and the dorsal region was selected for study. This region was sampled specifically in each animal to avoid any possible discrepancies due to regional variation (Susheela & George, 1964). Small pieces of both tissue about 1 mm³ were taken for electron microscopy and were fixed in 25 g glutaraldehyde/ml 0·1 M-phosphate buffer, pH 7·5. After being washed with buffer the muscle samples were post-fixed in 1 g osmium tetroxide/l overnight, block-stained, dehydrated and embedded in Araldite. Thin sections were stained with lead citrate and examined in an AEI 801 electron microscope.

Muscle samples for histochemical examination were supported by blocks of chilled liver, placed on small pieces of cork, covered in talcum powder and frozen in liquid nitrogen. In order to establish histochemical profiles of the muscles, transverse cryostat serial sections (10 μm) were cut and stained. The metabolic nature of the fibres was determined using methods for succinate dehydrogenase (EC 1·3·99·1; SDH) (Pearse, 1972), Sudan black B (Chayen et al. 1973) and L-glycerol-3-phosphate menadione oxidoreductase (EC 1·1·99·5; GPOX) (Lobley et al. 1977). With a histochemical method which is considered to indicate the intrinsic speed of contraction of the fibres, i.e. Ca²⁺-activated myofibrillar adenosine triphosphatase (EC 3·6·1·3; ATPase) pH 9·5 (Hayashi & Freiman, 1966), it was possible to describe the fibres as fast or slow. A profile of fibre types based on each of the above stains was determined for each of at least 200 fibres per muscle in each animal. Distinct fibre types were identified on the basis of their staining reaction (Table 1): fast-twitch glycolytic (FG), fast-twitch oxidative glycolytic (FOG), fast-twitch oxidative (FO) and slow-twitch oxidative (SO). The system of nomenclature used was based on that described by Peter et al. (1972). The values from each dietary group were pooled and the mean taken. Fibre diameters were measured from transverse sections stained by the ATPase method and using the method described by Dubowitz & Brooke (1973). Photomicrographs were used at a final magnification of ×67 for all determinations.

Samples of soleus muscles from animals from each group were taken for biochemical analysis. DNA estimations were made by the colorimetric method of Burton (1956) and protein was determined by the method of Lowry et al. (1951).
Table 1. Summary of response to stains of different muscle fibre types in rat diaphragm

<table>
<thead>
<tr>
<th>Fibre type... Stain</th>
<th>Fast-twitch glycolytic</th>
<th>Fast-twitch oxidative glycolytic</th>
<th>Fast-twitch oxidative</th>
<th>Slow-twitch oxidative</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>++ ++</td>
<td>++ ++</td>
<td>++ ++</td>
<td>+</td>
</tr>
<tr>
<td>SDH</td>
<td>+</td>
<td>++ ++</td>
<td>++ ++</td>
<td>++ ++</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>+</td>
<td>++ ++</td>
<td>++ ++</td>
<td>++ + +</td>
</tr>
<tr>
<td>$\alpha$-GPOX</td>
<td>++ ++</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

+, Weak staining reaction; ++ + +, intense staining reaction.

ATPase, Ca$^{2+}$-activated myofibrillar adenosine triphosphatase (EC 3.6.1.3); SDH, succinate dehydrogenase (EC 1.3.99.1); $\alpha$-GPOX, $\alpha$-glycerol-3-phosphate menadione oxidoreductase (EC 1.1.99.5).

RESULTS

Dietary Zn deficiency resulted in reduced weight gain (Fig. 1) and both a reduction and a 'cycling' of food intake similar to that described by Chesters & Quarterman (1970). At the end of the 4-week period the mean body-weight of the Zn-deficient animals was about 33% of the ad lib. controls and 72% of the pair-fed control animals. The animals deprived of dietary Zn had plasma Zn concentrations which were less than half of those in the Zn-supplemented control animals.

Soleus muscle

Soleus muscles from Zn-deficient animals showed an over-all reduction in wet weight, fibre number and fibre size in comparison with both the pair-fed and ad lib.-fed control animals. These results are similar to those described by O'Leary et al. (1979). The application of the four histochemical methods showed that the soleus muscles in all animals comprised three fibre types, SO, FOG and FO. The relative frequency of the fibre types are shown in
Table 2. Relative fibre type frequencies, fibre diameters and percentage areas of fibre types in soleus muscle from zinc-deficient and control rats
(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Relative frequency (%)</th>
<th>Fibre diameter (μm)</th>
<th>Percentage area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SO</td>
<td>FOG</td>
<td>FO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>ZD</td>
<td>8</td>
<td>56**</td>
<td>0.95</td>
<td>38</td>
</tr>
<tr>
<td>PF</td>
<td>8</td>
<td>61</td>
<td>1.25</td>
<td>37</td>
</tr>
<tr>
<td>AL</td>
<td>4</td>
<td>63</td>
<td>2.4</td>
<td>35</td>
</tr>
</tbody>
</table>

ZD, Zn-deficient; PF, pair-fed control; AL, ad lib.-fed control; SO, slow-twitch oxidative; FOG, fast-twitch oxidative glycolytic; FO, fast-twitch oxidative; S, ATPase⁺; F, ATPase⁺⁺ (FOG combined with FO; see Table 1). Mean values were statistically significantly different from those for PF group (Mann-Whitney U test): ** P < 0.01.

Table 3. DNA concentrations and protein concentrations from soleus muscle from zinc-deficient and control rats
(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DNA (mg/g tissue)</th>
<th>Protein (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>ZD</td>
<td>6</td>
<td>1.43</td>
<td>0.15</td>
</tr>
<tr>
<td>PF</td>
<td>5</td>
<td>1.12</td>
<td>0.36</td>
</tr>
<tr>
<td>AL</td>
<td>4</td>
<td>1.13</td>
<td>0.18</td>
</tr>
</tbody>
</table>

ZD, Zn-deficient; PF, pair-fed control; AL, ad lib.-fed control.

Table 2. There were significantly greater numbers of FO fibres and fewer SO fibres in the deficient animals compared with either of the control groups. This observation would suggest that the soleus muscles from the deficient animals were 'faster' than those in controls.

For comparison of fibre size the fibres were classed entirely on the basis of their ATPase reaction (see Table 1) as either ATPase⁺⁺, fast fibres or ATPase⁺, slow fibres. Comparative values based on these criteria are shown in Table 2. Note the similarity in percentage areas due to the larger numbers of small fast fibres in Zn-deficient rats. The mean 'atrophy factor', which reflects smallness, was calculated as described by Dubowitz & Brooke (1973) and was found to be greatest in the fast ATPase⁺⁺ fibres in the solei from Zn-deficient animals. This implies a more extreme reduction in size than that attributable to the growth-restricted pair-fed controls and that Zn deficiency may either directly or indirectly selectively reduce the size of fast fibres.

DNA concentration expressed as mg/g tissue was measured in solei from animals in each group. The results are shown in Table 3. There was a slight tendency towards higher levels of DNA in the solei of the deficient animals. Protein determinations (Table 3) revealed no significant differences in protein content of the muscles between the groups.
Muscle fibre types in zinc deficiency

Table 4. Mean fibre diameter (µm) of four fibre types from diaphragm of zinc-deficient and control rats

(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Fibre type...</th>
<th>FG</th>
<th>FOG</th>
<th>FO</th>
<th>SO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>ZD</td>
<td>8</td>
<td>23.92*</td>
<td>4.30</td>
<td>22.43*</td>
</tr>
<tr>
<td>PF</td>
<td>8</td>
<td>22.62*</td>
<td>5.64</td>
<td>21.50*</td>
</tr>
<tr>
<td>AL</td>
<td>3</td>
<td>34.81*</td>
<td>2.35</td>
<td>28.60*</td>
</tr>
</tbody>
</table>

There was no significant difference between ZD and PF groups.

ZD, Zn-deficient; PF, pair-fed control; AL, ad lib.-fed control; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative glycolytic; FO, fast-twitch oxidative; SO, slow-twitch oxidative; NS, not significant (P = 0.1).

Mean values were statistically significantly different from AL group (two-tailed Mann–Whitney U test): * P < 0.05.

![Relative frequency of the four fibre types in diaphragm muscle from the ad lib., pair-fed and zinc-deficient animals.](https://www.cambridge.org/core/coreimage)

Diaphragm muscle

There was no significant difference in the mean fibre diameter of any of the fibre types in the Zn-deficient groups compared with the pair-fed groups (Table 4). However, the mean fibre diameters in both these groups were significantly less than those from ad lib.-fed controls (Table 4). It was not practical to measure muscle weight or estimate total fibre number.

Four distinct fibre types were found in diaphragm (see Davies & Gunn, 1972). The relative frequencies of the various fibre types in the three groups of animals are shown in Fig. 2; a statistical analysis of these values based on the two-tailed Mann–Whitney U test is shown in Table 5. There was a significant increase (P < 0.001) in the proportion of FOG fibres in the Zn-deficient animals compared with both groups of controls. The frequency of FG fibres was significantly reduced (P < 0.05) in the deficient rats, but the frequencies of SO and FO fibres were not significantly altered. The 'shift' from FG to FOG fibres represents an increase in the apparent oxidative capacity of the diaphragm muscle from the
Table 5. Statistical significance of differences in relative frequencies of fibre types in diaphragm

<table>
<thead>
<tr>
<th>Fibre type...</th>
<th>FOG</th>
<th>FG</th>
<th>SO</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZD v. PF</td>
<td>***</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PF v. AL</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ZD v. AL</td>
<td>***</td>
<td>***</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

ZD, zinc deficient (n 15); PF, pair-fed control (n 13); AL, ad lib.-fed control (n 4); FOG, fast-twitch oxidative glycolytic; FG, fast-twitch glycolytic; SO, slow-twitch oxidative; FO, fast-twitch oxidative; NS, not significant.

* P < 0.05, *** P < 0.001 (two-tailed Mann–Whitney U test).

deficient animals. Oxidative capacity was indicated by two markers of aerobic metabolism (SDH which reflects the mitochondrial enzyme, and Sudan black B which colours fat) with a large number of fibres staining densely for these two stains. Electron microscopy did not reveal any clear increase in mitochondrial numbers, so the dense reaction with SDH presumably indicates higher levels of enzyme activity within the mitochondria of these fibres.

The Sudan black B stain in combination with the other histochemical methods suggested that the FOG fibres in the deficient animals contained the highest levels of fat, while in the control groups highest levels of fat were seen in the FO fibres. Electron microscopy revealed that the fibres in pair-fed animals had few small lipid droplets, whereas considerable numbers were evident in the diaphragm of the deficient animals (Plates 1 and 2). The lipid in these animals was associated with groups of intermyofibrillar mitochondria (Plate 2). In both control groups the lipid occurred in conjunction with single rows of intermyofibrillar mitochondria (Plate 3). Both pair-fed and Zn-deficient animals exhibited less lipid than found in the ad lib. controls, which had numerous large lipid droplets. The lipid present in all groups of animals showed no osmiophilia and probably comprised saturated fatty acids.

**DISCUSSION**

Dietary Zn deficiency resulted in a change in the proportions of fibre types in both soleus and diaphragm muscles. The soleus muscles in the deficient animals comprised more fast fibres which stained intensely for the ATPase reaction. In the diaphragm muscles from such animals there was an increase in aerobic fibre types, with a concomitant decrease in the proportion of anaerobic fibre types.

Although Zn-deficient and pair-fed controls had the same food intakes, the animals given the Zn-deficient diet showed greater growth retardation. This retardation presumably represents a less-efficient feeding and might reflect undernutrition of the Zn-deficient animals.

Undernutrition in early life has been reported not only to reduce muscle wet weight and to change fibre size, but also to alter the relative proportions of the muscle fibre types. These changes appear to affect both SO and FG fibres (Haltia et al. 1978; Howells et al. 1978; Goldspink & Ward, 1979) and are reported to be reversed on rehabilitation (Bedi et al. 1982). Several arguments suggest that our observed changes in fibre-type frequencies are, however, not an effect of undernutrition but an effect of the deficiency of Zn; the distinctive changes in histochemical profile in the Zn-deficient rats cannot be ascribed to undernutrition as such, since they were not seen in either the pair-fed or ad lib.-fed control groups, despite the difference in weight between these groups. Furthermore, the changes were largely different from those reported in studies of undernutrition.

The observed changes in fibre types are consistent with some imposed metabolic stress,
Muscle fibre types in zinc deficiency

either as a direct or an indirect effect of the deficiency. Lactate dehydrogenase (EC 1.1.1.27) activity is significantly reduced in heart and gastrocnemius muscle from Zn-deficient animals (Huber & Gershoff, 1973). This Zn metalloenzyme catalyses the conversion of pyruvate to lactate with a concomitant oxidation of NADH to NAD, so a decrease in enzyme activity might limit lactate production thereby increasing acetyl coenzyme A production and cytoplasmic NADH. If these pathways are accentuated in Zn deficiency then the increased aerobic capacity and lipid in the diaphragm might be explained. However, this appears unlikely since lactate dehydrogenase is not considered to be rate-limiting in muscle glycolysis (Newsholme & Crabtree, 1979) and a substantial decrease in enzyme activity would be necessary to change the pyruvate $\Rightarrow$ lactate equilibrium.

As the rat’s soleus grows and develops there is a transformation of type II (fast) fibres, which are numerous in the neonate, to type I (slow) fibres of which the adult muscle is largely comprised (Kugelberg, 1976). Kugelberg (1976) has also related the percentage of type I (SO) fibres in rat soleus to body-weight. In the present investigation, however, a differential effect on muscle fibre types attributable to body-weight was not seen when the relatively heavy rats, fed to appetite (Fig. 1), were compared with the lighter rats that were pair-fed with the Zn-deficient animals (Table 2). Furthermore, while both Zn-deficient and pair-fed animals had smaller fibres than the ad lib. group (Table 2), probably reflecting reduced body size and weight, only the Zn-deficient animals showed any significant difference in fibre type frequency. It seems unlikely that the observed effects in soleus are merely due to the lightness of the Zn-deficient group, unless a change occurs at a critical weight between the weights of the Zn-deficient group, unless a change occurs at a critical weight between the weights of the pair-fed and Zn-deficient groups.

It seems more likely that impaired development or differentiation might explain the present findings. The lower numbers of slow fibres in the soleus from the Zn-deficient rats would accord with the threefold increase in need for Zn (compared with fast fibres; see Cassens et al. 1967). This response, in soleus, might be considered as reflecting the relative Zn requirement for the development of each fibre type. Furthermore, the somewhat higher levels of DNA in the solei of the deficient animals support the contention of delayed development (Layman et al. 1980).

In diaphragm there was no significant difference between the fibre diameters in the Zn-deficient and pair-fed groups (Table 4). However, the fibre frequencies of the Zn-deficient group showed some significant differences compared with the other groups. Thus the results from diaphragm are consistent with those from soleus in that it is unlikely that the differences in the Zn-deficient group are entirely due to animal size.

Preliminary experiments (C. A. Maltin, L. Duncan and A. B. Wilson, unpublished results) suggest that the histochemical profile of the rat diaphragm muscle also appears to change with age. The percentages of FOG and FO fibres show a significant decrease, while the percentages of FG and SO fibre increase significantly, over the period from weaning to 4 weeks post-weaning. The changes in the histochemical profile in the diaphragms of the deficient rats might therefore be consistent with a perturbation of development.

Zn is thought to play a functional role in microtubules (Hesketh, 1981) and also influences neuromuscular transmission (Sandow & Bien, 1962). Since it has been demonstrated that the motor neurone is largely responsible for the fibre changes during development (Kugelberg, 1976) it is conceivable that Zn deficiency may modify motor neuronal control of muscle development.

The authors would like to thank Mrs Christina Grant and Miss Diane Yeoman for their expert technical assistance.
REFERENCES


EXPLANATION OF PLATES

Plate 1. Diaphragm from pair-fed control rat (longitudinal section). Small lipid droplets (L) are seen amongst the rows of mitochondria.

Plate 2. Diaphragm from zinc-deficient rat (longitudinal section). Note the number of small lipid droplets (L) associated with the group of mitochondria.

Plate 3. Diaphragm from *ad lib.*-fed control rat (longitudinal section). Note the size of the lipid droplets (L). The mitochondria occur in rows between the myofibrils.