Effects of dietary copper deficiency on male offspring of heterozygous brindled mice

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Female C57BL mice heterozygous for the brindled gene were mated to normal males and fed on a purified diet low in copper throughout gestation and lactation with (+Cu) or without (−Cu) Cu-supplemented drinking water. Male offspring of two genotypes (control, +/y and brindled, Mo^{br}/y) were compared when 10–12 d old. Brindled mice from dams on the −Cu treatment were smaller and had lower packed cell volumes than brindled mice from dams on the +Cu treatment. The −Cu brindled mice were smaller than their littermate brothers (+/y) but had equivalent biochemical features consistent with severe Cu deficiency. Compared with control mice from dams on the +Cu treatment, caeruloplasmin (*EC* 1.16.3.1) activity was lower in offspring of all three other groups including Mo^{br}/y mice who were not anaemic. Iron levels were similar in organs and bone marrow from all four groups of offspring. When dietary Cu is limiting in brindled mice a more severe Cu deficiency ensues. Thus, appropriate Cu nutriture is important to the management of Menkes' disease in humans, a genetic analogue of the brindled mouse.

Copper deficiency: Brindled mouse.

The trace element copper is required by cells, and is essential for a number of important enzymes involved in most aspects of metabolism. Limitation of the dietary supply of Cu leads to a number of pathophysiological conditions which, in part, can be explained by changes in these cuproenzymes (Prohaska, 1988*a*).

The anaemia of Cu deficiency in domestic animals, laboratory animals and humans appears to be the result of Cu–iron interaction, although the exact aetiology is unknown (Mills, 1980). One widely accepted theory suggests that failure to mobilize Fe is due to low ferroxidase (*EC* 1.16.3.1) activity, since the conversion of Fe^{2+} to Fe^{3+} is needed for Fe to bind to transferrin, and the oxidation of ferrous-Fe is known to be catalyzed by ferroxidase (a plasma Cu-protein also known as caeruloplasmin) (Osaki *et al.* 1966; Roeser *et al.* 1970; Evans & Abraham, 1973).

The 'ferroxidase theory' does not explain why adequate dietary Fe minimizes the anemia of Cu deficiency, and both low (Cohen *et al.* 1985) and high (Johnson & Hove, 1986) dietary Fe exacerbate the anaemia, or why dietary carbohydrate influences the anaemia of Cu deficiency without affecting ferroxidase activity (Fields *et al.* 1984).

Cu deficiency in lambs may lead to haemolytic anaemia (Suttle *et al.* 1987) and, in part, explain the accelerated turnover of erythrocytes in Cu deficiency (Bush *et al.* 1956). Thus, the accumulation of Fe in liver may be due to an enhanced catabolism. The Fe would accumulate in the reticulo-endothelial cells of liver and the release of this Fe may be dependent on ferroxidase, whereas release of Fe from hepatic parenchymal cells apparently is not (Baker *et al.* 1980). Cu-deficient liver cells also exhibit impairment in intracellular Fe metabolism (Williams *et al.* 1976). This may also contribute to the accumulation of hepatic Fe following dietary Cu deficiency (Mills, 1980).

Other factors involved in the expression of anaemia following Cu deficiency include sex (Prohaska & Lukasewycz, 1981; Fields *et al.* 1987) and genotype (Lukasewycz *et al.* 1987). Clearly, the aetiology of anaemia following Cu deficiency is not known completely.

Human neonates that are Cu-deficient develop anaemia (Danks, 1988). The ferroxidase theory of the anaemia for humans, however, has been challenged. Shokeir (1972) points out that normal neonates have lower ferroxidase activity, but higher diferric transferrin levels, than their mothers. Also low ferroxidase activity is not consistently associated with anaemia. Humans with genetic disorders of Cu metabolism (Wilson's and Menkes' diseases) have low ferroxidase levels, but absence of anaemia (Prohaska, 1986).

A murine model, the brindled mutation, was used to investigate the aetiology of anaemia following Cu deficiency (Prohaska, 1981). These mice, like humans with Menkes' disease, do not develop anaemia but have low ferroxidase activity. The mutation in both humans and mice is on the X-chromosome and analogous (Prohaska, 1986). Earlier studies comparing brindled offspring to offspring from dams that were fed on Cu-deficient diets suggested that there was milder Cu deficiency in erythropoietic tissues of brindled mice (Prohaska, 1983). The current experiments were designed to study a combination of dietary and genetic Cu deficiency.

MATERIALS AND METHODS

Animal care and diets

Experiments were conducted with C57BL mice (the original breeding pairs were kindly provided by Dr Douglas Grahn, Argonne National Laboratory, Argonne, IL). Hemizygous brindled males (Mo^{br}/y) were obtained by mating heterozygous females ($Mo^{br}/+$) with normal males ($+/y$). The mutant Mo^{br}/y mice were compared with normal $+/y$ mice when the offspring were 10–12 d old. Females were not under investigation in these experiments. Throughout gestation and lactation dams were fed on a purified diet low in Cu, modified AIN-76A (Teklad Laboratories, Madison, WI), which omitted cupric carbonate from the salt mix. Major dietary components of the AIN-76A diet are (g/kg): sucrose 500, casein 200, maize starch 150, maize oil 50, cellulose 50, mineral mix 35, vitamin mix 10 (American Institute of Nutrition, 1977). The low-Cu diet contained 0·47 mg Cu/kg and 50 mg Fe/kg when analysed by flame atomic absorption spectroscopy after wet digestion in nitric acid. Half the dams received deionized water, and their offspring are referred to as Cu-deficient (−Cu); the remaining dams were given cupric sulphate in the drinking water, 20 μg Cu/ml, and their offspring are referred to as Cu-adequate (+Cu). All mice were maintained in polycarbonate boxes with solid bottoms and stainless-steel tops at 23° and 55% relative humidity. At 2 d after parturition, litter size was adjusted to eight pups per dam. Four groups of male offspring were examined from the brindled dams on two dietary treatments: group 1 (+Cu, $+/y$), group 2 (+Cu, Mo^{br}/y), group 3 (−Cu, $+/y$), group 4 (−Cu, Mo^{br}/y). Mice in groups 1 and 2 were brothers as were mice in groups 3 and 4.

Tissue sampling

Mice were killed by decapitation and blood samples were drawn into heparinized microhaematocrit tubes. Livers, kidneys, hearts and spleens were removed, rinsed with deionized water, blotted and weighed. Marrow was removed from both femurs with a 25 μl Hamilton syringe (Hamilton Co., Reno, NV) that had been rinsed previously with an EDTA solution (10 g/l) followed by deionized water. The bone marrow was added to a small acid-washed plastic tube containing 100 μl water and vortexed thoroughly.

Biochemical analyses

Tissues for trace-metal analysis were transferred to acid-washed 50 ml Erlenmeyer flasks. Total tissue Cu and Fe were determined by flame atomic absorption spectroscopy (model 2380; Perkin-Elmer, Norwalk, CT). Individual liver samples were processed, but pools of

Table 1. *Effect of dietary copper deficiency in brindled mouse dams ($Mo^{br}/+$) during gestation and lactation on body-weight, packed cell volume and Cu status of 10–12 d old male offspring**

(Mean values and standard deviations)

	Body-wt (g)	Packed cell volume	Caeruloplasmin (*EC* 1.16.3.1) (units/l)	Liver Cu,Zn-SOD (units/mg)
			Cu-adequate	
Group 1 (+/y)				
Mean	5·48	0·348	32·0	144
SD	0·86	0·0254	4·14	13·9
n	17	17	11	4
Litters	9	9	9	3
Group 2 (Mo^{br}/y)				
Mean	4·02†‡	0·387†‡	2·48†	122
SD	0·30	0·0193	3·44	11·6
n	17	17	17	4
Litters	11	11	11	4
			Cu-deficient	
Group 3 (+/y)				
Mean	5·58‡	0·264†‡	0·40†	94·5†
SD	0·64	0·0261	1·45	18·9
n	11	11	11	4
Litters	6	6	6	3
Group 4 (Mo^{br}/y)				
Mean	2·94	0·345	5·88	94·8
SD	0·64	0·0378	6·14	15·9
n	9	6	4	3
Litters	5	4	3	2

Cu,Zn-SOD: Cu-zinc superoxide dismutase (*EC* 1.15.1.1). One unit of Cu,Zn-SOD activity is that amount of enzyme which inhibits the auto-oxidation of pyrogallol by 50%.

* Values were analysed for individual mice (*n*) sampled from the indicated number of litters by one-way ANOVA and four specific mean comparisons were tested by the Dunn–Bonferroni procedure: (1 *v.* 2), (1 *v.* 3), (2 *v.* 4), (3 *v.* 4).

† Mean values were significantly different from those for group 1 ($P < 0·01$).

‡ Mean values were significantly different from those for group 4 ($P < 0·01$).

kidney pairs, hearts and spleens (three to five organs each) were used to provide sufficient metal for accurate analyses. Tissues were wet-ashed with 4 ml HNO_3 (Instra-analysed grade; J. T. Baker Chemical Co., Phillipsburg, NJ), and the residue was brought to 4·0 ml with 0·1 M-HNO_3. A portion of the bone-marrow sample was wet-digested in a similar manner and analysed for total Fe. Analyses were satisfactorily checked with a certified standard, US National Bureau of Standards (NBS) 1577 bovine liver.

Livers for enzyme assay were transferred to small vials containing 19 vol. cold 0·32 M-sucrose, 0·1 mM-EDTA (pH 7), and disrupted vigorously for 30 s using a Tissumizer and microprobe (SDT-080EN; Tekmar Co., Cincinnati, OH). Portions of the liver homogenate and bone-marrow samples were used to determine total protein by a modified Lowry procedure with bovine albumin as a standard (Markwell *et al.* 1978).

Plasma obtained from the microhaematocrit tubes was used to determine caeruloplasmin (*EC* 1.16.3.1) activity using *o*-dianisidine as substrate at 37° in 0·1 M-sodium acetate (pH 5·5) containing 10 μM-diethylenetriaminepentaacetic acid (DETAPAC) as detailed previously (Prohaska, 1983). Cu–zinc superoxide dismutase (*EC* 1.15.1.1) (Cu, Zn-SOD) activity was measured in liver by following inhibition of pyrogallol autoxidation at 320 nm

Table 2. *Effect of dietary copper deficiency in brindled mouse dams ($Mo^{br}/+$) during gestation and lactation on organ Cu levels of 10–12 d old male offspring**

(Mean values and standard deviations)

	Liver Cu (μg/g)	Kidney Cu (μg/g)†	Heart Cu (μg/g)†	Spleen Cu (μg/g)†
		Cu-adequate		
Group 1 (+/y)				
Mean	26·8	2·33	3·49	2·08
SD	14·1	0·23	0·39	0·37
n	8	4	4	5
Litters	8	7	7	9
Group 2 (Mo^{br}/y)				
Mean	2·59‡	7·66‡	1·34‡	0·63‡
SD	0·34	0·29	0·29	0·25
n	10	4	4	5
Litters	9	7	7	9
		Cu-deficient		
Group 3 (+/y)				
Mean	1·60‡	1·28‡	1·04‡	0·56‡
SD	0·31	0·12	0·80	0·46
n	8	4	4	5
Litters	4	7	7	8
Group 4 (Mo^{br}/y)				
Mean	1·83	3·17	0·37	0·73
SD	0·54			
n	6	1	1	1
Litters	3	3	3	3

* Values were analysed for individual mice (*n*) sampled from the indicated number of litters by one-way ANOVA and four specific mean comparisons were tested by the Dunn–Bonferroni procedure: (1 *v.* 2), (1 *v.* 3), (2 *v.* 4), (3 *v.* 4).

† Only two mean comparisons were tested (1 *v.* 2) and (1 *v.* 3), because group 4 values were from a single pool of organs from four mice. Values from groups 1–3 were from the number (*n*) of pooled organ samples each obtained from three or four mice. The total number of litters sampled is indicated.

‡ Mean values were significantly different from group 1 ($P < 0·01$).

as described previously (Prohaska, 1983). Liver homogenates were treated with ethanol–chloroform to inactivate manganese-SOD.

Statistical analysis

Values were collected from mice over a period of several months. Values from individual mice from multiple litters were analysed by one-way ANOVA. Specific mean comparisons were tested using the Dunn-Bonferroni procedure (an *a priori* technique for multiple comparisons that uses a modified *t* statistic) (Wallenstein *et al.* 1980). Significance was tested at $\alpha = 0·05$ and 0·01.

RESULTS

Pregnancy and parturition were not affected by the Cu-deficient treatment. The double challenge of the brindled mutation and dietary Cu deficiency of dams lowered the viability of group 4 mice (−Cu, Mo^{br}/y). Theoretically, 25% of the offspring studied in a given litter should have been of genotype Mo^{br}/y. Surviving group 4 offspring represented 9% (9/104) of thirteen litters on the −Cu treatment compared with 23% (22/97) for group 2 offspring from twelve litters on the +Cu treatment.

Table 3. *Effect of dietary copper deficiency in brindled mouse dams ($Mo^{br}/+$) during gestation and lactation on organ iron levels of 10–12 d old male offspring**

(Mean values and standard deviations)

	Liver Fe		Marrow Fe	Kidney Fe	Heart Fe	Spleen Fe
	μg/g	μg/liver	(μg/mg)†	(μg/g)‡	(μg/g)‡	(μg/g)‡
			Cu-adequate			
Group 1 (+/y)						
Mean	25·8	5·02	1·39	14·8	34·2	59·2
SD	2·75	0·68	0·60	1·02	1·76	1·70
n	8	8	7	4	4	5
Litters	8	8	7	7	7	9
Group 2 (Mo^{br}/y)						
Mean	50·2§	5·75	1·67	15·2	36·7	71·0
SD	13·0	1·08	0·78	0·26	8·62	10·8
n	10	10	7	4	4	5
Litters	9	9	7	7	7	9
			Cu-deficient			
Group 3 (+/y)						
Mean	26·6 ‖	5·8	1·48	13·2	24·8	49·7
SD	4·50	0·65	0·56	1·21	2·83	9·15
n	8	8	5	4	4	5
Litters	4	7	5	7	7	8
Group 4 (Mo^{br}/y)						
Mean	56·8	5·4	1·63	28·7	75·2	67·8
SD	29·0	0·82	0·09			
n	6	6	3	1	1	1
Litters	3	3	3	3	3	3

* Values were analysed for individual mice (*n*) sampled from the indicated number of litters by one-way ANOVA and four specific mean comparisons were tested by the Dunn–Bonferroni procedure: (1 *v.* 2), (1 *v.* 3), (2 *v.* 4), (3 *v.* 4).

† Femur marrow Fe is expressed per mg protein.

‡ Only two mean comparisons were tested (1 *v.* 2) and (1 *v.* 3), because group 4 values were from a single pool or organs from four mice. Data from groups 1–3 were from the number (*n*) of pooled organ samples each obtained from three or four mice. The total number of litters sampled is indicated.

§ Mean value was significantly different from those for group 1 ($P < 0{\cdot}01$).

‖ Mean value was significantly different from those for group 4 ($P < 0{\cdot}01$).

Body-weight of the offspring was affected by an interaction between diet and genotype (Table 1). Brindled mice weighed less than their brothers. Supplementing the dams with Cu improved the weight of brindled but not normal offspring.

Packed cell volume was influenced independently by diet and genotype. Brindled mice had higher packed cell volumes than their brothers (Table 1). Dietary Cu deficiency in dams lowered packed cell volume levels in both brindled mutants and their brothers.

Plasma caeruloplasmin activity was altered markedly, but not additively, by diet and genotype. Caeruloplasmin activity was much lower in offspring of group 2 and group 3 compared with genotypically normal mice nursed by dams on the Cu-adequate treatment (group 1). The plasma caeruloplasmin activity of offspring in group 4 was not statistically different from activity measured in plasma from mice of either group 2 or 3 (Table 1).

Offspring of group 3 had Cu, Zn-SOD activity 66% of that in group 1. Activity of Cu, Zn-SOD in liver of mice in group 4 was equivalent to mice in groups 2 and 3.

Cu levels were determined in liver, kidney, heart and spleen of mice from all four treatment groups (Table 2). The brindled mutation and Cu-deficient treatment each

lowered liver Cu levels, but the effects were not additive. Heart and spleen Cu changes were qualitatively similar. Both diet and genetics lowered Cu levels in these organs of mice of groups 2 and 3 compared with group 1, a trend noted in liver. It is possible that the combination of Cu-deficient diet and genetics (group 4 offspring) resulted in lower heart Cu levels. Unlike liver, heart and spleen, kidney Cu was higher in group 2 than in group 1. However, dietary Cu deficiency lowered kidney Cu in normal offspring (group 3) and appeared to lower Cu in the brindled mutants (group 4).

Organ Fe levels were also determined on the same samples used for Cu analyses and, in addition, in marrow from femurs (Table 3). The concentration of Fe was higher in livers from brindled mice (groups 2 and 4) than in liver from normal mice (groups 1 and 3). When total liver Fe was compared all four groups had equivalent levels, suggesting that the increased concentration observed in livers of brindled mice was due to slower depletion of stores because of impaired growth (Table 1). In these suckling mice Cu-deficient treatment had no effect on liver Fe. Fe levels in bone marrow, kidney, heart and spleen of offspring were not different between the groups.

DISCUSSION

The anaemia which accompanies dietary Cu deficiency has a long history and unclear aetiology. The ferroxidase theory argues that Fe availability limits the synthesis of haemoglobin (Osaki *et al.* 1966, 1971; Roeser *et al.* 1970). Others argue that intracellular Fe metabolism may be the limiting factor in Cu deficiency (Williams *et al.* 1976; Mills, 1980). Results of the current experiments favour the latter, a metabolic or hormonal factor, since there was no evidence of an Fe deficit in the marrow of anaemic mice, nor accumulation of liver Fe, despite evidence for low caeruloplasmin (ferroxidase) activity.

The presence of anaemia (lower packed cell volume) in these studies is related to Cu deficiency. When comparing offspring with normal genotype (+/y) this is quite clear. Offspring from dams fed on a Cu-deficient diet expressed many features of Cu deficiency such as lower caeruloplasmin activity, liver Cu,Zn-SOD activity, and organ Cu concentrations compared with non-anaemic control (+/y) offspring from dams on a Cu-adequate treatment. No differences in Fe levels were evident between these two groups.

One feature of the Cu-deficient +/y offspring from brindled dams that was different from earlier studies was the normal body-weight. In previous studies, dietary Cu deficiency in normal dams (+/+) resulted in male offspring that were smaller (Prohaska & Smith, 1982; Prohaska, 1983, 1984). The Cu-deficient males were anaemic as haemoglobin levels were 73, 79 and 76% of those measured in offspring from Cu-adequate dams respectively (Prohaska & Smith, 1982; Prohaska, 1983, 1984). The larger size of the +/y Cu-deficient offspring in these studies might be due to a competitive feeding advantage when compared with their mutant brothers, since the brindled offspring nursed by the same dams had the lowest body-weight of the four groups studied.

One of the anomalies of brindled mice is their failure to develop anaemia even though signs of severe Cu deficiency are evident (Camakaris *et al.* 1979; Prohaska, 1981). The presence of a lower packed cell volume in brindled mice whose dams were on the Cu-deficient treatment illustrates that this mutation does not afford complete protection against anaemia. Although rare, cases of anaemia in infants with Menkes' disease support the same conclusion in humans (Aguillar *et al.* 1966; Dorn *et al.* 1973; Lott *et al.* 1975; Williams *et al.* 1978). In the current studies it is difficult to point out major biochemical differences between the brindled offspring from dams on Cu-deficient treatment *v.* Cu-adequate treatment. The lower body-weight and packed cell volume in the Cu-deficient group 4 was suggestive of a more severe Cu deficiency, as were the modest changes in liver

Cu,Zn-SOD, and in kidney and heart Cu levels. Normally, brindled mice have higher packed cell volumes compared with Cu-adequate mice, as was the case for these experiments and many others previously (Prohaska & Smith, 1982; Prohaska, 1983, 1984). This may be due partly to reduced size. Thus, the lower packed cell volume of brindled mice in group 4 with the smallest body-weight is suggestive of a more severe Cu deficiency, perhaps limiting erythropoiesis.

Changes in Cu distribution in brindled offspring have been reported many times, initially by Hunt (1974). Others confirmed that liver Cu is lower and kidney Cu higher in brindled males compared with their littermate brothers (Camakaris *et al.* 1979; Prohaska, 1981). Furthermore, +/y males from brindled dams had lower Cu levels compared with +/y males from control dams (Prohaska, 1983, 1984). In those studies brindled dams and control dams were fed on two different diets. However, in the current study brindled dams were fed on the same diet as control dams used previously, and the +/y offspring in the current experiments had liver Cu levels that were lower than normal (Prohaska, 1983, 1984). This shows that even normal offspring of brindled dams have a modest alteration in Cu stores. It should be noted that despite the lower liver Cu levels, no evidence of growth impairment, lower packed cell volumes or changes in functional Cu pools (caeruloplasmin or liver Cu,Zn-SOD activity) were noted. The altered Cu status of offspring from brindled dams may be due to altered placental transfer of Cu (Mann *et al.* 1980).

Limiting dietary Cu of brindled dams resulted in a more severe Cu deficiency in the brindled male offspring compared with brindled offspring from dams given adequate Cu. A similar finding in a study with post-lactational Cu deficiency in young heterozygous brindled females supports the notion that adequate Cu nutriture is especially important in management of Menkes' disease (Prohaska, 1988*b*).

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