The various faces of copper in laboratory animals

Ingeborg de Wolf

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Ingeborg de Wolf

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De diverse aspecten van koper bij laboratoriumdieren

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 20 september 2001 des middags te 14.30 uur

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Ingeborg Désirée de Wolf

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promotores: Prof. Dr. Ir. A.C. Beynen
(Afdeling Voeding, Faculteit der Diergeneeskunde, Universiteit Utrecht)

Prof. Dr. L.F.M. van Zutphen
(Hoofdafdeling Proefdierkunde, Faculteit der Diergeneeskunde, Universiteit Utrecht)

co-promotor: Dr. H.A. van Lith
(Hoofdafdeling Proefdierkunde, Faculteit der Diergeneeskunde, Univeristeit Utrecht)

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Maybe it's just the dream in me
Maybe it's just my style
Maybe it's just the freedom that I've found
Given the possibility
Of living up to the dream in me
You know that I'll be reaching for higher ground

J. DEUTSCHENDORF & J. HENRY
HIGHER GROUND

Voor papa en mama

Contents

My Diary.
My Unexpected Journey.
There and Back Again.
And What Happened After.

J.R.R. TOLKIEN

THE LORD OF THE RINGS

Chapter 1	General introduction	9
Chapter 2	Assessment of dietary copper requirement of the laboratory mouse (NMRI) as based on reproductive performance in four successive generations	17
Chapter 3	The possible role of copper in the development of oxidative stress and associated disease	31
Chapter 4	Lack of oxidative damage in mice after high copper intake	47
Chapter 5	High copper copper intake does not affect longevity of the laboratory mouse	57
Chapter 6	Liver copper content of rats hypo- or hyperresponsive to dietary cholesterol	67
Chapter 7	Genetic and correlation analysis of hepatic copper content in the rat	81
Chapter 8	Quantitative trait loci influencing hepatic copper in rats	93
Chapter 9	Cholesterol and copper in the liver of rabbit inbred strains with	105

differences in dietary cholesterol response

Chapter 10 Mapping of QTLs for hepatic copper in rabbits 119

Chapter 11 Conclusions and summary 131

Nederlandse samenvatting voor niet-vakgenoten 139

Dankwoord 145

Curriculum vitae 151

Chapter 1

General Introduction

General introduction

Copper and copper containing compounds have been associated with maintenance of health and the treatment of diseases for a very long time: as early as 400 BC, copper was used for medical reasons, such as disinfecting [1, 2]. However, it took until the nineteenth century, until its presence in plants and animals was well-recognised [3]. Only in 1926, the evidence was presented that copper is an essential trace element in animal nutrition [4]. The essentiality of copper for humans was first shown during the 1960s in malnourished children in Peru [5].

In the course of time, copper appeared to be involved in many biochemical processes, e.g. the formation of haemoglobin [6] and the biosynthesis of elastin and collagen [7-9]. Copper metabolism plays a central role in these biochemical processes. Disturbance of or disorders in the copper metabolism may have serious consequences, such as liver cirrhosis and necrosis or death, as is evident from Wilson and Menkes disease [10].

Copper metabolism actually starts with the copper entering the mammal through the alimentary tract [11, 12]. After the digestion of the food, the absorption of copper probably occurs primarily in the small intestine. This process can be disturbed by other nutritional factors such as high intakes of zinc, iron, or ascorbic acid decreasing copper absorption [13-16]. Some adaptation of absorption relative to need takes place [17, 18]. After the absorption has taken place, copper enters the interstitial fluid and blood plasma. The process of copper distribution can be divided into three phases, i.e. transport of copper to the hepatocytes of the liver and to a lesser extent to the kidneys, uptake of copper into the hepatocytes followed by incorporation of copper into several enzymes (e.g. ceruloplasmin and Cu-Zn superoxide dismutase), and transport of copper to and distribution over the other tissues [17, 19]. Finally, most of the copper has to find its way back to the liver. How this happens is unclear. Probably, ceruloplasmin, transcuprein and albumin are involved in this process. The homeostasis of copper is primarily maintained by biliary excretion via the faeces; little is excreted via the urine [20].

In order to be able to maintain copper homeostasis, the amount of copper that is excreted must be compensated for by the absorption of copper from the food or vice versa. The amount that should be compensated for differs during the various stages of life. In the adult stage, compensation for endogenous loss of copper is needed, which can be expressed as the requirement for maintenance. During growth, reproduction and lactation, more copper is

needed to supply these processes, implying an additional need for copper in the form of a certain requirement for 'production'.

The copper requirements of different animal species, including mouse, rat and human, have been published by the National Research Council (NRC) in the form of minimal requirements or allowances [21]. However, the requirements given for the mouse are not based on thorough research and, therefore, the reliability can be questioned.

Based on similarities with the rat and research of Reeves *et al*. [22], Mulhern and Koller [23], Knapka *et al*. [24] and Hurley and Theriault Bell [25], the NRC estimates the mouse's copper requirement for growth and maintenance to be 6 ppm of Cu and for pregnancy and lactation to be 8 ppm of Cu [21]. However, none of these experiments had the intention to make an estimation of the mouse's copper requirement during the various stages of its life. Reeves *et al.* [22] limited their research to adult male mice, using only biochemical parameters with sustainment of maximum serum copper and serum ceruloplasmin activity as criterion. No zootechnical parameters, such as reproductive performance, were studied in order to arrive at an estimation. Mulhern and Koller [23] followed mice from birth till 8 weeks of age, examining the influence of copper status on the immune response. Knapka *et al.* [24] formulated an open formula diet and examined whether differences in the results of biological research occurred when this open formula was fed instead of a closed formula diet. Copper concentrations in both the open formula as well as the closed formula diet were considerably higher than the estimated requirement. The experiment of Hurley and Theriault Bell [25] was designed to examine genetic influence on the effects of a dietary manganese deficiency during prenatal development. This study did not have the purpose to propose a copper requirement or allowance.

It may be obvious that more thorough research is needed in order to come to a proper and reliable estimation of the mouse's copper requirement. Therefore, an experiment was designed to study both biochemical and zootechnical parameters over several generations of mice in order to get more information about the copper requirement during the various stages of life (chapter 2). Main criteria for proposing a copper allowance for the mouse are the reproductive outcome, growth performance and sustainment of maximum plasma and hepatic copper concentrations and of plasma ceruloplasmin.

However, knowing the level of copper required in the mouse's diet does not necessarily mean that this is the exact copper concentration found in commercially available diets. In fact, analyses show that commercially available diets often contain much more copper than is required by the mouse. Copper appears to be involved in the production of free radicals and

reactive oxygen species (ROS), which are very reactive particles, through the Haber-Weiss reaction, which may result in oxidative stress (chapter 3). The hypothesis investigated in chapters 4 and 5 states that copper overload, caused by higher dietary copper concentrations than required, may shorten life span and increases oxidative damage to macromolecules by inducing oxidative stress.

Under normal conditions, a balance exists between the radical generating and the radical-scavenging systems and free radicals and reactive oxygen species are playing an integral part in normal cell physiology [26]. However, they are also capable of damaging biological macromolecules such as DNA and proteins. In the case of oxidative stress, which is the result of an imbalance between the radical-generation and general-scavenging system, more free radicals are being generated than being scavenged, resulting in damage to DNA, proteins, saccharides and lipids [27]. Oxidative stress has been suggested to be associated with accelerating ageing. A number of age-related diseases, such as atherosclerosis and cataractogenesis, and various neurological disorders, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, are also associated with oxidative stress [28]. Chapter 3 provides a more detailed review on the role of copper in oxidative stress.

Within the range of possible dietary copper concentrations, a copper overload is one extreme; the extreme at the opposite site is a copper deficiency. Several factors may influence the copper status. One of these is dietary cholesterol concentration. Feeding cholesterol to rabbits and rats may alter the metabolism of copper and may result in decreased liver copper concentrations, though not inevitably in a copper deficiency [29, 30]. A relationship between copper and cholesterol is also indicated by the observation that experimental copper depletion with a copper-deficient diet induced hypercholesterolemia in rats [31-33]. This was the impetus to compare the hepatic copper content of dietary cholesterol resistant (animals showing only a slight response to dietary cholesterol and therefore also called hyporesponders) and dietary cholesterol susceptible (animals showing an enormous increase in plasma and/or liver cholesterol levels and therefore also called hyperresponders) inbred rat and rabbit strains on a diet with or without added cholesterol. Based on literature, it was anticipated that on a cholesterol-rich diet the hyperresponding rat and rabbit inbred strains would have a lower liver copper content and thus would require a higher copper intake than their hyporesponding counterparts. The results of the experiment are described in chapters 6 and 9.

In order to search for possible causative factors that might be involved in these strain-specific differences, we have performed a genetic analysis in both species. The aim of these genetic analyses was to identify the chromosomal regions that may be involved in controlling liver

copper content after a cholesterol-rich diet. Identifying the chromosomal regions may provide clues as to possible candidate genes that may be involved in controlling liver copper content. Quantitative trait locus (QTL) analyses were performed in two sets of recombinant inbred rat strains (derived from SHR/OlaIpcv and BN-*Lx*/Cub progenitors) and in an F_2-intercross progeny of a cross between hyporesponding and hyperresponding rats (derived from LEW/OlaHsd and BC/CpbU inbred strains) that had been fed a cholesterol-rich diet (chapters 7 and 8). QTL-analysis was also performed in the F_2-intercross progeny of a cross between hyporesponding IIIVO/JU and hyperresponding AX/JU rabbits (chapter 10).
An overview of the results found during this PhD project is given in the Conclusions section of this thesis (chapter 11). A short description of the results is reported in the summary; a short description in more plain terms can be found in the 'Nederlandse samenvatting voor niet-vakgenoten'.

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Chapter 2

Assessment of dietary copper requirement of the laboratory mouse (NMRI) as based on reproductive performance in four successive generations

I.D. de Wolf [1]
X.M. Fielmich-Bouman [1]
L.F.M. van Zutphen [1]
A.C. Beynen [1, 2]

Departments of [1] Laboratory Animal Science and [2] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

submitted

Assessment of dietary copper requirement of the laboratory mouse (NMRI) as based on reproductive performance in four successive generations

Abstract

The copper requirement of the laboratory mouse has not been well established, the current minimal requirement being set at 6 mg Cu/kg diet for maintenance and 8 mg Cu/kg diet for pregnant and lactating animals. We have attempted to estimate the copper requirement of the mouse by performing an experiment in which the effects of four different levels of dietary copper (1, 2, 4 or 8 mg Cu/kg diet) were studied in four generations of NMRI mice. The effects of copper intake on reproductive outcome, growth performance and sustainment of maximum plasma and hepatic copper concentrations and of plasma ceruloplasmin have been evaluated. This study shows that in NMRI mice, a semipurified diet containing 1 ppm copper had a marked depression on reproductive performance. Plasma, hepatic and carcass copper concentrations were not or only moderately indicative as to the copper requirement. To take into account the various factors affecting copper requirement and the availability of dietary copper, we suggest the general copper allowance of laboratory mice to be set at 4 ppm.

Introduction

The mouse is the most commonly used laboratory animal, but its copper requirement is not well established. The National Research Council (NRC, 1995) has set the minimal requirement for both immature and adult mice at 6 mg Cu/kg diet and for pregnant and lactating animals at 8 mg Cu/kg diet, but acknowledges that specific studies to determine the copper requirement of mice have not been published. The recommendations of the NRC are based on four experiments with mice (Hurley and Theriault Bell, 1974; Knapka *et al.*, 1974; Mulhern and Koller, 1988; Reeves *et al.*, 1994) and two experiments with rats (Johnson *et al.*, 1993; Klevay and Saari, 1993). Only one experiment was designed to estimate the minimal requirement of adult male mice using the sustainment of maximum serum copper and serum ceruloplasmin activity as criterion (Reeves *et al.*, 1994). The copper requirement of mice during life stages other than maintenance remains unknown. Given this lack of information we decided to estimate the copper requirement of the mouse by performing an experiment in which the effects of four different levels of dietary copper (1, 2, 4 or 8 mg Cu/kg diet) were studied in four generations of NMRI mice. Based on the effects of copper intake on reproductive outcome, growth performance and sustainment of

maximum plasma and hepatic copper concentrations and of plasma ceruloplasmin, we attempted to estimate the dietary copper requirement of the NMRI mouse.

Materials and methods

The experimental protocol was approved by the animal experimentation committee of the Utrecht Faculty of Veterinary Medicine.

Animals

Male and female SPF-derived outbred NMRI mice (HsdWin:NMRI, Harlan Cpb, Zeist, The Netherlands) were used. On arrival, the mice (212 females and 152 males) were aged 3 weeks. They were weighed and 8 males and 8 females were killed to obtain zero-time control values. The remaining mice were marked individually and assigned to one of the four experimental, dietary groups. The mice were gradually transferred from a commercial diet (RMH-1110, Hope Farms, Woerden, The Netherlands) to the experimental diets over a period of four days.

Housing

The mice were housed under conventional conditions. They were kept in Makrolon type II cages (UNO BV, Zevenaar, The Netherlands) with a layer of sawdust as bedding. A controlled 12-h light/dark cycle (light: 7.00-19.00 h), controlled temperature (19-21ºC) and relative humidity (50-55%) were maintained in the room.

Diets

The semipurified, pelleted diets complied with the recommendations for mice (NRC, 1995), and contained either 1, 2, 4 or 8 ppm Cu (Table 1). During the course of the experiment, separate batches of diets were prepared. Each batch was analyzed for its copper level. The analyzed levels were 0.66 ± 0.10, 2.00 ± 1.18, 2.80 ± 0.41 and 5.80 ± 0.92 ppm Cu, respectively. In the Tables (Results) the concentrations as added to the experimental diets are used. The diets were stored at 4ºC until feeding. Diets and demineralised water were administered *ad libitum* throughout the whole experiment. Feed intake was assessed for intervals of 7 days by weighing the amount added onto the feed hopper and also weighing the leftovers and spilled feed that could be recovered from the bedding. Body weights were determined at regular intervals.

Experimental design

Fig. 1 shows the experimental design. As from the age of 3 weeks, the mice were housed three or four of the same sex in Macrolon II cages. Feed intake and weight were recorded weekly. At various intervals (Fig. 1), seven or eight mice of each sex from the various generations were selected at random and killed for analyses. In a randomized order, the animals were anaesthetized by exposure to diethyl ether. Blood was collected via aorta puncture or orbital puncture into heparinized tubes. The animals were killed while they were still under anesthesia and the liver was excised. The liver and carcass were weighed and frozen at -20ºC. Blood was centrifuged at 1000x g for 15 min, after which plasma was collected and frozen at -20ºC.

When the mice were aged 9 weeks, reproduction was started to yield the F_1, F_2 and F_3 generations (Fig. 1). One male mouse was housed with one female of the same dietary group in a Makrolon II cage for a period of three days. In total, 24 breeding couples per generation per dietary group were formed. After the three days of housing together, the males were separated from the females and housed in their original cases. The females remained to be housed individually in the Makrolon II cages until the pups were weaned. At parturition, the number of

Table 1: Ingredient composition of the experimental diets

Ingredient (g)	
Corn oil	25.0
Coconut fat	25.0
Casein	200.0
Starch and dextrose (1:1 w/w)	668.9
Cellulose	30.0
$CaCO_3$	12.5
$MgCO_3$	2.4
$NaH_2PO_4.2H_2O$	10.4
KCl	3.8
Mineral premix[1]	10.0
Vitamin premix[2]	12.0
$CuSO_4.5H_2O$:	
1 ppm Cu, or	0.000
2 ppm Cu, or	0.004
4 ppm Cu, or	0.0118
8 ppm Cu, or	0.0276
Total	1000.0

[1] The mineral premix consisted of (mg/10 g): FeSO4.7H2O: 174.4; MnO2: 15.8; ZnSO4.H2O: 82.3; KI: 0.1962; Na2SeO3.5H2O: 0.4996; Na2MoO4.2H2O: 0.378; Corn starch: 9726.4

[2] The vitamin premix consisted of (mg/12 g): Retinol: 4.8 (≡ 2400 IU/kg); Cholecalciferol: 2.0 (≡ 1000 IU/kg); *RRR*-α-tocopherol: 44.0 (purity: 50%); Phylloquinone: 1.0; Biotine: 0.2; Choline Chloride: 4000.0 (purity: 50%); Folium Acid: 0.5; Niacine: 15.0; Calcium Panthothenate: 80.0 (purity: 45%); Riboflavin: 7.0; Thiamine: 5.0; Vitamin B6: 8.0; Vitamin B12: 10.0 (purity: 0.1%); Corn starch: 7822.5

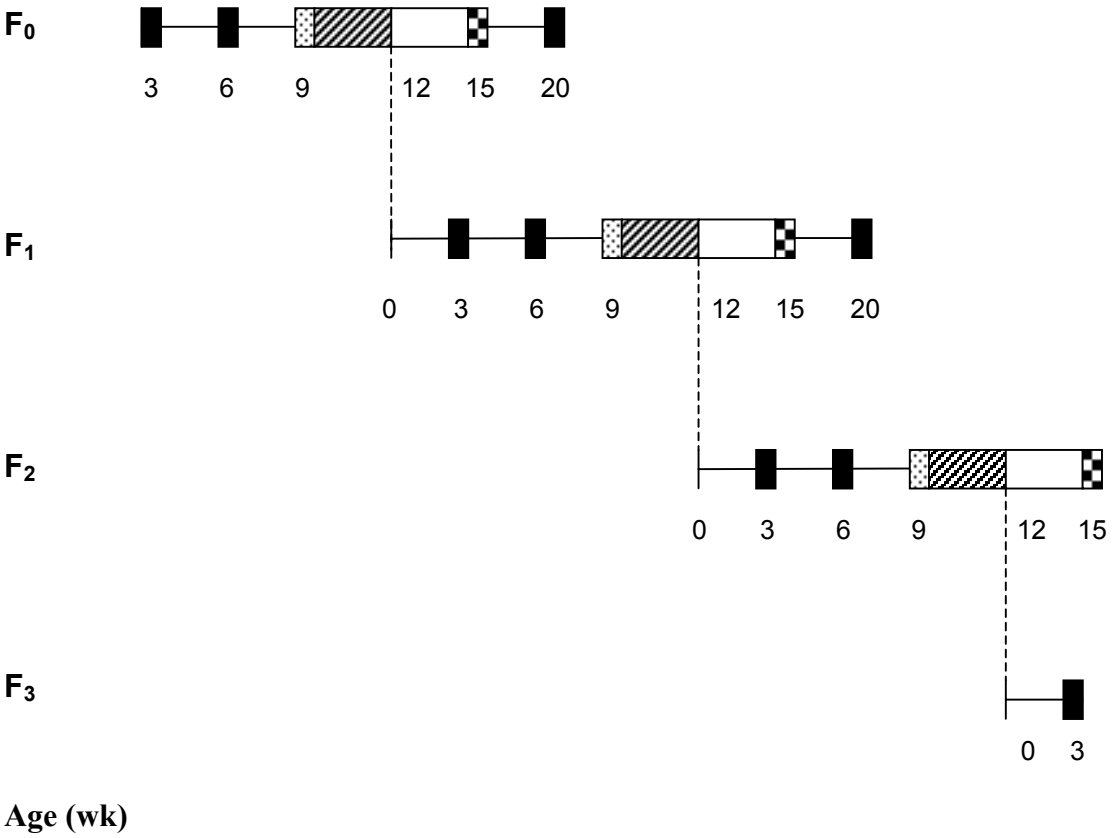

Figure 1: Schematic presentation of breeding and sampling schedule to which mice were subjected in a parallel feeding trial. The mice were fed semipurified diets containing either 1, 2, 4 or 8 ppm copper. Numbers below the horizontal lines indicate the age of the mice. F_0, F_1, F_2 and F_3 indicate the subsequent generations. F_1, F_2 and F_3 mice remained on the diet of their dams. Explanation of symbols: ■: 7 or 8 male and female mice per dietary group were killed and plasma and tissues were collected; ▩ : 7 or 8 male and female mice per dietary group were killed with consecutive collection of plasma and tissues and start of conception; ▨ : gestation in selected females of each dietary group; □ : lactation in selected females of each dietary group; ▣ : 7 or 8 male and female mice per dietary group were killed with consecutive collection of plasma and tissues and weaning of offspring.

pups was registered. Subsequently, the litters were standardized to 11 (F_1) or 12 (F_2) pups per dam. Pups were exchanged between litters of the same dietary group. Weaning was performed at three weeks after parturition and sex of the pups was registered.

Analysis of copper in diets, liver and carcass

Carcasses were freeze-dried and ground with a coffee mill. Diet pellets were also homogenized using the coffee mill. Diet, liver and carcass samples were dried overnight at 105ºC and the dry weights were measured. Subsequently, the samples were ashed at 200ºC for one hour, 300ºC for two hours, 400ºC for three hours and 500ºC for ten hours. The remaining ash was dissolved in

1.0 ml of concentrated $HClO_4$, which was then evaporated at 225ºC, this procedure being repeated until the ash was completely white. The ash was then dissolved in 1.0 ml of 6 M HCl. Copper was measured by flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia). As a reference sample we used bovine liver sample (NBS 1577b, National Institute of Standards Technology, Gaithersburg, USA). Accuracy was 103% (mean: 164 mg Cu/kg; reference value: 160 mg Cu/kg) and precision, expressed as s.e.m., was 9 mg Cu/kg.

Analysis of blood plasma

Plasma samples of the individual mice had to be pooled to determine the plasma copper and ceruloplasmin concentration. Generally, seven or eight plasma samples of mice of the same age, sex, and diet were pooled. Plasma copper was measured colorimetrically using a test kit (Boehringer Mannheim GmbH, Mannheim, Germany). The assay was performed on a Cobas Bio Auto-analyser (Roche Diagnostic Systems, Hoffman-La Roche, Basle, Switzerland). The concentration of ceruloplasmin was determined with the method of Sunderman and Nomoto (1970), also using the auto-analyser.

Statistical analysis

Analysis of variance (ANOVA) was performed with dietary copper concentration, gender, age and generation as main effects. A chi square analysis was performed with reproductive performance as main effect of dietary copper concentration, except for the average litter size which was analysed with ANOVA. The level of significance was pre-set at $P<0.05$. Statistical analyses were carried out according to Petrie and Watson (1999) using a commercially available statistical package (SPSS, 1990).

Results

Reproduction outcome

Reproductive performance of the group fed the diet with 1 ppm copper was poor as based on conception rate, percentage of litters born alive and pup mortality (Table 2). The conception rate was only 68%, but an identical conception rate was found in the F_2 females receiving the diet with 8 ppm Cu. Litter size in the F_0 group fed the diet with 1 ppm Cu was normal, but the percentage of litters born alive was low. The low conception rate and low percentage of litters born alive resulted in a relatively small number of offspring born alive (data not shown). The

Table 2: Effects of dietary copper level on reproduction in NMRI mice

Diet (ppm Cu)	F_0	F_1	F_2	Statistics[1]
	Percentage of pregnant females			
1	68	n.m.[2]	n.m.	
2	82	86	82	
4	86	77	86	
8	90	86	68	
	Percentage of litters born alive			
1	87[a]	n.m.	n.m.	p=0.0594
2	94	100	100	
4	100	100	100	
8	100	100	100	
	Average litter size at birth			
1	11.6	n.m.	n.m.	
2	12.1	11.4	11.8	
4	12.4	12.5	10.1	
8	11.9	11.5	12.7	
	Percentage of pups died between birth and weaning			
1	96[b]	n.m.	n.m.	p=0.000
2	0	2	2	
4	0	2	18	
8	0	0	4	

[1] In a number of chi square analyses, an expected value <5 resulted in less discriminating power; values bearing a superscript letter are significantly different from the other values in the column.
[2] n.m. means 'not measured'.

Table 3: Body weight (in g) of male and female mice aged 5 weeks and fed diets containing 1, 2, 4 or 8 ppm of copper[1]

Gender	Diet	F_0	F_1	F_2	Statistics[2]
M[3]	1 ppm	28.1 ± 2.6	n.m.[3]	n.m.	
M	2 ppm	27.9 ± 2.4	31.4 ± 2.1	31.4 ± 2.9	
M	4 ppm	28.1 ± 2.5	32.0 ± 2.6	30.3 ± 2.3	
M	8 ppm	27.6 ± 2.4	31.7 ± 1.9	31.1 ± 2.4	
F[3]	1 ppm	24.0 ± 1.9	n.m.	n.m.	
F	2 ppm	23.7 ± 2.2	25.5 ± 1.9	26.0 ± 2.0[a]	p=0.009
F	4 ppm	23.7 ± 2.2	26.0 ± 2.1	25.9 ± 2.2[b]	p=0.019
F	8 ppm	24.2 ± 1.8	26.2 ± 1.9	24.8 ± 2.3[a,b]	

[1] Means ± s.d. for 7 or 8 animals per group.
[2] Statistical analysis is show for dietary effects only; values bearing the same superscript letter are significantly different.
[3] M means 'male', F means 'female' and n.m. means 'not measured'.

most dramatic effect, however, occurred between birth and weaning: pup mortality was 96% in the F_0 group fed the diet with 1 ppm Cu. The reproductive study could not be continued for the diet with 1 ppm Cu. Remarkable pup mortality as high as 18% also occurred in the F_2 group receiving the diet with 4 ppm Cu. This pup mortality probably was unrelated to diet because in the F_2 group receiving the diet with 2 ppm Cu mortality was only 2%.

Weight and feed intake

Dietary copper level did not significantly affect growth as illustrated by the values of body weight for the mice aged 5 weeks (Table 3). Body weight increased with age (not shown), males having higher body weights than females of the same age. Feed intake was not affected by dietary copper concentration (not shown). Feed intake increased with age until 15 weeks. Males generally ate more than females.

Copper in plasma, liver and carcass

Plasma copper and ceruloplasmin concentrations were not significantly affected by dietary copper concentration, gender, sex or generation (results not shown). Likewise, there was no significant effect of dietary copper on plasma copper and ceruloplasmin concentrations of female breeders.

The carcass copper concentration in the mice on arrival (F_0 generation, aged 3 weeks) was 11.5 ± 2.1 µg Cu/g dry weight (means ± SD, n=8) for the males and for the females the value was 10.0 ± 1.3. Lower dietary copper levels were associated with lower copper concentrations in carcass.

Table 4: Copper concentration (in µg/g dry weight) in carcass of mice fed diets containing 1, 2, 4 or 8 ppm of copper[1]

Gender	Diet	F_0, 6 wk	F_1, 3 wk	F_1, 6 wk	F_2, 3 wk	F_2, 6 wk	F_3, 3 wk
M[2]	1 ppm	$2.7 \pm 0.4^{a,b,c}$	4.0[3]	n.m.[2]	n.m.	n.m.	n.m.
M	2 ppm	4.2 ± 1.1^{a}	$3.9 \pm 1.1^{d,e}$	7.7 ± 1.9	$5.2 \pm 1.1^{f,g}$	4.3 ± 1.4	7.0 ± 1.8
M	4 ppm	4.5 ± 0.8^{b}	6.6 ± 1.3^{d}	7.5 ± 2.2	$6.4 \pm 0.6^{f,h}$	5.6 ± 2.1	8.8 ± 1.7
M	8 ppm	5.2 ± 0.5^{c}	7.3 ± 1.4^{e}	9.1 ± 2.4	$9.1 \pm 1.9^{g,h}$	5.4 ± 1.9	9.5 ± 2.2
F[2]	1 ppm	$3.6 \pm 0.7^{i,j}$	$3.3 \pm 0.8^{4,k,l}$	n.m.	n.m.	n.m.	n.m.
F	2 ppm	4.7 ± 1.1	4.1 ± 0.8^{m}	7.7 ± 3.0	6.3 ± 1.2	4.5 ± 0.4^{n}	7.2 ± 2.0^{o}
F	4 ppm	4.8 ± 0.5^{i}	$5.8 \pm 0.9^{k,m}$	8.0 ± 1.8	7.0 ± 1.0	5.4 ± 1.4	8.7 ± 2.7
F	8 ppm	5.5 ± 0.4^{j}	6.4 ± 1.1^{l}	8.2 ± 2.0	7.8 ± 2.0	7.0 ± 1.9^{n}	10.9 ± 1.5^{0}

[1] Means ± s.d. for 7 or 8 animals per group.
[2] M means 'male', F means 'female' and n.m. means 'not measured'; letters indicate significant differences ($p<0.05$) between dietary groups based on ANOVA.
[3] Data of the F_1 at the age of 3 weeks receiving 1 ppm Cu are based on 1 male and 5 females.

Table 4 documents the results for the mice aged 3 and 6 weeks. Copper concentration in carcass was not influenced by gender; age and generation did influence copper concentration in carcass in a number of cases significantly ($p<0.044$ and $p<0.014$, respectively), but no causal relationship was found between age and generation, and copper concentration in carcass .

Liver copper concentration in the mice on arrival (F_0 generation, aged three weeks) was 90.2 ± 63.6 µg Cu/g dry weight (means ± SD, n=8) for the males and for the females the value was 17.7 ± 2.8. Liver copper at the age of six weeks in the F_0 males receiving the diet with 1 ppm Cu was statistically significant lower than that in the other mice ($p=0.001$) (Table 5). Liver copper at the age of three weeks in the F_1, F_2 and F_3 groups receiving the diet with 2 ppm Cu was significantly lower than that in the groups receiving 4 or 8 ppm Cu ($p<0.037$) (Table 5). There was no systematic dose-response relationship between copper intake in mice fed 4 or 8 ppm Cu and liver copper concentration. The males of the F_0 (except the males fed 1 ppm Cu), F_1 and F_2 generations had significantly higher values for liver copper than the females, except for the three week old mice (Table 5, $p<0.046$). In the F_2 generation, liver copper for the groups fed the diet with either 4 or 8 ppm Cu was lower at the age of six weeks than at three weeks. In the breeders no effect of dietary copper level on the liver copper concentration was found (results not shown).

Discussion

A dietary copper concentration of 1 ppm (analysis: 0.66 ppm Cu) resulted in a markedly depressed reproductive performance so that it is obvious that the mice were copper deficient. The dietary copper concentration of 2 ppm appeared (analysis: 2.00 ppm Cu) to sustain reproduction in the NMRI mice and thus seemed to meet their copper requirement. Mulhern and Koller (1988) fed mice semipurified diets containing either 0.5, 1, 2 or 6 ppm copper. The mice were studied from birth until the age of 8 weeks and the authors also concluded, with the immune status of the mice as criterion, that dietary copper concentrations of 0.5 and 1 ppm resulted in copper depletion.

There are differences in copper requirement in relation to gender (Prohaska and Lukasewycz, 1981; Mulhern and Koller, 1988; Lynch and Klevay, 1994) or genetic background (Doyle, 1980; Hurley *et al.*, 1980) There also are interactions between copper and other nutrients that affect copper absorption (Doyle, 1980). We used semipurified diets to which copper sulphate was added so that it may be anticipated that the copper was highly available. In practice, diets based on natural ingredients are used. The copper in those diets may be less available. Given the factors affecting the copper requirement and availability, we suggest that the dietary copper allowance of the laboratory mouse should be set at 4 ppm Cu rather than at 2 ppm. The allowance that we

propose is lower than the NRC's current recommendation of 6 ppm Cu for maintenance and 8 ppm for pregnant and lactating mice. Reeves *et al.* (1994) also estimated the copper requirement of the adult male Swiss-Webster mouse to be between 2.5 and 4 mg Cu/kg diet depending on the parameter used to estimate copper requirement.

The copper concentration in the diets did not affect body weight and feed intake of the mice. Likewise, Prohaska and Lukasewycz (1989) reported no differences in body weight between copper deficient and copper adequate mice, but reduced weight gain due to copper deficiency was found by Blakley and Hamilton (1987). Reduced weight gain thus appears to be an ambiguous indicator of copper deficiency and other indicators of copper deficiency may be more appropriate to underpin the proposed allowance.

The concentration of copper and ceruloplasmin in plasma did not differ between the dietary groups, but the within-group variation was considerable. A decrease in ceruloplasmin activity has been described for copper-deficient mice and their offspring (Mulhern and Koller, 1988; Prohaska and Lukasewycz, 1989; Arce and Keen, 1992; Kang *et al.*, 2000), as well as a significant decrease in serum copper concentration (Reeves *et al.*, 1994). According to Milne (1988), serum copper concentration and ceruloplasmin activity is not the best indicator of copper status. Instead, enzyme activities of erythrocyte superoxide dismutase and platelet cytochrome-c oxidase may be better indicators of metabolically active copper and copper stores than plasma concentrations of copper or ceruloplasmin. The activities of these enzymes are sensitive to changes in copper stores and are not as sensitive to factors not related to nutritious copper (Milne, 1988).

Arce and Keen (1992) found that tissue copper levels were decreased in the offspring of copper-deficient breeders. Our data show that copper intake was directly related with the copper content of carcass. Low liver copper concentrations in mice fed a copper-deficient diet and their offspring have been described by various authors (Blakley and Hamilton, 1987; Prohaska, 1991; Arce and Keen, 1992; Reeves *et al.*, 1994; Kang *et al.*, 2000). Lynch and Klevay (1994) reported that hepatic copper was significantly reduced in copper-deficient females, but not in males. Our data show that liver copper at the age of six weeks in the F_0 males receiving the diet with the lowest Cu concentration was statistically significant lower than that in the other mice and that liver copper at the age of three weeks in the F_1, F_2 and F_3 groups receiving the diet with 2 ppm Cu was significantly lower than that in the groups receiving the higher concentrations. At dietary copper concentrations higher than 2 ppm, copper intake had no systematic impact on liver copper. Although at the age of three weeks, liver copper appears to be lower in the groups receiving 2 ppm Cu, the observed liver copper levels do not point at copper deficiency. At

dietary copper concentrations of at least 2 ppm, an equilibrium may be established at which sufficient copper is supplied to tissues in spite of low concentrations of copper in liver and carcass. This idea is confirmed by the normal reproductive performance of the groups receiving 2 ppm Cu.

In conclusion, this study shows that NMRI mice fed a semipurified diet containing less than 2 ppm copper has a marked depression of reproductive performance. Plasma copper concentration and ceruloplasmin activity were not indicative as to the copper requirement of the mice, whereas hepatic copper concentrations were only indicative at lower dietary copper levels. To take into account the various factors affecting copper requirement and the availability of dietary copper, we suggest the general copper allowance of laboratory mice to be set at 4 ppm.

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Table 5: Copper concentration (in µg/g dry weight) in liver of mice fed diets containing 1, 2, 4 or 8 ppm of copper[1]

Gender	Diet	F_0, 6 wk[2]	F_1, 3 wk	F_1, 6 wk	F_2, 3 wk	F_2, 6 wk	F_3, 3 wk
M[2]	1 ppm	$11.4 \pm 2.7^{a,b,c}$	18.6[3]	n.m.[2]	n.m.	n.m.	n.m.
M	2 ppm	19.0 ± 4.3^{a}	$13.4 \pm 1.3^{d,e}$	22.1 ± 3.4	$15.0 \pm 2.9^{f,g}$	25.0 ± 1.4^{h}	16.4 ± 0.8^{j}
M	4 ppm	19.6 ± 3.0^{b}	20.7 ± 6.0^{d}	20.1 ± 5.5	35.6 ± 11.7^{f}	$21.5 \pm 2.5^{h,i}$	27.7 ± 13.1
M	8 ppm	22.5 ± 4.4^{c}	26.0 ± 6.7^{e}	23.3 ± 5.0	31.9 ± 6.3^{g}	25.7 ± 3.2^{i}	33.8 ± 10.0^{j}
F[2]	1 ppm	13.7 ± 2.4	$12.9 \pm 1.7^{3,k}$	n.m.	n.m.	n.m.	n.m.
F	2 ppm	15.5 ± 1.6	$13.6 \pm 0.8^{l,m}$	14.1 ± 1.7	$17.6 \pm 3.6^{n,o}$	16.8 ± 2.3	17.6 ± 2.8^{p}
F	4 ppm	14.9 ± 1.6	23.9 ± 6.3^{l}	14.1 ± 2.4	46.2 ± 13.4^{n}	15.4 ± 1.7	31.6 ± 16.0
F	8 ppm	14.5 ± 2.2	$27.0 \pm 10.4^{k,m}$	14.8 ± 1.0	44.4 ± 15.3^{o}	15.3 ± 0.9	39.7 ± 16.4^{p}

[1] Means ± s.d. for 7 or 8 animals per group

[2] M means 'male', F means 'female' and n.m. means 'not measured'; values bearing the same superscript letter are significant different ($p<0.05$) between dietary groups based on ANOVA

[3] Data of the F_1 at the age of 3 weeks receiving 1 ppm Cu are based on 1 male and 5 females

Chapter 3

The possible role of copper in the development of oxidative stress and associated disease

I.D. de Wolf [1]
L.F.M. van Zutphen [1]
A.C. Beynen [1, 2]

Departments of [1] Laboratory Animal Science and [2] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

submitted

The possible role of copper in the development of oxidative stress and associated disease

Abstract

Reactive oxygen species (ROS) and free radicals are chemical substances that play a role in normal cell physiology. However, these molecules may also cause damage to biological molecules such as DNA, proteins, and phospholipids, and thus leading to disease. Copper, as a transition metal, is suggested to be critical in the formation of ROS and free radicals. Although the body possesses an antioxidant defence and repair system, it can not always protect itself against free radicals and ROS. Imbalance between the production of free radicals and ROS and the antioxidant defence and repair system in favour of the free radicals and ROS results in oxidative stress. In this review, we focus on the role of copper in the development of oxidative stress. Evidence of the role of copper in the generation of free radicals and ROS has been obtained mainly by *in vitro* research, in which combinations of copper and a reducing agent were used. The results of these studies implicate an indirect, facilitating role of copper in the development of oxidative stress. Suggestive evidence for copper facilitating oxidative stress under *in vivo* conditions comes mainly from relationships between copper and various multifactorial diseases. Because of the multifactorial character of these diseases, it is difficult to determine if and, if yes, what role copper plays in the development of diseases that are associated with oxidative stress. It is concluded that until now, no material proof exists for copper being involved in the development of oxidative stress *in vivo*.

Introduction

This paper scrutinizes the possible role of copper in the development of oxidative stress and associated disease. The basic concepts concerning free radicals, reactive oxygen species (ROS), the antioxidant defence and repair system, and oxidative stress are described. The pro-oxidant role of the transition metal copper is reviewed. It is stressed that the alleged involvement of copper in the development of oxidative stress is based on *in vitro* experiments mainly. Suggestive evidence for copper facilitating oxidative stress under *in vivo* conditions comes from relationships between copper and various multifactorial diseases.

In this review we have chosen to describe a limited number of examples that may illustrate the possible role of copper in the process of oxidative stress rather then trying to give a complete overview of the literature in this field.

What are free radicals and reactive oxygen species?

A free radical can be defined as any chemical species that can independently exist and contains one or more unpaired electrons [1]. Free radicals may be formed by the homolysis of covalent bonds, the addition of an electron to a neutral atom or its loss of a single electron [2]. Reactive oxygen species (ROS) include oxygen-containing radicals as well as non-radical derivatives of oxygen. Both free radicals and ROS, the terms being used interchangeably, are formed *in vivo* in normal cell metabolism [1, 3]. They play a crucial role in processes such as the 'respiratory burst' of phagocytically active cells and they may function as messengers and also regulate oxidative inactivation of enzymes [4-8]. However, free radicals may attack biological molecules such as DNA, proteins and phospholipids, and thus cause damage at the cellular level, for instance by affecting signal transduction, cell membrane functions and gene expression [2, 8]. DNA is a critical target because of its central role in cellular metabolism [9]. Some radicals and ROS are more damaging than others. The hydroxyl radical ($OH^{\bullet}$) is considered to be the most reactive radical, attacking all biological molecules present at its site of formation and usually setting off free radical chain-reactions [8, 10].

Formation of free radicals in the presence of transition metals

As stated by Saran *et al.* [11] "there is probably not a single radical chain process *in vivo* that proceeds without the participation of some metal in loose or bound form, whether as a side effect or even playing a dominant role".

Transition metals are metals that have a variable oxidation number, which allows them to participate in single electron transfer reactions [1]. These transition metals are incorporated as functional redox centres in antioxidant enzymes such as catalase and superoxide dismutase. Therefore, these metals are often classified as antioxidant nutrients [12, 13]. However, transition metals may also be considered pro-oxidant nutrients because of their capability to facilitate free radical reactions by converting H_2O_2, which is a product of normal cell physiology [14], via the so-called Fenton reaction [1]:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^{\bullet} + OH^{-} \quad (1)$$

where M^{n+} can be Ti^{3+}, Cu^{+}, Fe^{2+}, Co^{2+}, Cr^{4+} or Ni^{2+}.

The Fenton reaction is the first step of the Haber-Weiss reaction, which results in the formation of $OH^{\bullet}$ and other reactive oxygen species [1, 15]:

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe} OH^{\bullet} + OH^{-} + O_2 \quad (2)$$

This reaction has also been shown to occur in the presence of other metals such as copper and chromium [16].

The reactive oxygen species produced in the Haber-Weiss reaction, especially the $OH^{\bullet}$ produced in the Fenton reaction, may cause damage at (sub)cellular and macromolecular levels, such as enhanced lipid peroxidation, DNA damage and protein damage [16-19].

A major argument against the occurrence of Fenton reactions *in vivo* is the unavailability of metal catalysts under *in vivo* conditions [16, 20]. Research, however, has indicated that metal catalysts are available *in vivo* [16, 21-24]. Another argument against the occurrence of the Fenton reaction *in vivo* is the very low rate constant of the reaction. However, calculations suggest $OH^{\bullet}$ may be generated in the cell at a considerable rate [16, 20]. Criticism regarding the Fenton reaction also concerns the production of $OH^{\bullet}$, that critics believe not to be a product of the Fenton reaction [16, 20]. During the interaction of transition metal compounds with H_2O_2 metal oxo and peroxo species, such as ferryl and perferryl, are formed as well, which are also capable of damaging DNA and proteins in a site-specific manner [16, 20, 25-35]. The ferryl ion is kinetically indistinguishable from free $OH^{\bullet}$, which hinders determining whether the ferryl ion, free $OH^{\bullet}$ or both species are formed [24].

Certain metal ions catalyse the production of ROS in ways other than the Fenton reaction. Metal ions such as mercury, cadmium and nickel react with and thereby deplete free sulfhydryl groups [36]. The decrease in free sulfhydryl groups may lead to the formation of oxidative stress, resulting in tissue-damaging effects [37].

A single transition metal may initiate or catalyse the formation of ROS by more than one mechanism involving more than one organelle or cell type. Transition metals are not only involved in the formation of free radicals, but may also react with free radicals [10, 16, 24, 38-50], yielding intermediary molecules with a metal-carbon σ-bond, which could also be a key step in the induction of free radical damage [46-48].

The antioxidant defence and repair system

To protect the body against free radicals and ROS, there is the so-called antioxidant defence and repair system. An antioxidant is any substance that at low concentrations, when compared with that of an oxidisable substrate, significantly delays or inhibits oxidation of the substrate [10]. Three different ways in which the antioxidant defence and repair system acts can be distinguished:

1. Antioxidants may scavenge free radicals and in the process be transformed into new, but less reactive free radicals. The consequential chain reaction will be terminated when a free radical reacts with the free radical form of the antioxidant [1]. Examples of such antioxidants are vitamin E, beta-carotene, quercetin and enzymes such as superoxide dismutase [2].
2. Sequestration of transition metals into forms incapable of stimulating free radical reactions and thus preventing the formation of free radicals [1]. Proteins with sequestering activity are e.g. ceruloplasmin, metallothionein, ferritin and transferrin [2].
3. Repairing, if possible, the biological damage caused by ROS and free radicals. An example is the DNA repair process, which repairs most damage to DNA.

Under normal *in vivo* conditions, the antioxidant defence system is able to neutralise the ROS generated. In response to increased oxidative stress, antioxidant defence can be induced [51, 52].

Oxidative stress

Oxidative stress can be defined as a situation in which the production of free radicals and ROS overwhelms the antioxidant defence and repair system [53]. The imbalance may be due to an increased production of free radicals and ROS and/or a decreased functioning of the antioxidant defence and repair system.

Oxidative stress is considered an important factor in the process of ageing [2, 10, 54-61]. It may also be involved in the development of diseases such as atherosclerosis, cancer and neurodegenerative disorders such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease [2, 54, 62].

Evidence for a role of copper in oxidative stress

Most studies investigating the role of copper in the generation of free radicals and ROS describe oxidative damage to DNA and lipid peroxidation as the main effects of oxidative stress. Copper is thought to participate in the development of various multifactorial diseases. Although the body possesses an antioxidant defence system to counteract oxidative stress,

research on the role of copper in oxidative stress suggests that antioxidants exhibit pro-oxidant characteristics in the presence of copper.

Copper-mediated damage to DNA

Copper has been found to bind to non-histone nuclear matrix proteins at sites where DNA loops anchor, suggesting that the electron-rich DNA might be a target of copper-mediated oxidative damage [43, 63-67]. *In vitro* research showed that mixtures of Cu^{2+} and H_2O_2 caused strand scission and modification of the bases in DNA, whereas H_2O_2 or Cu^{2+} alone did not cause any of this damage [25, 68-73]. Addition of a copper-reducing agent caused a very large increase in base damage and strand breakage [68, 69, 74-77]. The products of base damage in the presence of Cu^{2+}, cytosine glycol, thymine glycol, 8-hydroxyadenine and especially 8-hydroxyguanine, suggest the involvement of $OH^{\bullet}$ [68]. Since DNA breakage often occurs near guanine residues, it has been suggested that Cu^{2+} ions bind to DNA at guanine sites, where it reacts with H_2O_2 and a reducing agent to generate $OH^{\bullet}$, which attacks the DNA bases in a site-specific manner [9, 68, 78]. Controversy exists about the nature of the ultimate DNA damaging species. Some investigators consider $OH^{\bullet}$ to be the culprit, whereas others suggest that an oxidised metal-oxygen complex is responsible for the DNA damage [79]. Copper-containing complexes may also act as catalyst in the formation of ROS, which is evident from the reaction of Cu^{2+}-ethylenediamine with H_2O_2 [70].

In vitro research indicates that copper ions not only react with endogenous agents to generate free radicals and ROS, but also can mediate the conversion of xenobiotics, leading to the formation of reactive species and resulting in oxidative damage to DNA [28, 29, 80-82]. Hydroquinone is a polyphenolic constituent of tobacco smoke and a tumor promotor for the development of lung carcinomas [83]. During copper-mediated oxidation of hydroquinone in benzoquinone and quinone, a copper-hydroquinone complex may be formed, this complex enhancing the formation of ROS, resulting in site-specific DNA cleavage [84-86]. It is questionable whether $OH^{\bullet}$ is formed during the Cu^{2+}-mediated oxidation of hydroquinone. A copper-peroxide complex with similar reactivity as singlet oxygen, rather than free $OH^{\bullet}$ may participate in the production of DNA damage *in vitro* [87].

Gossypol is a polyphenolic binaphthyl dialdehyde found in cotton seeds. In *in vitro* experiments, gossypol may be oxidised to a gossypol radical in the presence of Cu^{2+}. The gossypol radical may react with DNA, causing strand breakages in double and single stranded

DNA [88]. DNA cleavage by the gossypol-Cu^{2+} complex may also be the result of the generation of active oxygen species, mainly singlet oxygen [89].

Discrepancy exists between the results of *in vitro* and *in* vivo results, since in mice fed increasing concentrations of copper no indication of increased oxidative damage to DNA in the form of 8-oxo-7,8-dihydro-2□-deoxyguanosine was found [90].

Copper-mediated lipid peroxidation

Cu^{2+} is suggested to catalyses lipid peroxidation. Although copper has been reported to catalyse lipid peroxidation in human erythrocytes and of membrane lipids [91, 92], studies on the role of copper in lipid peroxidation mainly focus on copper-mediated LDL oxidation. In order to study oxidation of LDL *in vitro*, copper is often added to the reaction mixture to oxidise LDL [93]. Evidence for copper-mediated LDL oxidation i*n vivo* is merely indirect. *In vivo*, oxidised LDL mediates pathological events that are important in the development of atherosclerosis [94-96]. Copper is speculated to be involved in LDL oxidation as accelerated progression of atherosclerosis and elevated levels of auto-antibodies against oxidised LDL are associated with high serum copper concentrations [97-103]. Furthermore, increased levels of copper ion have been detected in advanced atherosclerotic lesions [104].

A very indirect indication on the role of copper in lipid peroxidation *in vivo* has obtained from research in humans. In humans, serum total copper concentration is in the range of 17 ± 3 μM, but increases with ageing [105-107]. An increase in serum copper concentration may be associated with higher levels of oxidative damage. Indeed, elderly have higher levels of systemic oxidative stress, resulting in lipid peroxidation as measured by the indicators plasma TBARS (thiobarbituric acid reactive substances) and FPL (fluorescent products of lipid peroxidation) [107]. Whether the relationship between the increase in serum total copper and the higher levels of oxidative damage is causally related needs to be investigated.

In vivo research in mice fed increasing concentrations of copper did not reveal increased lipid peroxidation as no significant difference in the levels of malondialdehyde between the dietary groups occurred [90].

The role of copper in diseases associated with oxidative stress and life span

Given the fact that free radicals and ROS can cause damage at (sub)cellular and macromolecular level, it is not surprising that many diseases have been associated with oxidative stress. One of them was already briefly mentioned in this review, viz. atherosclerosis.

Another intriguing disease, of which some pieces of the puzzle are already found but many pieces still remain to be discovered, is Alzheimer's disease. Copper may be of particular importance in the development of this neurodegenerative disease as excellently reviewed by Multhaup and Masters (1999)) [108]. In Alzheimer's disease, the Cu^{2+} binding amyloid precursor protein (APP) accumulates in neurites [109-113]. The accumulation of APP may lead to disruption of copper compartmentalisation and thus to copper toxicity [114]. The oxidative modification of APP, following the binding of Cu^{2+}, results in the formation of Cu^{1+} and cystines. During the generation of cystine, electrons are liberated, which results in an enhanced production of $OH^{\bullet}$, resulting in oxygen radical-induced neuronal damage and death through lipid peroxidation [108]. Research suggests that reaction between H_2O_2 as generated by extracellular forms of Cu,Zn-SOD, and the APP-Cu^{1+} complex on the surface of neurons, leads to amyloidogenic C-terminal fragmentation of APP [10, 115, 116]. Recently, it was shown that intact amyloid Aβ peptide (Aβ), which can be deposited in the brains as plaques, can be generated by non-specific proteases from amyloidogenic C-terminal fragments of APP [117]. Aβ has been proposed to cause an overproduction of H_2O_2 or related peroxides and thus may act on APP as a feedback reaction, thereby increasing oxidative stress [108, 118].

Oxidative stress is considered an important factor in the process of ageing [2, 10, 54-61]. If copper can induce oxidative stress by generating free radicals and ROS, the copper-mediated oxidative stress may result in a shorter life span. However, no significant difference in life span of mice fed increasing copper concentrations was found [119].

The various faces of the antioxidant defence system

As described above, the body possesses an antioxidant defence system to counteract the generation of free radicals and ROS. *In vitro* research indicates that in the presence of copper, some antioxidants may exert pro-oxidative behaviour.

Copper ion-binding to amino groups of proteins and other molecules, such as ascorbate, may represent an antioxidant effect, because it prevents copper ions from entering into the redox cycles necessary for $OH^{\bullet}$ formation. In the presence of H_2O_2 or ascorbate, copper that is bound can still be catalytically active and catalyse multihit oxidative reactions on the molecule or amino acid it is bound to or on other biomolecules in its close vicinity [120-128]. In *in vitro* studies, Cu^{2+}-dependent site-specific DNA damage has been found if ascorbate was added to the reaction mixture. It has been suggested that a copper-peroxide complex with reactivity similar to $OH^{\bullet}$ rather than $OH^{\bullet}$ itself participates in the DNA cleavages produced

[129]. Research performed *in vivo* in rats exposed to a combination of $CuSO_4$ and ascorbic acid, however, showed through electron spin resonance spin-trapping technologies that copper induced the production of $OH^{\bullet}$ [130].

Glutathione (GSH) is another endogenous compound exerting a dual role in the antioxidant defence system. GSH can induce site-specific oxidative DNA damage in the presence of Cu^{2+}, the addition of H_2O_2 to the *in vitro* systems increasing the DNA damage even further [129, 131, 132]. At higher GSH:Cu ratios, GSH has, however, an effective antioxidant functionality [131].

A third example of an endogenous compound that, based on *in vitro* research, is thought to exert both a antioxidant and a pro-oxidant function is the copper-binding enzyme Cu,Zn-SOD, which catalyses the dismutation of two $O_2^{\bullet -}$ ions into O_2 and H_2O_2 [133-135]. Using its own dismutation product, H_2O_2, as a substrate, the enzyme can generate $OH^{\bullet}$ by a Fenton-like reaction involving its bound copper ions [136-138]. The $OH^{\bullet}$ may react with the Cu,Zn-SOD molecule itself or with other molecules in the vicinity of its generation site. If $OH^{\bullet}$ reacts directly with Cu,Zn-SOD, copper ions can be released from the damaged enzyme, which in turn can enhance the Fenton-like reaction by reacting with H_2O_2 [136, 139]. According to Hodgson and Fridovich [140], bound Cu^{2+}-$OH^{\bullet}$ rather than free $OH^{\bullet}$ is generated during the reaction of intact Cu,Zn-SOD with H_2O_2. This Cu^{2+}-$OH^{\bullet}$ intermediate may be responsible for the inactivation of Cu,Zn-SOD by being scavenged intramolecularly, thereby producing destruction of ligands for Cu^{2+} in Cu,Zn-SOD and fragmentation of SOD [139, 140].

Even biological antioxidants, such as α-tocopherol, of which the importance is recognised widely may possess pro-oxidant properties [141-148]. *In vitro* research indicated that each α-tocopherol molecule can reduce two Cu^{2+} ions bound to DNA to Cu^{1+}. The DNA-Cu^{1+} complex reacts with H_2O_2 to generate $OH^{\bullet}$ or species of similar reactivity, leading to DNA base oxidation and backbone cleavage [149].

Conclusion

Copper is suggested to play a role in the generation of free radicals and ROS through the Fenton reaction:

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-} \qquad (3)$$

The free radicals and ROS generated in this reaction are thought to cause damage at the (sub)cellular and macromolecular level.

Evidence of the role of copper in the generation of free radicals and ROS has been obtained mainly by *in vitro* research. In these studies, the role of copper in generating ROS and free radicals was often studied in the presence of reducing agents. Much of the studies were focussed on oxidative damage to DNA and lipid peroxidation as the main effects of oxidative stress. The results of the *in vitro* studies implicate an indirect, facilitating role of copper in the development of oxidative stress

Evidence for copper facilitating oxidative stress under *in vivo* conditions comes mainly from relationships between copper and various multifactorial diseases. Because of the multifactorial character of these diseases, it is difficult to determine if and what role copper plays in the development of diseases that are associated with oxidative stress. Until now, no material proof exists for copper being involved in the development of oxidative stress *in vivo*.

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Chapter 4

Lack of oxidative damage in mice after high copper intake

I.D. de Wolf [1]
X.M. Fielmich-Bouman [1]
A. Wellejus [3]
B. Daneshvar [4]
M. Hansen [4]
S. Loft [3]
L. Dragsted [4]
L.F.M. van Zutphen [1]
A.C. Beynen [1, 2]

Departments of [1] Laboratory Animal Science and [2] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [3] Institute of Public Health, Panum Institute, University of Copenhagen, Copenhagen and [4] Institute of Food Safety and Toxicology, Veterinarian and Food Administration, Søborg, Denmark

submitted

Lack of oxidative damage in mice after high copper intake

Abstract

On the basis of *in vitro* experiments, copper (II) has been suggested to be involved in the development of reactive oxygen species. We have tested whether high copper intakes would cause oxidative stress in intact animals. Mice were fed diets containing either 5, 25, 125 or 625 ppm Cu. Oxidative damage to DNA, proteins and lipids was assessed by determining the levels of 8-oxodG, oxidised lysine residues and MDA as indicators, respectively. In addition, total antioxidant status was measured. The liver copper concentration was increased only in the group fed the highest level of dietary copper. Increasing dietary copper levels did not significantly influence levels of 8-oxodG, MDA and oxidised lysine residues in plasma proteins, however there was a trend towards higher levels of protein and lipid oxidation with increasing doses of copper. Likewise, the total antioxidant status was not affected by the dietary copper level. We conclude that exposure to high copper levels does not result in significant oxidative damage in plasma and liver under *in vivo* conditions.

Introduction

Copper (II) not only is an essential trace element for animals and human [1, 2], but it may also be involved in the development of reactive oxygen species [3-10]. An increased formation of reactive oxygen species may lead to oxidative stress due to an imbalance in the oxidant – antioxidant system [11]. Oxidative stress causes oxidative damage to DNA, proteins and lipids [12, 13], which in turn is associated with ageing and disorders, such as ischemic heart disease and Parkinson disease [12, 14]. In diseases associated with oxidative stress, copper might play a role through its participation in the formation of reactive oxygen species [15]. In Alzheimer's disease, high concentrations of copper as well as oxidative stress markers are found near amyloid A beta peptide deposits. It has been put forward that copper may exacerbate and facilitate amyloid A beta-mediated oxidative damage in Alzheimer's disease [16-18].

We wished to test our idea that high dietary copper levels would produce oxidative stress and reduce antioxidant status. We performed an experiment with mice fed diets containing either 5, 25, 125 or 625 ppm Cu for a period of 6 weeks. At the end of the experimental period, samples were taken to assess oxidative damage to DNA, proteins and lipids and to measure

total antioxidant status. To assess copper status of the mice, we measured the hepatic copper concentration.

Materials and methods

The protocol of the experiment was approved by the animal experimentation committee of the Utrecht Faculty of Veterinary Medicine.

Experimental protocol

Sixty male SPF-derived outbred NMRI mice (HsdWin:NMRI, Harlan Cpb, Zeist, The Netherlands), aged 10 weeks, were used. The mice were divided into five groups so that body weight distributions of the groups were similar. Each group was assigned to one of the experimental diets. The animals were housed under conventional conditions. The animal room had a regulated temperature (19-21°C), relative humidity (55-65%) and controlled lighting (12 h/day, light 07.00 to 19.00 h). The mice were housed three per cage in Makrolon

Table 1: Ingredient composition of the experimental diets

Ingredient	Weight (g)
Corn oil	25.0
Coconut fat	25.0
Casein	200.0
Starch + dextrose (1:1 w/w)	668.9
Cellulose	30.0
$CaCO_3$	12.5
$MgCO_3$	2.4
$NaH_2PO_4.2H_2O$	10.4
KCl	3.8
Trace element premix[1]	10.0
Vitamin premix[2]	12.0
$CuSO_4.5H_2O$:	
5 ppm Cu, or	0.0158
25 ppm Cu, or	0.0944
125 ppm Cu, or	0.4876
625 ppm Cu	2.4538
Total	1000.0

[1] Composition of trace element premix (mg/10 g): $FeSO_4.7H_2O$: 174.4; MnO_2: 15.8; $ZnSO_4.H_2O$: 27.4; KI: 0.1962; $Na_2MoO_4.2H_2O$: 0.378; Corn starch: 9781.8

[2] Composition of the vitamin premix (mg/12 g): Retinol: 4.8 (≡ 2400 IU/kg); Cholecalciferol: 2.0 (≡ 1000 IU/kg); Phylloquinone: 1.0; Biotin: 0.2; Choline chloride: 4000.0 (purity: 50%); Folic Acid: 0.5; Niacine: 15.0; Calcium Panthothenate: 35.6 (purity: 45%); Riboflavin: 7.0; Thiamin: 5.0; Vitamin B_6: 1.0; Vitamin B_{12}: 10.0 (purity: 0.1%); Corn starch: 7917.9

type II cages (UNO BV, Zevenaar, The Netherlands).

Over four days the mice were gradually transferred from a commercial, pelleted, natural-ingredient diet (RMH-B, Hope Farms BV, Woerden, The Netherlands) to the experimental diet. The experimental, semi-purified, pelleted diets complied with the nutrient requirements of mice [19], but contained as variable either 5, 25, 125 or 625 ppm Cu. Pure selenium and *RRR*-α-tocopherol were not added to the diets because of their antioxidant properties and possible attenuating influence on copper-induced antioxidant status. The ingredient composition of the diets is described in Table 1. The analysed copper concentrations in the experimental diets with 5, 25, 125 and 625 ppm added copper were 4, 23, 127 and 705 ppm Cu, respectively. The diets were stored at 4°C until feeding. Diets and demineralised water were administered *ad libitum*.

At the end of the 6 weeks during experimental period all animals were anaesthetised with chloroform so as to reach the surgical phase. Subsequently, blood samples were taken by heart puncture or orbital puncture and collected in heparin-coated tubes. Then, the animals were killed while still under anaesthesia and livers were removed and frozen immediately at -80ºC. Plasma was prepared by centrifuging the blood samples (1000x g, 15 min) and was then stored at -20ºC until use.

Biochemical analysis

Liver copper concentration was determined as described [20]. The total antioxidant status was determined in plasma using the Randox total antioxidant status colorimetric assay kit (Randox, Crumlin, UK). The test was performed on a Cobas-BIO automatic micro-centrifugal analyser (Roche Diagnostics Systems, Hoffmann-La Roche, Basel, Switzerland).

The level of hepatic 8-oxo-7,8-dihydro-2□-deoxyguanosine (8-oxodG), an indicator of oxidation of DNA, was analysed according to the method of Helbock et al. [21]. Total malondialdehyde (MDA) in plasma, an indicator of lipid oxidation, was determined as described by Lauridsen et al. [22]. Plasma protein 2-adipic semialdehyde residues, a measure of oxidised lysine residues in plasma protein, was measured according to Daneshvar et al. [23].

Statistical analysis

The Kolmogorov-Smirnov one-sample test was used to check normality of the data. All results within groups were found to be normally distributed. The data were subjected to one-way analysis of variance (ANOVA). If the ANOVA showed a significant effect ($p<0.05$), the

group means were further compared with the unpaired Student's *t* test using pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested using a F-test. All statistical analyses were carried out according to Petrie and Watson [24] using a SPSS PC+ computer program [25].

Results

Liver copper concentration was markedly increased in the group exposed to the highest dietary copper concentration, but at lower copper intakes there were no differences between the groups (Table 2).

Total antioxidant status was not affected by the dietary copper level (Table 2). Likewise, dietary copper concentration had no significant effect on plasma levels of the indicators of oxidative damage, i.e. MDA and plasma protein 2-adipic semialdehyde residues (Table 2) although these markers tended to increase with increasing doses of copper. Hepatic 8-oxodG, an indicator of DNA damage, was not affected by copper intake (Table 2).

Discussion

As based on the observed similar hepatic copper concentrations, the mice were able to maintain copper homeostasis when fed the diets with copper levels of either 5, 25 or 125 ppm. There was a dramatic increase in hepatic copper concentration after feeding the diet containing 625 ppm of copper. Apparently, at the high copper intake the liver's excretory mechanisms were saturated, resulting in copper accumulation.

The level of oxidative DNA damage in the liver reported in this study is about 10 times lower than the level of hepatic oxidative DNA damage reported by Bialkowski *et al.* [26] in

Table 2: Hepatic copper, levels of indicators of oxidative damage and antioxidant status in mice fed diets with different copper concentrations[1]

Dietary copper (ppm)	Liver copper concentration (μg/g liver)	Total antioxidant status (mmol/l)	8-oxodG (8-oxodG/10^6 dG)	Specific oxidised amino acids (pmol/mg protein)	MDA (pmol/mg protein)
5	26.59 ± 6.61	0.74 ± 0.09	3.50 ± 1.74	42.16 ± 6.40	90.2 ± 9.8
25	31.96 ± 5.21	0.73 ± 0.05	2.89 ± 1.92	43.29 ± 5.45	97.5 ± 11.8
125	34.19 ± 18.84	0.73 ± 0.09	3.92 ± 1.49	47.34 ± 10.44	97.5 ± 9.8
625	547.35 ± 403.55[2]	0.73 ± 0.05	3.47 ± 0.83	45.57 ± 13.04	100.0 ± 12.7

[1] Results expressed as means ± SD for 12 mice per dietary group

[2] Statistically significant difference ($p<0.05$) in liver copper concentration between mice fed the diet with 625 ppm Cu and the mice fed the other diets

maternal healthy Swiss mice. This difference may be due to strain differences in the susceptibility for oxidative damage or in the efficiency of the repair system. Differences in dietary composition may also contribute to the observed difference in oxidative DNA damage. Furthermore, the Swiss mice used in the study of Bialkowski *et al.* [26] were older than the mice used in our experiment. Aging has been associated with increased 8-oxodG levels in nuclear [27] but particularly mitochondrial DNA in rats and mice [28]. As far as we know, up to now no data have been published on the total antioxidant status in the mouse *in vivo*. Likewise, no comparable data on mouse plasma MDA or on plasma protein lysine residue oxidation in the mouse have been previously published, but the results are in accordance with other ongoing studies in the authors' laboratories. The levels of the latter marker in this study are approximately 5-10 times lower than expected since this marker is known in general to be related to the basal metabolic rate and to be inversely related to the maximal life-span potential of various animal species [23]. Plasma MDA in the mouse seems also to be 4-5 times lower than in the rat, indicating major differences in the redox regulation in plasma between these two species.

Copper could play a role in the development of oxidative damage as it may catalyse the formation of reactive oxygen species and free radicals through its participation in the Haber-Weiss reaction [29, 30]. The liver is the main site of copper storage [31] so that we expected the liver to be most susceptible to copper-induced oxidative damage. However, in the mice fed the diet with 625 ppm of copper, no increase in hepatic 8-oxodG, an indicator of DNA damage, was found. Thus, we did not find any evidence for oxidative damage in the liver, despite the massive accumulation of copper. Evidence that copper increases the level of 8-oxodG in tissue or plasma comes from studies in which copper is used as an intermediate to produce free radicals [32-34]. Thus, evidence for the oxidative role of copper is obtained mainly indirectly [29]. To our knowledge, no *in vivo* experiments studying the effect of feeding mice increasing copper concentrations on oxidative DNA damage have been performed before. The copper-loaded rat liver has been observed to be minimally affected and to have increased resistance to galactosamine-induced inflammation and to the oxidation-mediated toxicity by carbon tetrachloride [35, 36]. Thus, copper-mediated oxidative toxicity in rodents was previously found to be minimal in accordance with our results.

Increasing copper intakes will cause increasing fluxes of copper through the blood [31]. Therefore, copper-mediated oxidative damage could occur in blood plasma. Evidence for this hypothesis comes from *in vitro* studies and from *in vivo* studies, in which a combination of copper and a reducing agent was used to induce free radicals [37]. As far as we know, no

experiments studying a direct relationship between copper and oxidative damage in plasma of mice have been performed. Plasma MDA, an indicator of lipid peroxidation, and the level of oxidised lysine residues, an indicator of plasma protein oxidation, only increased non-significantly with higher dietary copper levels. Thus, there was only limited evidence for oxidative stress.

The outcome of this study is at variance with the concepts advanced in the literature in that copper plays a role in the development of oxidative damage. The putative role of copper is based on *in vitro* studies [29]. Under *in vivo* conditions, as in our study with intact mice, various mechanisms may be operative in counteracting any copper-induced oxidative stress. In the body, there is an active antioxidant defence and repair system capable of preventing and repairing oxidative damage [38].

Our results do not exclude the possibility that copper is involved in free radical formation and the occurrence of oxidative damage under specific conditions. In humans, serum copper concentration increases with ageing [39, 40]. In the elderly, a high copper level could contribute to their higher level of systemic oxidative stress as these people may have less protective capacity [40]. However, our study could not confirm the pro-oxidant role of copper *in vivo*, despite the copper accumulation in the liver at high copper intakes. More research concerning the potential role of copper in oxidative stress under *in vivo* conditions is needed.

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Chapter 5

High copper intake does not affect longevity of the laboratory mouse

I.D. de Wolf [1]
X.M. Fielmich-Bouman [1]
L.F.M. van Zutphen [1]
A.C. Beynen [1, 2]

Departments of [1] Laboratory Animal Science and [2] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

submitted

High copper intake does not affect longevity of the laboratory mouse

Abstract

There is suggestive evidence that high copper intake promotes the generation of reactive oxygen species through the Haber-Weiss reaction, resulting in oxidative stress, which may cause accelerated ageing. Commercial rodent diets generally contain copper concentrations that are up to 10 times higher than the copper requirement of mice. We investigated whether high copper intake affects longevity. Male and female NMRI mice were fed semipurified diets containing either 5, 25 or 125 ppm copper. There was no effect of copper intake on lifespan of the mice. It is suggested that oxidative stress caused by high copper intake was nullified by the body's antioxidant defence and repair system.

Introduction

Copper is a transition metal and thus is involved in the formation of free radicals and reactive oxygen species (ROS) through the Haber-Weiss reaction (De Wolf *et al.,* submitted a). Mammalian cells possess antioxidant defence and repair mechanisms to scavenge free radicals and ROS (Halliwell, 1993; Knight, 1998), but oxidative stress will arise if these mechanisms are overwhelmed (Sies, 1991). Oxidative stress is considered to accelerate the process of ageing (Knight, 1998; Halliwell and Gutteridge, 1989; Harman, 1998; Kohn, 1985; Upton, 1977; Harman, 1993; Harman, 1956; Harman, 1981; Harman, 1992).

In a feeding trial with mice we did not find evidence for high copper intake causing oxidative stress (De Wolf *et al.,* submitted b). However, oxidative stress was assessed using indicators and it cannot be excluded that inappropriate indicators had been selected. In addition, endpoint measures, such as longevity, would be more convincing as to negative effects of high copper intake, if shown to be affected.

Commercial rodent diets generally contain copper concentrations that are up to 10 times higher than the copper requirement of mice. We thus hypothesised that the high copper intakes occurring in practice would reduce longevity of the laboratory mouse. The hypothesis was tested in the present experiment.

Materials and methods

The protocol of the experiment was approved by the animal experimentation committee of the Utrecht Faculty of Veterinary Medicine.

Experimental protocol

78 Male and 77 female SPF-derived outbred NMRI mice (HsdWin:NMRI, Harlan Cpb, Zeist, The Netherlands), aged 4 weeks, were used. The mice were divided into three groups so that body weight distributions of the groups were similar. Each group was assigned to one of the three experimental diets.

The animals were housed under conventional conditions. The animal room had a regulated temperature (19-21°C) and relative humidity (55-65%), and controlled lighting (12 h/day, light 07.00 to 19.00 h). The mice were housed three or four per cage in Makrolon type II cages (UNO BV, Zevenaar, The Netherlands).

During four days upon their arrival, the mice were gradually transferred from a commercial, pelleted, natural-ingredient diet (RMH-1110, Hope Farms BV, Woerden, The Netherlands) to the experimental diets. The experimental, semi-purified pelleted diets complied with the nutrient requirements of mice (National Research Council, 1995), but contained as variable either 5, 25 or 125 ppm Cu. The ingredient composition of the diets is given in Table 1. The analysed copper concentrations in the experimental diets with 5, 25 and 125 ppm added Cu were 3.76 ± 0.34, 23.52 ± 1.56 and 129.47 ± 5.83 ppm Cu (means ± SD for 5 batches per diet), respectively. The diets were stored at 4°C until feeding. Diets and demineralised water were administered *ad libitum*. Food consumption was registered on a weekly basis. Body weights were registered weekly until the age of 10 weeks and then every four weeks.

The mice remained in the experiment until dying spontaneously. If severe suffering of a mouse was detected and death was expected within 24 hours, the animal was killed by cervical dislocation. After 80 weeks, the experiment was stopped because the surviving mice suffered from severe skin lesions, the incidence of the lesions being unrelated to the type of diet. A piece of liver of each mouse was stored -20ºC for copper analysis as described by De Wolf *et al.* (submitted c).

Statistical analysis

Differences between the dietary groups in longevity were statistically evaluated using Kaplan Meier Survival Analysis. Diet was included as a factor; statistical significance was defined as

$p<0.05$. The Kolmogorov-Smirnov one-sample test was used to check normality of the liver copper concentrations. The results within groups were normally distributed. The data were subjected to one-way analysis of variance (ANOVA). If the ANOVA showed a significant effect ($p<0.05$), the group means were further compared with the unpaired Student's *t* test using pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested using a F-test. All statistical analyses were carried out according to Petrie and Watson (1999) using a SPSS PC+ computer program (SPSS, 1990).

Results

Hepatic copper concentration

In the males, there was no effect of dietary copper concentration on liver copper. The concentrations were 17.60 ± 8.01, 13.90 ± 3.81 and 19.21 ± 8.09 μg Cu/g liver (means ± SD) for the groups fed the diets containing 5, 25 or 125 ppm of Cu, respectively.

Liver copper concentrations in the female mice fed diets with 5, 25 or 125 ppm Cu were

Table 1: Ingredient composition of the experimental diets

Ingredient	Weight (g)
Corn oil	25.0
Coconut fat	25.0
Casein	200.0
Starch + dextrose (1:1 w/w)	669.6
Cellulose	30.0
$CaCO_3$	12.5
$MgCO_3$	1.7
$NaH_2PO_4.2H_2O$	10.4
KCl	3.8
Trace element premix[1]	10.0
Vitamin premix[2]	12.0
$CuSO_4.5H_2O$:	
5 ppm Cu, or	0.0158
25 ppm Cu, or	0.0944
125 ppm Cu	0.4876
Total	1000.0

[1] Composition of trace element premix (mg/10 g): $FeSO_4.7H_2O$: 174.4; MnO_2: 15.8; $ZnSO_4.H_2O$: 27.4; KI: 0.1962; $Na_2MoO_4.2H_2O$: 0.378; Corn starch: 9781.8

[2] Composition of the vitamin premix (mg/12 g): Retinol: 4.8 (≡ 2400 IU/kg); *RRR*-α-tocopherol: 44.0 (purity: 50%); Cholecalciferol: 2.0 (≡ 1000 IU/kg); Phylloquinone: 1.0; Biotin: 0.2; Choline Chloride: 4000.0 (purity: 50%); Folic Acid: 0.5; Niacine: 15.0; Calcium Panthothenate: 35.6 (purity: 45%); Riboflavin: 7.0; Thiamin: 5.0; Vitamin B_6: 1.0; Vitamin B_{12}: 10.0 (purity: 0.1%); Corn starch: 7873.9

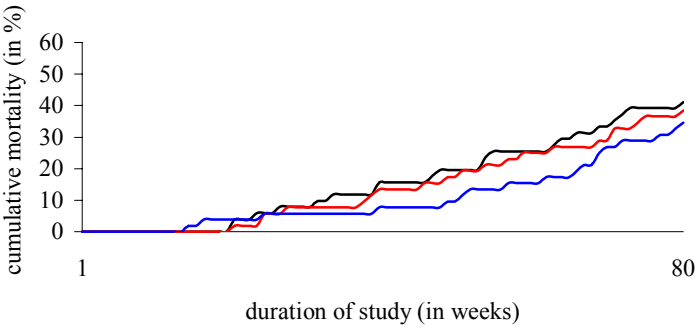

Figure 1: Mortality among male and female mice fed diets with 5, 25 or 125 ppm Cu. Black: 5 ppm of Cu added to the diet; red: 25 ppm of Cu; blue: 125 ppm of Cu.

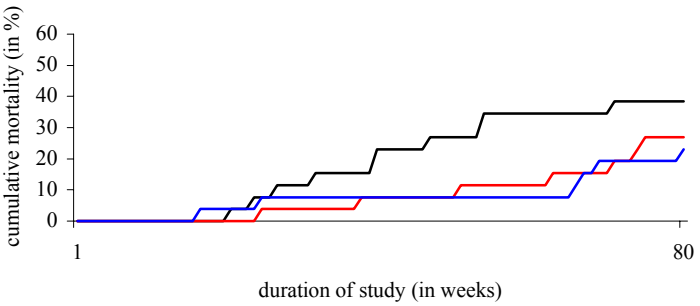

Figure 2: Mortality among male mice fed diets with 5, 25 or 125 ppm Cu. Black: 5 ppm of Cu added to the diet; red: 25 ppm of Cu; blue: 125 ppm of Cu.

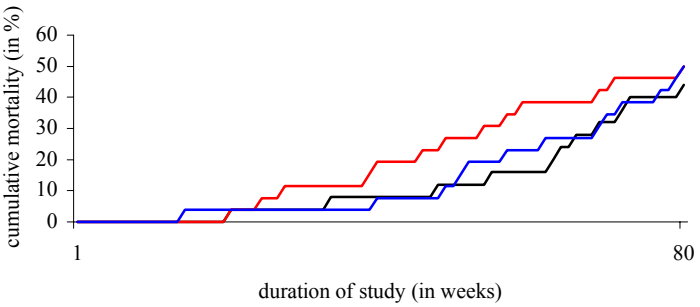

Figure 3: Mortality among female mice fed diets with 5, 25 or 125 ppm Cu. Black: 5 ppm of Cu added to the diet; red: 25 ppm of Cu; blue: 125 ppm of Cu.

11.20 ± 3.71, 9.48 ± 3.46 and 19.30 ± 9.19 µg Cu/g liver, respectively. Liver copper concentration in mice fed the diet containing 125 ppm Cu was statistically significantly higher than liver copper concentrations in mice fed diets containing either 5 or 25 ppm Cu ($p<0.01$ and $p<0.005$, respectively).

Body weight and feed intake

No statistically significant differences for body weight and feed intake were found in the mice fed the diets containing either 5, 25 or 125 ppm of Cu.

Longevity

After 80 weeks, when about 62% of the mice were still alive, we stopped the experiment because the surviving mice suffered from severe skin lesions. There were no differences in the frequency or severity of skin lesions between the dietary groups. Known causes of skin lesions could be ruled out so that the aetiology of the skin lesions remained unknown.

We did not observe significant differences in mortality between the three dietary groups when males and females were pooled (Figure 1). Although not statistically significant, mortality tended to be lowest in mice receiving the diet with 125 ppm of Cu, except for the period up to 25 weeks. This tendency is stronger, though not significant, for the males as a separate group (Figure 2). In the females, mortality in those mice receiving diets with 5 or 125 ppm of Cu tended to be somewhat lower than in their counterparts fed the diet containing 25 ppm of Cu, but cumulative mortality at 80 weeks did not differ between the groups (Figure 3).

Discussion

The hypothesis that high copper intake in the laboratory mouse reduces longevity was rejected by this experiment. On the contrary, mortality in the mice fed the diet with 125 ppm of Cu tended to be lower than in the groups with lower copper intake. Pathological examination of the mice did not reveal any differences between the dietary groups (data not shown). There is substantial evidence that copper is involved in the generation of reactive oxygen species, which can result in oxidative stress (De Wolf *et al.,* 2001a). There are at least two explanations for this apparent discrepancy. First, the alleged role of copper in accelerating the process of ageing is based on indirect evidence. As far as we know, this study is the first controlled experiment on the effect of copper intake on the development of oxidative stress in intact animals. Possibly, any oxidative stress caused by high copper intake was nullified by the body's antioxidant defence and repair system (De Wolf *et al.,* submitted a and b).

Secondly, copper metabolism in the mice was able to maintain copper homeostasis so that copper did not accumulate and thus could not cause oxidative damage. This reasoning is supported by the fact that in the male mice liver copper concentrations were similar for the three dietary treatments and in the female mice liver copper concentration was only slightly increased in the mice fed the diet with 125 ppm.

Commercial rodent diets generally contain levels of nutrients that are much higher than the physiological requirements. The high dietary concentrations of protein and phosphorus contribute to the development of glomerulonephrosis (Beynen and Meijer, 1995). So far, there is no evidence that the high copper concentrations in commercial diets, which range from 6 to 43 ppm (data not shown), have undesirable effects on the health of mice.

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Chapter 6

Liver copper content of rats hypo- or hyper-responsive to dietary cholesterol

I.D. de Wolf [1]
X.M. Fielmich-Bouman [1]
Æ. Lankhorst [1]
B.A. van Oost [2, 3, 4]
A.C. Beynen [1, 5]
V. Křen [6, 7]
M. Pravenec [6, 7]
L.F.M. van Zutphen [1]
H.A. van Lith [1]

Departments of [1] Laboratory Animal Science, [2] Clinical Sciences of Companion Animals, [3] Equine Sciences, [4] Farm Animal Health and [5] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [6] Department of Genetics of Model Diseases, Institute of Physiology, Academy of Sciences of the Czech Republic and [7] Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Prague, Czech Republic

submitted

Liver copper content of rats hypo- or hyperresponsive to dietary cholesterol

Abstract

The question addressed is whether cholesterol intake reduces the hepatic copper content in rats. For this purpose we have compared the hepatic copper content of two selected rat inbred strains after feeding the animals a control or a high fat, high cholesterol diet. One strain was dietary cholesterol resistant (SHR/OlaIpcv), whereas the other strain was susceptible to dietary cholesterol (BN-*Lx*/Cub). Dietary cholesterol-susceptible rats have a lower baseline hepatic copper content when compared with their resistant counterparts. The consumption of a hypercholesterolemic diet decreased liver copper concentration (expressed in μg/g dry weight) to about the same extent in both strains. However, dietary cholesterol did not reduce the absolute (expressed as μg/whole liver) and relative (expressed as μg/whole liver/100 g body weight) copper store of rats. The decrease of liver copper concentration after the high fat, high cholesterol diet is probably not caused by a decrease in hepatic copper amount, but rather due to dietary-induced hepatomegaly.

Introduction

Copper is an essential trace element for living systems, because it is used as a co-factor for key-enzymes involved in various fundamental biochemical processes but copper can be very toxic as well (1). Maintaining copper homeostasis via a well-functioning copper metabolism is thus a critical process. Copper metabolism, however, is affected by numerous internal and external factors, among which the pH in the gastrointestinal system, the hepatic and biliary function and the composition of the diet (2, 3). One of the nutrients associated with copper metabolism is cholesterol. For the rat, a substantial decrease in liver copper concentration has been shown after feeding a cholesterol-rich diet (4). Moreover, feeding rats a copper-deficient diet results in hypercholesterolemia (5, 6).

The mutual relationship between cholesterol and copper metabolism prompted us (i) to compare the hepatic copper content of dietary cholesterol resistant (hyporesponding) and dietary cholesterol susceptible (hyperresponding) rat inbred strains on a diet without added cholesterol and (ii) to test whether hyperresponding rats show a more marked decrease in hepatic copper content that their hyporesponding counterpart when fed a diet with added cholesterol.

Materials and methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals, housing, diets and preparation of samples

Rats (*Rattus norvegicus*) of the SHR/OlaIpcv and BN-*Lx*/Cub inbred strain were used. The spontaneously hypertensive rat (SHR) is a model of essential hypertension (7) and the normotensive Brown Norway congenic strain, BN-*Lx*, is a model of genetically determined leg malformation, the polydactyly-luxate syndrome (8). Furthermore, BN-*Lx*/Cub is a dietary cholesterol susceptible (hyperresponding) strain and SHR/OlaIpcv is a dietary cholesterol resistant (hyporesponding) strain (9).

After weaning up to an age of 7 weeks male SHR/OlaIpcv and BN-*Lx*/Cub rats were fed a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands), containing 21.57 mg Cu/kg diet. The chemical composition of this commercial diet has previously been described (10). After this pre-experimental period, the rats were divided into two groups per strain so that within-strain body weight distributions of the groups were similar. One group of each strain was transferred to a high-fat, high-cholesterol diet (SHR/OlaIpcv, n=5; BN-*Lx*/Cub, n=7), while the other remained to be fed on the commercial, control diet (SHR/OlaIpcv, n=4; BN-*Lx*/Cub, n=4). The high-fat, high-cholesterol diet was made from the commercial diet by addition of 5.0% (w/w) olive oil (Reddy, Van de Moortele NV, Oudenbosch, The Netherlands) and 2.0% (w/w) cholesterol (USP, Solvay-Parmaceutical BV, Weesp, The Netherlands). The cholesterol and olive oil were mixed into the diet by the manufacturer (Hope Farms BV). The high-fat, high-cholesterol diet was provided in pelleted form and was stored in the freezer until use. The test period lasted 4 weeks.

The animals were housed and studied in the Institute of Physiology, Czech Academy of Sciences under natural lighting conditions, temperature of 18-21^{o}C and relative humidity of 55-65%. The animals were housed as pairs or as groups of three animals in wire-topped Macrolon type III cages with a layer of sawdust as bedding. The rats had free access to food and tap water. Body weight was recorded at the beginning (day 0) and at the end (day 28) of the experimental period.

Blood samples were taken on days 0, 14 and 28 in random order between 08.00 and 10.00 h after a 16 hour fasting period. Orbital puncture was performed, while the rats were under light diethyl ether anesthesia. Blood was collected in tubes without anticoagulant. After clotting, serum was prepared by low-speed centrifugation and stored in the freezer until use. At the end of the experimental period the animals were anesthetized with diethyl ether, exsanguinated via the

inferior vena cava and the livers were removed and weighed. For each animal aliquots of liver (0.5 g) were immediately frozen (11).

Chemical analyses

Lipids were extracted from liver homogenates according to a modification of the method of Abell *et al.* (12). The pieces of the liver were homogenized on ice in ten volumes 12.5% (v/v) ethanol with a 180 s burst of an UltraTurrax tissue homogenizer (Janke and Kunkel, Staufen, Germany) at 20000 rev./min. The homogenates were then frozen at -20^{o}C, thawed and firmly stirred. From this homogenate 200 µl was taken and 2.0 ml of an ethanol-solution containing KOH (ethanolic alkali: 6 ml of 50%-KOH in a final volume of 100 ml absolute-ethanol) was added. The saponification was carried out in closed tubes overnight at 50^{o}C. After this reaction the tubes were adjusted to room temperature and 2.0 ml distilled water plus 4.0 ml petroleum ether (40^{o}C-60^{o}C) was added. The tubes were closed and shaken for 10 minutes with a frequency of 500 movements/min. The liquids were allowed to separate for 10 minutes. Three ml of the petroleum-ether fraction was evaporated under nitrogen at 70^{o}C. The residue was dissolved in 0.5 ml of absolute-ethanol. A sample from this final solution was taken for cholesterol determination.

Total cholesterol in the liver and the serum was measured enzymatically according to Siedel *et al.* (13), using a kit (Monotest®) supplied by Boehringer Mannheim GmbH (Mannheim, Germany). Cholesterol analyses were performed on a Cobas-BIO automatic micro-centrifugal analyser (Roche Diagnostics Systems, Hoffmann-La Roche, Basel, Switserland). For each individual animal the area under the curve (AUC) for the total experimental period was derived from the measured concentrations by the trapezoidal rule.

Copper in the liver was determined by drying pieces of the liver overnight at 105^{o}C, after which the dry weights were measured. Subsequently, the samples were ashed at 200^{o}C for one hour, 300^{o}C for two hours, 400^{o}C for three hours and 500^{o}C for ten hours. The remaining ash was dissolved in 1.0 ml concentrated $HClO_4$ which was then evaporated at 225^{o}C. This step was repeated until the ash was completely white. The ash was then dissolved in 1.0 ml 6 M HCl. Copper was measured by using flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia).

Statistical analyses

The animals were housed as pairs or as groups of three in a cage. Strictly speaking, a cage is the experimental unit. However, in the statistical analyses we treated these data as if an individual rat

was the experimental unit. Based on daily inspection of the rats (no signs of fighting among the animals in one cage etc.) we felt that this could be justified. In fact, diet and strain were the only important factors that discriminate between the individual rats.

The Kolmogorov-Smirnov one-sample test was used to check normality of the data. All results within groups were normally distributed. The significance of the differences between groups was calculated by a two-way analysis of variance (ANOVA). Homogeneity of the variances was tested using Bartlett's test. When necessary, the variances were equalized by logarithmic (14) transformation of the data. After transformation the variances were similar and the transformed within-group data were still normally distributed. Thus, application of an analysis of variance on the (transformed) data is then straightforward. If the analyses of variance showed significant effects the group means were further compared with the unpaired Student's *t* test. These tests were performed with pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was then tested using a F-test. To take into account the greater probability of a type I error due to multiple comparisons, the level of significance for the unpaired Student's *t* tests was pre-set at $P<0.05$/times a group is used for a comparison (i.e. $P<0.05/2 = 0.025$) instead of $P<0.05$, according to Bonferroni's adaptation. In all other cases, the probability of a type I error <0.05 was taken as the criterion of significance. Between selected parameters, Spearman's coefficient of rank correlation (R) was calculated; significance was assessed by a two-tailed test. Two-side probabilities were estimated throughout. All statistical analyses were carried out according to Petrie and Watson (15) using a SPSS PC+ computer program (15).

Results

Growth performance

At the beginning of the test period all rats were of the same age, but group mean body weight of SHR/OlaIpcv rats, when compared to BN-*Lx*/Cub rats, was slightly higher (data not shown). As would be expected, body weight increased, in an identical fashion, in the two rat inbred strains during the course of the experiment. Thus, as a consequence the strain difference in body weight was also present at the end of the experimental period (Table 1).

Serum cholesterol

At the end of the pre-experimental period, serum cholesterol levels (initial values) of the BN-*Lx*/Cub rats (81±9 mg/dL n=11) were slightly, but significantly higher than those of HR/OlaIpcv rats (72±8 mg/dL n=9) (Student's *t* test, p=0.029). The serum cholesterol response is

expressed as the AUC (Table 1). The high-fat, high-cholesterol diet when compared with the control diet produced in the SHR/OlaIpcv and BN-*Lx*/Cub strain a 1.3 and 2.5 times higher AUC, respectively. On the control diet BN-*Lx*/Cub and SHR/OlaIpcv had a similar AUC, whereas on the diet with added cholesterol the AUC of the BN-*Lx*/Cub strain was 1.7 times higher than that of SHR/OlaIpcv strain.

Liver weight

Irrespective of the diet, SHR/OlaIpcv rats have higher absolute and relative liver (wet and dry) weights than BN-*Lx*/Cub rats (Table 1). Previously we reported similar differences in liver wet weight between other substrains of SHR and BN (SHR/Cpb and BN/Cpb) (16). The consumption of fat and cholesterol raised in both strains absolute and relative liver (wet and dry) weights. This may be caused by hepatic lipodystrophy as suggested previously for rats (17).

Table 1: Body weight, serum cholesterol content and liver weight of hypo- and hyperresponsive rats fed diets with or without added cholesterol[1]

	Diet without added cholesterol		**Diet with added cholesterol**		
Measure	**SHR/OlaIpcv (n=4M)**	**BN-*Lx*/Cub (n=4M)**	**SHR/OlaIpcv (n=5M)**	**BN-*Lx*/Cub (n=7M)**	**Sign.[2]**
Final body weight *(g)*	217±4[a4]	171±11[a]	213±17[b]	175±14[b]	S
Serum cholesterol content *(AUC, day 0 to day 28)*					
(mg.day/dL)	2114±114[b5]	1881±156[a]	2713±177[bc]	4685±1127[ac]	S,D,SxD[3]
Liver wet weight					
Absolute (g)	6.77±0.16[ac]	4.32±0.20[ad]	7.80±0.56[bc]	5.40±0.28[bd]	S,D
Relative (g/kg body wt.)	31.21±0.70[ac]	25.20±1.23[ad]	36.60±0.64[bc]	30.96±1.39[bd]	S,D
Liver dry weight					
Absolute (g)	2.01±0.04[ac]	1.28±0.09[ad]	2.84±0.26[bc]	2.19±0.13[bd]	S,D,SxD[3]
Relative (g/kg body wt.)	9.29±0.18[ac]	7.46±0.40[ab]	13.33±0.47[c]	12.57±0.80[b]	S,D

1 Values are means ± SD; n is the number of male (M) animals per group

2 Significance (P<0.05) based on two-way ANOVA with main factors *strain* and *diet*: S: effect of strain, D: effect of diet, SxD: interaction.

3 ANOVA after logarithmic transformation of the data

4 Contrast significance (Student's *t* test; P<0.025); within rows, values bearing the same superscript letter are significantly different

5 From this group there was no day 28-serum available for determination of serum cholesterol concentration. Therefore, the AUC from day 0 to day 28 for this group was estimated by doubling the calculated AUC from day 0 to day 14. We felt that this was allowed, since for the BN-*Lx*/Cub group on the diet without added cholesterol the serum cholesterol concentration on day 0, 14 and 28 were not significantly different from each other and the serum cholesterol level on day 0 and day 14 of the low-fat, low-cholesterol SHR/OlaIpcv dietary group were also not significantly different.

Liver cholesterol

The consumption of cholesterol and fat drastically raised liver cholesterol concentration (mg/g wet weight and mg/g dry weight) in both strains (magnitude of the increase: SHR/OlaIpcv, 15 times; BN-*Lx*/Cub, 25 times) (Table 2). This corroborates previous work with inbred and outbred strains of the rat (10, 18). This diet effect was also borne out if liver cholesterol content is expressed as mg/whole liver or as mg/whole liver/100 g body weight (Table 2). BN-*Lx*/Cub rats when compared with SHR/OlaIpcv rats had on both diets a higher liver cholesterol concentration. On the high-fat, high-cholesterol diet absolute and relative liver cholesterol pool of BN-*Lx*/Cub rats was significantly enlarged when compared with the pools of SHR/OlaIpcv rats. However, on the control diet BN-*Lx*/Cub rats when compared with the SHR/OlaIpcv counterparts had a lower absolute pool of liver cholesterol. The two strains had similar relative pools of liver cholesterol on the low-fat, low-cholesterol diet.

Table 2: Liver cholesterol and copper content of hypo- and hyperresponsive rats fed diets with or without added cholesterol[1]

	Diet without added cholesterol		**Diet with added cholesterol**		
Measure	**SHR/OlaIpcv (n=4M)**	**BN-*Lx*/Cub (n=4M)**	**SHR/OlaIpcv (n=5M)**	**BN-*Lx*/Cub (n=7M)**	**Sign.[2]**
Liver cholesterol concentration					
(mg/g wet weight)	2.87±0.08^{ac4}	3.49±0.21^{ad}	38.65±3.59^{bc}	70.71±7.71^{bd}	S,D[3]
(mg/g dry weight)	9.66±0.26^{ac}	11.81±0.81^{ad}	106.02±8.31^{bc}	174.05±18.49^{bd}	S,D[3]
Liver cholesterol amount					
(mg/whole liver)	19.47±0.80^{ac}	15.06±0.75^{ad}	302.36±42.75^{bc}	381.31±43.14^{bd}	D,SxD[3]
(mg/100 g body wt.)	8.97±0.38^{a}	8.80±0.64^{c}	141.43±13.26^{ab}	219.23±30.12^{bc}	S,D,SxD[3]
Liver copper concentration					
(µg/g wet weight)	9.20±2.46	7.72±1.82	7.14±0.87	6.83±0.63	-
(µg/g dry weight)	30.93±8.32	26.06±5.90	19.58±2.03^{a}	16.71±1.05^{a}	S,D[3]
Liver copper store					
(µg/whole liver)	62.19±16.21^{a}	33.40±8.27^{a}	55.90±9.45^{b}	36.76±2.77^{b}	S[3]
(µg/100 g body wt.)	28.66±7.47	19.30±3.72	26.15±3.44^{a}	21.10±1.69^{a}	S[3]

[1] Values are means ± SD; n is the number of male (M) animals per group.
[2] Significance (P<0.05) based on two-way ANOVA with main factors *strain* and *diet*: S: effect of strain, D: effect of diet, SxD: interaction.
[3] ANOVA after logarithmic transformation of the data.
[4] Contrast significance (Student's *t* test; P<0.025); within rows, values bearing the same superscript letter are significantly different.

Liver copper

There were no significant strain or diet effects on the levels of hepatic copper when the copper concentration is expressed as µg/g wet weight (Table 2). Although, the diet with added cholesterol produced lower group mean liver copper levels in both strains and the group means of the BN-*Lx*/Cub strain were lower than those of the SHR/OlaIpcv strain. If liver copper concentration is expressed as µg/g dry weight, there was a significant strain and diet effect. SHR/OlaIpcv when compared with BN-*Lx*/Cub rats had higher group mean liver copper levels, but in the multiple comparison procedure this strain effect reached the level of statistical significance only on the diet with added cholesterol. The high-fat, high-cholesterol diet produced in both strains lower group means of liver copper level (expressed as µg/g dry weight). After calculation of the absolute (µg/whole liver) and relative (µg/whole liver/100 g body weight) liver copper store and performing a statistical analysis only a strain effect was revealed: SHR/OlaIpcv when compared with BN-*Lx*/Cub rats had enlarged stores of liver copper.

Of the parameters for liver copper content only the variable liver copper concentration expressed as µg/g dry weight was significantly (negatively) correlated with the AUC ($R=-0.8842$, $n=20$, $p<0.001$). Liver copper concentration expressed as µg/g dry weight was significantly (negatively) associated with each parameter for liver cholesterol content (Table 3). If liver copper concentration is expressed as µg/g wet weight there was a significant assocation with liver cholesterol concentration (expressed as mg/g dry weight) and liver cholesterol amount (expressed as mg/whole liver/100 g body weight) (Table 3). Absolute liver copper store was, in contrast with relative copper store, significantly associated with liver cholesterol concentration expressed as µg/g dry weight.

Discussion

In the literature substantial evidence for a relationship between cholesterol levels and copper metabolism has been described. The aim of the present work was to determine whether cholesterol in the diet influences liver copper content differently in rats which differ in their hypercholesterolemic response to dietary cholesterol.

On the control diet the mean liver copper concentration was 26.06 and 30.93 µg/g dry weight for BN-*Lx*/Cub and SHR/OlaIpcv, respectively (Table 2). This is in line with the range of hepatic copper concentrations reported in Takahashi *et al*. (19) (10.7 - 29.9 µg/g dry weight) and Veenendaal *et al.* (20) (31.0 - 41.0 µg/g dry weight).

A high fat, high cholesterol diet produced in both rat inbred strains significantly lower liver copper levels when expressed as μg/g dry weight (Table 2). We may assume that under the conditions of *ad libitum* food consumption the animals maintain relatively constant intakes of energy, regardless of the composition of the diet (21). Therefore on the test diet, when compared with the control diet, copper intake was lower. In addition, the olive oil in the high fat, high cholesterol diet, may also modify copper absorption: long-chain fatty acids may reduce the copper absorption rate (22). However, the absolute and relative liver copper stores were not influenced by the high fat, high cholesterol diet. Thus, the diet effect on hepatic copper levels cannot readily be explained by a reduction in copper intake or absorption. Since the high fat, high cholesterol diet induced hepatomegaly in both strains (Table 1), the decrease in liver copper concentration might be explained by an enlargement of the liver. The decrease in liver copper concentration in rats fed a high cholesterol diet or a diet supplemented with L-histidine as reported by Abu-el-Zahab *et al.* (4) and Harvey *et al.* (23), respectively, is most likely caused also by hepatomegaly.

The BN-*Lx*/Cub when compared with the SHR/OlaIpcv strain has, irrespective of the diet, lower levels of hepatic copper and also lower liver copper stores (Table 2). As to the mechanism accounting for the strain difference in liver copper content between these two rat strains we can only speculate. Part of this effect may be due to lower feed intake by the BN rats when compared with the SHR rats. A possible explanation for the observed strain difference in hepatic copper may also be found in the relatively high glucocorticoid levels of SHR rats (24). Glucocorticoids can stimulate synthesis of metallothionein and the copper-containing protein ceruloplasmin in the liver (25). Copper, absorbed from the diet through the intestine, is transported to and taken up by the liver. In part it is incorporated into newly synthesized ceruloplasmin that is excreted into the plasma. Besides incorporation of copper in copper-containing proteins, part is stored as metallothionein. The remaining copper is excreted into the bile. Thus, once ceruloplasmin and metallothionein are synthesized, there might be both an increase in liver copper content and in plasma copper concentration. It is interestingly to note that Berthelot *et al.* (26) reported that plasma copper levels were similar in SHR and WKY rats at 5 and 7 weeks of age, but thereafter plasma copper levels increased significantly more in SHR than WKY. Schedl *et al.* (27) described a trend of increased serum copper concentrations in the SHR relative to the WKY control rat. Furthermore, Apostolova *et al.* (28) found that SHR showed significantly higher values of hepatic metallothionein when compared with WKY rats.

In conclusion, SHR/OlaIpcv and BN-*Lx* rat inbred strains differ in liver copper content. Consumption of a high fat, high cholesterol diet reduced liver copper concentration, but not liver

copper store. The strain effect may be explained by differences in circulating glucocorticoid levels.

Acknowledgements

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Table 3: Associations between liver cholesterol and liver copper in rats[1]

	Liver cholesterol concentration				**Liver cholesterol amount**			
	mg/g wet weight		***mg/g dry weight***		***mg/whole liver***		***mg/100 g body weight***	
	Spearman's R	**p-value**	**Spearman's R**	**p-value**	**Spearman's R**	**p-value**	**Spearman's R**	**p-value**
Liver copper concentration								
(µg/g wet weight)	-0.3940	0.086	-0.4481	0.048	-0.2376	0.313	-0.4541	0.044
(µg/g dry weight)	-0.8752	<0.001	-0.8857	<0.001	-0.8165	<0.001	-0.9038	<0.001
Liver copper store								
(µg/whole liver)	-0.4165	0.068	-0.4481	0.048	-0.0812	0.734	-0.3459	0.135
(µg/100 g body wt.)	-0.3383	0.145	-0.4060	0.076	-0.0767	0.748	-0.2977	0.202

1 Association based on 20 animals.

Chapter 7

Genetic and correlation analysis of hepatic copper content in the rat

I.D. de Wolf [1]
X.M. Fielmich-Bouman [1]
B.A. van Oost [2, 3, 4]
A.C. Beynen [1, 5]
V. Křen [6, 7]
M. Pravenec [6, 7]
L.F.M. van Zutphen [1]
H.A. van Lith [1]

Departments of [1] Laboratory Animal Science, [2] Clinical Sciences of Companion Animals, [3] Equine Sciences, [4] Farm Animal Health and [5] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [6] Department of Genetics of Model Diseases, Institute of Physiology, Academy of Sciences of the Czech Republic and [7] Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Prague, Czech Republic

submitted

Genetic and correlation analysis of hepatic copper content in the rat

Abstract

Thirty recombinant inbred (RI) strains derived from the spontaneous hypertensive rat (SHR/OlaIpcv) and the Brown Norway (BN-*Lx*/Cub) progenitors were used to search for quantitative trait loci (QTLs) that are responsible for differences in liver copper between these two strains. The heritability of liver copper concentration (expressed as µg/g liver w.w. and µg/g liver d.w.) and liver copper store (µg/whole liver) was estimated to be 57%, 57% and 46%, respectively. In a total genome scan of the RI strains, involving over 600 genetic markers, suggestive association was found between liver copper store (µg/whole liver) and the *D16Wox9* marker on chromosome 16 (lod score = 2.8), and between liver copper concentration (µg/g dry weight) and the *D10Cebrp1016s2* marker on chromosome 10 (lod score = 3.0). These putative QTLs are responsible for nearly 34% and 40% of the additive genetic variability for liver copper store and concentration, respectively.

Introduction

Differences between inbred strains of rats have been reported for liver copper content (1-4), which suggests an important role for genetic factors. For instance, the Long-Evans Cinnamon (LEC) mutant rat shows an excessive copper accumulation in the liver (5). Genetic analysis revealed that a deletion mutation in the *Atp7b* gene, which codes for an ATPase that can either transport copper into or out of the cell, is responsible for hepatic copper accumulation in the LEC strain (6). This mutation of the *Atp7b* gene is the rat counterpart of the Wilson's disease gene in humans (5) and is located on rat chromosome 16. However, QTL analyses have not yet been carried out with other strains that differ for liver copper content. This prompted us to perform a genetic analysis with the BN-*Lx*/Cub and SHR/OlaIpcv as progenitor strains. In a previous study (1) we found that the hepatic copper content of the SHR/OlaIpcv strain was about 1.5 times higher when compared with the BN-*Lx*/Cub strain. The aim of the present study was to locate the QTLs influencing the liver copper content in these strains and to investigate how liver copper content correlates with parameters for insulin resistance, fatty acid metabolism and blood pressure. The results of the QTL-analysis and possible candidate genes located in the vicinity of these QTLs will be discussed.

Material and methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals

Genetic studies were performed in 30 recombinant inbred (RI) strains derived from spontaneously hypertensive rats (SHR/OlaIpcv) and normotensive Brown Norway rats (BN-*Lx*/Cub) (7). The SHR progenitor strain descends from inbred SHR originally obtained from the National Institutes of Health. The BN-*Lx* progenitor is a congenic strain that carries a segment of chromosome 8 from the polydactylous PD/Cub strain (7). All strains have been maintained in Prague by inbreeding for more than 15 years. The RI strains were derived from an (SHR x BN-*Lx*) F_2 population: the F_2 rats were paired off at random, and each of these F_2 pairs was used to generate a new inbred strain by repeated brother x sister mating of the offspring for at least 20 generations. Currently, most of the RI strains reached more than 45 generations of brother-sister inbreeding. From three to six males of each RI strain the liver copper content was determined.

Experimental protocol

The animals were housed and studied in the Institute of Physiology, Czech Academy of Sciences under natural lighting conditions, temperature of 18-21^{o}C and relative humidity of 55-65% .The animals were housed as pairs or as groups of three animals in wire-topped Macrolon type III cages with a layer of sawdust as bedding. The rats had free access to food and tap water.

After weaning up to an age of 7 weeks, the animals were fed a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands). The chemical composition of this commercial diet has previously been described (8). Then, the rats received a commercial diet supplemented with 5.0% (w/w) olive oil (Reddy, Van de Moortele NV, Oudenbosch, The Netherlands) and 2.0% (w/w) cholesterol (USP, Solvay-Parmaceutical BV, Weesp, The Netherlands). This diet had been fed for 4 weeks. In a previous experiment (1), we found that a high-fat, high-cholesterol diet has no effect on liver copper content in BN-*Lx*/Cub or SHR/OlaIpcv, whereas the decrease of liver copper concentration was identical in both strains (i.e. there was no interaction-effect between the factors "strain" and "diet") (1).

At the age of 11 weeks the animals were anesthetized with diethyl ether, exsanguinated via the inferior vena cava and the livers were removed and weighed. For each animal two liver specimen (0.5 g) were immediately frozen (9).

Chemical analyses

Liver copper concentration and liver copper content were determined by drying the liver specimen overnight at 105°C, after which the dry weights were measured. Subsequently, the samples were ashed at 200°C for one hour, 300°C for two hours, 400°C for three hours and 500°C for ten hours. The remaining ash was dissolved in 1 ml concentrated $HClO_4$ which was then evaporated at 225°C. This step was repeated until the ash was completely white. The ash was then dissolved in 1 ml 6 M HCl. Copper was measured by using flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia).

Genetic and statistical analyses

Heritability of liver copper content was estimated according to the method of Plomin and McClearn (10) using the variances in liver copper between and within the RI and progenitor strains. The additive genetic variance was estimated as 50% of the total variance between the means of the RI strains; the environmental variance was estimated to be the average variance in mean phenotypic values within the RI strains. Narrow heritability was calculated by dividing the additive genetic variance by the sum of the additive genetic variance and the environmental variance.

QTL mapping was performed using Map Manager QT (version b28) (11) and the strain distribution patterns of more than 600 genetic markers previously mapped in the RI strains (7). The marker data set covers approximately 1200 centiMorgans of the rat genome and has proved effective in genome scanning for QTL regulating a variety of complex traits (12). Map Manager QT was used to test for single locus associations by regression analysis and the significance of each potential association was measured using the likelihood ratio statistics (LRS) of Haley and Knott (13). The interval regression method of Map Manager QT was used to test for QTL within marker intervals. The significance threshold for the genome wide scan was empirically determined by the Map Manager QT permutation test, using the informative markers and 1000 permuted data sets as recommended by Doerge and Churchill (14). Significant linkage was defined in accordance with the guidelines of Lander and Kruglyak (15) as statistical evidence occurring by chance in the genome scan with a probability of 5% or less. Based on these criteria and the results of the permutation test, a LRS threshold value of 15.9 and 16.4 (corresponding to a lod score of 3.5 and 3.6, respectively) was established for significant linkage in the RI strain data set for liver copper concentration (expressed per g dry weight) and hepatic copper content (expressed as µg Cu/whole liver), respectively. The LRS threshold value for suggestive linkage

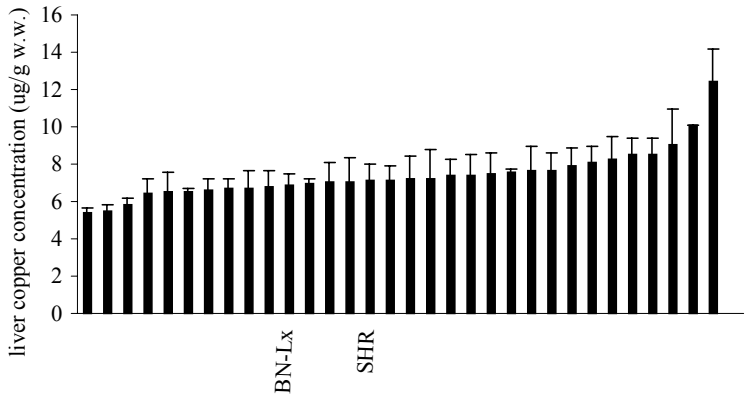
was found to be 9.1 and 9.4 (corresponding to a lod score of about 2.0) for liver copper concentration and hepatic copper content, respectively.

Results

Our previous studies with the progenitor strains revealed that liver copper content was higher in SHR/OlaIpcv when compared with BN-*Lx*/Cub (1). In the RI strains derived from the progenitors of these strains, the distribution of each parameter for liver copper content was continuous, suggesting a polygenic mode of inheritance of the trait (Fig. 1). The BN-*Lx*/Cub strain exhibited the lowest hepatic copper content, whereas the mean liver copper content of the SHR/OlaIpcv progenitor arrived somewhat right to the middle of the distribution of this parameter of the RI strains (Fig. 1c). For liver copper concentration neither the BN-*Lx*/Cub nor the SHR/OlaIpcv were at the extremes of the distribution of this parameter of the RI strains (Fig. 1a and 1b). The observation of lower and higher hepatic copper contents in certain RI strains than in either progenitor is consistent with multifactorial inheritance and suggests the possibility of gene-gene interactions.

Based on the variances in hepatic copper content within and between the RI strains, the narrow heritability of liver copper content was estimated to be 57%, 57% and 46% for hepatic copper expressed as μg/g wet weight, μg/g dry weight and μg/whole liver, respectively. Given the observed substantial genetic component to hepatic copper content in the RI strain model, we scanned for QTLs influencing liver copper phenotypes using the Map Manager QT program. Genome scanning of the RI strains revealed suggestive linkage of the hepatic copper phenotypes to the *D10Cebrp1016s2* marker on chromosome 10 (LRS = 13.9, lod score = 3.0, p = 0.00019) and the D16Wox9 marker (coding for *Mbpa*, serum mannose binding protein A) on chromosome 16 (LRS = 13.5, lod score = 2.9, p = 0.00035) (Table 1). Interval mapping across the critical regions of chromosome 10 and 16 revealed distinct LRS peaks near the *D10Cebrp1016s2* and *D16Wox9* marker, respectively (Fig. 2). We designated the chromosome 10 QTL region *Hcuc1* (hepatic copper content 1) and the chromosome 16 QTL region *Hcuc2* (hepatic copper content 2). *Hcuc1* accounts for 40% of the additive genetic variance of the hepatic copper concentration phenotype, whereas *Hcuc2* accounts for 34% of the hepatic copper store phenotype.

The HXB/BXH sets of RI strains are extensively used for mapping of genes involved in e.g. blood pressure regulation, insulin resistance and fatty acid metabolism (12), and quantitative data on these parameters are available. Several papers suggest for rats a relationship between copper metabolism and hypertension (16, 17), between copper metabolism and diabetes (18, 19) and between copper metabolism and fatty acid metabolism (20). Therefore, we searched for possible



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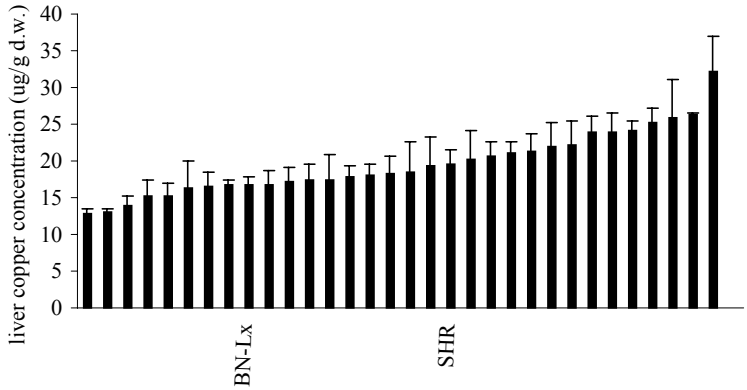
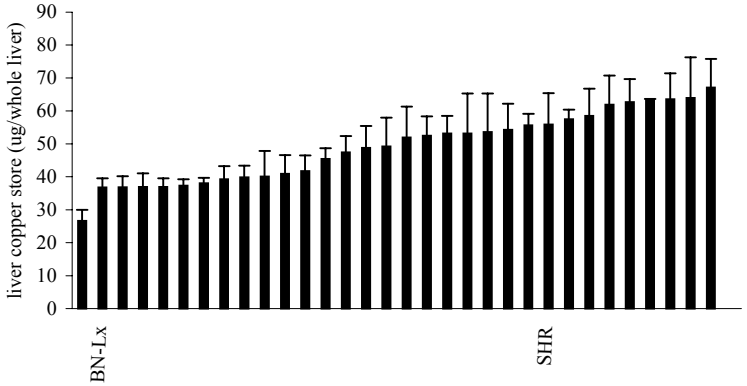


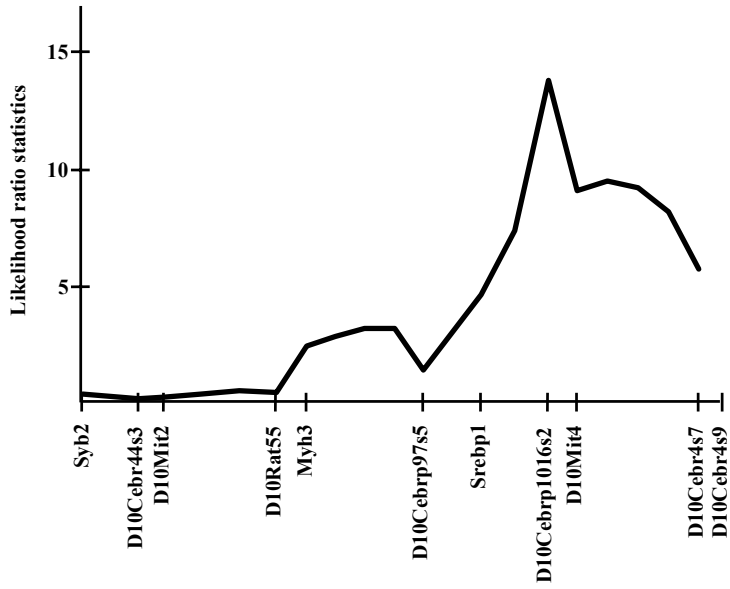
b.



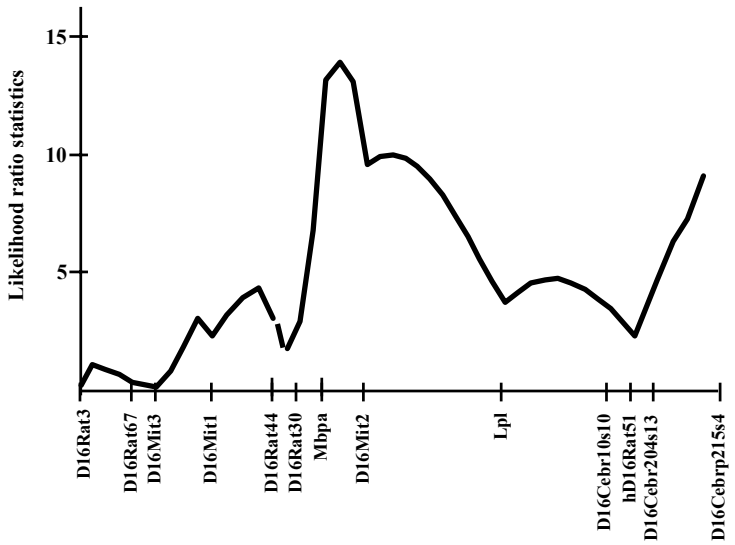
c.

Figure 1: The distribution of liver copper content (means ± SD) of recombinant inbred strains and the progenitor strains. Liver copper content has been expressed as (a) μg/g wet weight, (b) μg/g dry weight and (c) μg/whole liver.

a)


Likelihood ratio statistics
15
10
5
Syb2
D10Cebr44s3
D10Mit2
D10Rat55
Myh3
D10Cebrp97s5
Srebp1
D10Cebrp10l6s2
D10Mit4
D10Cebr4s7
D10Cebr4s9

b)


Likelihood ratio statistics
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10
5
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D16Rat67
D16Mit3
D16Mit1
D16Rat44
D16Rat30
Mbpa
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D16Cebr204s13
D16Cebrp215s4

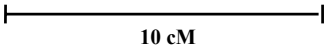
10 cM

Figure 2: Interval mapping of the QTL regulating (a) liver copper concentration (μg/g dry weight) or (b) liver copper store (μg/whole liver). Likelihood ratio statistics from the Map Manager QT linkage analysis are plotted across the segment of chromosome 10 (a) or chromosome 16 (b). Estimated distance between markers in centiMorgans were determined with the Haldane map function.

Table 1: Genetic markers associated with hepatic copper content in rats[1].

		Allele		Lod
Phenotype	Marker	BN-*Lx*/Cub	SHR/OlaIpcv	score
μg/g dry weight	*D10Cebrp1016s2*	17.48 ± 0.85	23.74 ± 1.35	3.0
μg/whole liver	*D16Wox9*	44.29 ± 2.35	57.78 ± 2.55	2.8

[1] Means (± SEM) of hepatic copper content in RI strains that inherited BN-Lx/Cub and SHR/OlaIpcv alleles of respective genetic markers.

correlations between parameters for liver copper content and blood pressure, insulin resistance or fatty acid metabolism parameters. In table 2 the significant associations are summarized.

Discussion

Genetic analysis of the liver copper content in the recombinant inbred strains indicated that the phenotypes are under polygenic control as suggested by the continuous variability (Fig. 1). The narrow heritability was estimated to be between 46% and 57%, which motivated us to search for responsible QTLs. Genome wide scanning for associations between marker genotypes and liver copper resulted in the localization of a QTL on rat chromosome 10 (near the *D10Cebrp1016s2* marker) for liver copper concentration (μg/g dry weight) and another one on chromosome 16 (near the *D16Wox9* marker) for liver copper store (μg/whole liver) (Table 1 and Fig. 2).

Based on homology between the segment of rat chromosome 10, where the putative QTL has been mapped (Fig. 2), and human chromosome 5q (21), it is possible that the *Atox1* gene, which codes for the antioxidant protein 1 (22), is a positional candidate for the regulation of liver copper concentration. It has been suggested that in the liver this protein binds and delivers cytosolic copper to the Wilson disease ATPase protein (ATP7B) in the trans-Golgi network. This ATPase is required for incorporation of copper into ceruloplasmin (the major copper binding protein in the circulation) during its formation and folding and/or to release hepatic copper into bile (5). As a result of differential activity of antioxidant protein 1, more or less copper may be excreted in bile or the circulation, implying that less or more copper may be stored in the liver.

Hiromura and Sakurai (23) reported that rat *Atox1* was found to be expressed at high levels in the liver.

On rat chromosome 16 the Lod score peaked at the *D16Wox9* marker (Fig 2). This marker codes for *Mbpa*, serum mannose bindig protein A. The *Atp7b* gene has been located at the telomeric part of the q-arm of rat chromosome 16 at a distance of about 55 cM from *Mbpa* (24). This location excludes *Atp7b* as a positional candidate gene for the observed variation in liver copper content in the present strains. The homologous human gene (*MBL2*) is on chromosome 10q (25). In the vicinity of this gene, the *COX15* gene is located (26). The cox15 protein is a constituent of the inner mitochondrial membrane and is most likely involved in the assembly of the cytochrome oxidase protein backbone (27). A mutation in the *Cox15* gene might result in an impaired assembly of the COX protein backbone. Since the normal COX protein backbone is bound to 2 copper-containing prosthetic groups, a mutation in the *Cox15* gene could result in a reduced capacity of the hepatic mitochondria to store copper.

The SHR strain is a frequently used model for hypertension studies. In the Dahl salt-sensitive rat, which is another model for hypertension, Clegg *et al*. (16) have found that an increase of the systolic blood pressure was associated with a decrease of the liver copper concentration. We also found a significant negative correlation between these two parameters. *D16Wox9* is located in the region of chromosome 16, which was previously found to be linked to inherited variation in salt-loaded blood pressure (28). Also, the *D10Cebrp1016s2* marker has previously been shown to indicate a QTL regulating blood pressure (29). It is not clear so far whether or not these two parameters are functionally related. The positive correlation, found between hepatic copper concentration and the parameters for insulin resistance and fatty acid metabolism, might be explained by the fact that *Srebf1* is closely linked to the *D10Cebrp1016s2* marker (30). In man this gene is a key regulator of genes that encodes enzymes in the biosynthetic pathways of cholesterol, fatty acids and triglyceride metabolism (31, 32). Moreover, it has been demonstrated that this gene plays a pivotal role in mediating the effects of insulin on the expression of genes that regulate hepatic glucose and lipid metabolism (33, 34).

In summary, the present study indicates that both chromosome 10 and 16 contain a locus that plays a role in controlling the hepatic copper content in rats. When considering the homology of the rat chromosomal regions that contain a hepatic copper content (*Hcuc*) locus with those of human, one might speculate about *Atox1* and *Cox15* genes as candidate loci for *Hcuc1* (RNO10) and *Hcuc2* (RNO16), respectively. Further experiments, including the development of congenic sublines of SHR into recombinant sublines of SHR.BN congenic strains, are necessary to confirm and precisely map the QTLs on rat chromosome 10 and 16.

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Table 2: Significant associations between liver copper and blood pressure, insulin resistance or fatty acid metabolism in rats[1]

	Blood pressure				Insulin resistance		fatty acid metabolism	
	systolic		*diastolic*		*insulin-stimulated glucose uptake in isolated adipocytes*		*isoproterenol-induced lipolysis in isolated adipocytes*	
	Pearson's r	p-value	Pearson's r	p-value	Pearson's r	p-value	Pearson's r	p-value
Liver copper concentration								
(µg/g wet weight)	-0.43	0.02	-0.36	<0.05	0.51	0.01	0.40	0.04
(µg/g dry weight)	-0.42	0.02	-0.46	0.01	not significant		not significant	

[1] Association based on 30 RI strains. For each parameter the means from the RI strains have been used for calculation of the correlation coefficients.

Chapter 8

Quantitative trait loci influencing hepatic copper in rats

I.D. de Wolf [1]
A.C.M. Bonné [1]
X.M. Fielmich-Bouman [1]
B.A. van Oost [2, 3, 4]
A.C. Beynen [1, 5]
L.F.M. van Zutphen [1]
H.A. van Lith [1]

Departments of [1] Laboratory Animal Science, [2] Clinical Sciences of Companion Animals, [3] Equine Sciences, [4] Farm Animal Health and [5] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

submitted

Quantitative trait loci influencing hepatic copper in rats

Abstract

Significant differences in liver copper content have been observed between rat inbred strains. To define loci controlling this trait, the offspring (n=190) from an (LEW/OlaHsd x BC/CpbU) F_2-intercross was genetically analyzed. From each F_2-animal liver copper content was determined and genomic DNA was screened with polymorphic microsatellite markers. We found a major quantitative trait locus (QTL) for liver copper content in females on chromosome 2 and in males on chromosome 10. Both QTLs accounted for approximately 20% of the genetic variance. In addition, suggestive linkage for liver copper content was found on rat chromosomes 1, 8, 12, 14 and 19. The regions on these chromosomes contain genes that are responsible for 9.0 to 15.5% of the genetic variance of liver copper content.

Introduction

Strain differences in rat liver copper content have been described by Yu *et al.* (1), Hayashi *et al.* (2), Schilsky *et al.* (3) and De Wolf *et al.* (4). Previously, we have searched for the genetic components associated with liver copper content using a set of recombinant rat inbred strains derived from SHR/OlaIpcv and BN-*Lx*/Cub since no QTL analyses, except for the Long-Evans Cinnamon (LEC) mutant rat, have been carried out with strains that differ in liver copper content. It was suggested that at least two regions, one on chromosome 10 and one on chromosome 16, are associated with liver copper content in male rats (5). However, the limited power of recombinant inbred strains for detecting QTLs prompted us to perform a total genome scan of an F_2-population to search for additional genetic factors controlling liver copper content. Furthermore, the previous study in which we used recombinant inbred strains included male rats only. In this study, we included both male and female rats to study whether gender-related differences in QTLs for liver copper concentration or liver copper content exist. The F_2-intercross from the LEW/OlaHsd and BC/CpbU inbred strains, previously used for testing the genetic basis of the differences in susceptibility for cholesterol, was available for this study.

Materials and methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals and housing

All animals were kept under SPF conditions and a 12 hours per day light-regimen (7.00 h - 19.00 h). The other laboratory conditions, temperature and humidity, were also controlled. From four males and four females of the parental strains LEW/OlaHsd (obtained from Harlan, UK) (LEW) and BC/CpbU (obtained from the Central Laboratory Animal Institute of the Utrecht University, The Netherlands) (BC), the liver copper content was determined as described in the experimental protocol (see below). The F_1-generation consisted of 17 males and 15 females and was derived by reciprocal matings of LEW and BC animals. The F_1-hybrids were intercrossed (brother-sister mating) producing F_2-progeny. From 90 F_2-males and 100 F_2-females, the liver copper content was determined.

Experimental protocol

The animals were housed as pairs or as groups of three animals in wire-topped Macrolon type III cages with a layer of sawdust as bedding. The rats had free access to food and tap water. After weaning up to an age of 7 weeks the animals were fed a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands). The chemical composition of this commercial diet has previously been described (6). Then, the rats received a commercial diet supplemented with 5.0% (w/w) olive oil and 2.0% (w/w) cholesterol. This diet had been fed for 4 weeks. In a previous experiment, we found that this diet did not influence the strain difference in liver copper content (4).

At the age of 11 weeks, body weight of the rats was determined. The animals were anesthetized with diethyl ether, exsanguinated via aorta punction and the livers and spleens were removed and weighed. For each animal, two pieces of liver (0.5 g) were immediately frozen (7). The spleen was used for DNA isolation.

Chemical analyses

Copper in the liver was determined by drying the pieces of the liver overnight at 105°C, after which the dry weights were measured. Subsequently, the samples were ashed at 200°C for one hour, 300°C for two hours, 400°C for three hours and 500°C for ten hours. The remaining ash was dissolved in 1 ml concentrated $HClO_4$, which was then evaporated at 225°C. This step was

repeated until the ash was completely white. The ash was then dissolved in 1 ml 6 M HCl. Copper was measured by using flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia).

Genome scan

A total of 239 autosomal microsatellite (SSLP) markers, polymorphic between the LEW and BC strain, were used for screening of the F_2 progeny (8).

Statistical and QTL analyses

Both for the parental strains and for the F_2-intercross rats, all statistical analyses were carried out according to Petrie and Watson (9) using a SPSS PC+ computer program (10).

I. Parental strains

The Kolmogorov-Smirnov one-sample test was used to check normality of the measured phenotypic characteristics of the BC and LEW rats. All results within groups were normally distributed. The significance of the differences between groups was calculated by a two-way analysis of variance (ANOVA) with strain and gender as main factors. Homogeneity of the variances was tested using Bartlett's test. When necessary, the variances were equalized by logarithmic transformation of the data (9). After transformation the variances were similar and the transformed within-group data were still normally distributed. Thus, application of an analysis of variance on the (transformed) data is then straightforward.

II. F_2-animals

Within each gender, all liver copper traits were normally distributed (tested by Kolmogorov-Smirnov one-sample test). The location of the QTLs affecting the measured quantitative traits and the variance explained by each locus was determined using the MapQTL computer package (11). The interval-mapping module was used (12). Results were expressed as Lod scores. Lod score thresholds to achieve the genome-wide significance levels of 5% (significant linkage) were, as proposed by Lander and Kruglyak (13), 4.3 when no model of gene action was specified ("free" genetics), 3.4 when gene action was restricted to recessive or dominant models, and 3.3 when gene action was restricted to an additive model. For suggestive linkage, Lod score values were 2.8 ("free" genetics), 2.0 (recessive or dominant) and 1.9 (additive), respectively.

Comparison of the liver copper traits of the F_2 animals after grouping by genotype of a microsatellite marker was also performed. If a microsatellite marker and the trait of interest are segregating independently, the values of the trait will be equally distributed among the homozygote and heterozygote genotypes. The Kolmogorov-Smirnov one-sample test was used to check normality of these data. All results within genotype groups were found to be normally distributed. For each gender, cosegregation of phenotypes with alleles at marker loci was evaluated by comparing the values between different genotypes by one-way ANOVA.

Results

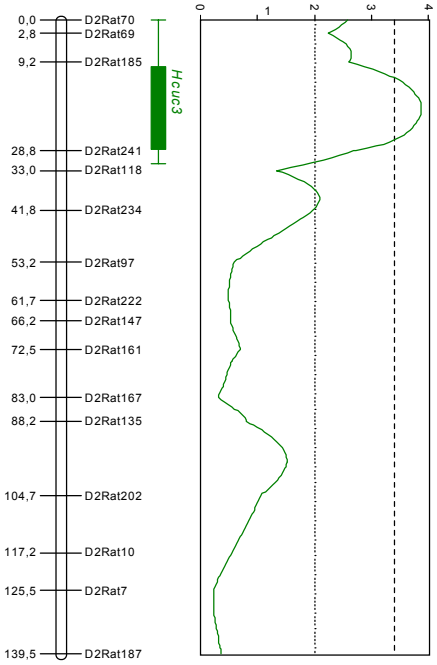
Parental strains

At the end of the test period the BC and LEW rats were of the same age, but LEW rats had a higher body weight than BC rats. The strain effect on body weight was more pronounced in the male rats. As would be expected males were significantly heavier than females (table 1).

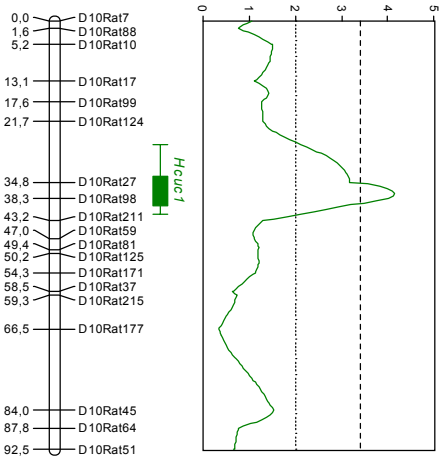
Table 1: Body weight, liver weight and liver copper content of BC/CpbU and LEW/OlaHsd rats[1]

	BC/CpbU		**LEW/OlaHsd**		
Measure	**Males (n=4)**	**Females (n=4)**	**Males (n=4)**	**Females (n=4)**	**Sign.[2]**
Final body weight *(g)*	220±21	177±5	352±34	224±12	S,G,SxG
Liver wet weight					
Absolute (g)	10.0±1.2	9.3±0.4	13.4±1.8	8.1±0.4	G,SxG
Relative (g/kg body wt.)	45.2±2.6	52.2±1.3	38.0±1.8	36.3±0.7	S,G,SxG
Liver copper concentration					
(µg/g wet weight)	8.7±2.0	8.1±2.4	6.3±0.4	7.9±2.0	-
(µg/g dry weight)	18.9±3.6	18.7±5.7	15.2±1.2	18.0±4.5	-
Liver copper store					
(µg/whole liver)	85.3±10.0	75.2±23.5	83.3±6.3	64.0±14.4	-
(µg/100 g body wt.)	39.3±8.0	42.4±13.0	23.7±0.7	28.8±7.8	S[3]

[1] Values are means ± SD; n is the number of animals per group.
[2] Significance (P<0.05) based on two-way ANOVA with main factors *strain* and *gender*; S: effect of strain, G: effect of gender, SxG: interaction.
[3] ANOVA after logarithmic transformation of the data.



a.



b.

Figure 1: Lod score plots of chromosomes exhibiting significant linkage of quantitative trait to microsatellite markers. Quantitative trait linkage analysis was performed by interval mapping using MapQTL on data collected from 190 (LEW/OlaHsd x BC/CpbU) F_2-intercross rats investigated in this study. (A) Chromosome 2, females: hepatic copper concentration (µg/g dry weight). (B) Chromosome 10, males: hepatic copper concentration (µg/g dry weight). The distances are indicated in cM. The positions of selected marker loci genotyped in the F_2 progeny are indicated on the x-axis of each panel. The most likely position for each QTL, determined by its 2.0-Lod support interval, is indicated by a solid, thick bar under the plot. The thin lines at both ends of the bar represent Lod scores between 1.0 and 2.0. Thin and thick dotted lines represent threshold value of the Lod score considered as suggestive and significant for linkage, respectively, in the model of inheritance chosen according to the Student's *t* test analysis (additive model).

For both strains male rats have higher absolute liver weights than female rats. This gender effect was higher in LEW than in BC rats. Relative liver weight was similar in male and female LEW rats. In contrast, group means for relative liver weight of male BC rats were about 13% lower when compared with those of female BC rats (table 1).

Group means of hepatic copper concentration (µg/g liver d.w.) and hepatic copper store (µg/whole liver) were higher in BC when compared with LEW rats. However, only if liver copper content was expressed relative to body weight (µg/whole liver/100 g body weight), the difference reached statistical significance (table 1).

Genetic mapping of quantitative traits

The liver copper content of F_2 rats was expressed in four different units, as µg/g wet weight, µg/g dry weight, µg/whole liver and µg/whole liver/100g body weight. Whenever a (suggestive) QTL was found using the MapQTL software, a one-way ANOVA with post hoc Student's *t* test was performed for the markers flanking the peak of the QTL or at the peak of the QTL. The mode of inheritance was chosen as free, additive, dominant or recessive according to the significance of differences in the mean values of the traits between rats that were homozygous LEW, heterozygous LEW:BC and homozygous BC. Results are shown in Table 2. For females we found a significant QTL on chromosome 2 and suggestive QTLs on chromosomes 1 and 19. For males a significant QTL was found on chromosome 10 and suggestive QTLs were detected on chromosomes 1, 8, 12 and 14. Figure 1 shows the Lod score curve across chromosome 2 (females) and 10 (males) for hepatic copper concentration (µg/g dry weight). As shown in Table 3, the LEW alleles in the *D2Rat185-D2Rat241* region increased liver copper concentration in female rats in a recessive manner. In male rats, the LEW alleles in the *D10Rat27-D10Rat98* region also increased the liver copper concentration in a recessive manner.

Discussion

In the present study, genome wide scanning for associations between marker genotypes and liver copper content resulted in the localization of two significant (on rat chromosomes 2 and 10) and eight suggestive QTLs (on rat chromosomes 1, 2, 8, 10, 12, 14 and 19) (Table 2, Fig. 1). There is evidence that some QTLs are recessive and other QTLs are dominant with respect to the LEW allele (Tables 2 and 3). Thus both progenitor strains contain "plus" and "minus" genes for hepatic copper content, meaning that these genes increase or reduce the liver copper content in one of the homozygous progenitor strains, whereas these genes reduce or increase the liver copper content in the other homozygous progenitor strain.

Previous genetic analysis of a large set of recombinant inbred strains derived from BN and SHR revealed that a QTL on the central part of chromosome 10, tentatively indicated as *Hcuc1*, influences hepatic copper concentration in male rats (5). In the present (LEW x BC) F_2-intercross, liver copper concentration expressed as μg/g dry weight in males was also found to be associated with this part of chromosome 10 (Table 2). Based on the previous results we hypothesized that the rat *Atox1* (antioxidant protein 1) gene might be a positional candidate for this QTL (5). It was suggested that in the liver the antioxidant protein 1 binds and delivers cytosolic copper to the Wilson disease ATPase protein (ATP7B) in the trans-Golgi network. This ATPase is required for incorporation of copper into ceruloplasmin (the major copper binding protein in the circulation) during its formation and folding or to release hepatic copper into bile (14). The present results support the presence of a candidate gene on chromosome 10.

The region of rat chromosome 2 that shows in female rats linkage to hepatic copper (Tables 2 and 3, Fig. 1) contains the gene *Hsd3b* coding for the enzyme 3beta-hydroxysteroid dehydrogenase-delta5-delta4 isomerase (15). This enzyme plays a crucial role in the biosynthesis of all classes of active steroids (16). It is known that glucocorticoids can stimulate synthesis of the copper-containing protein ceruloplasmin in the liver. Copper, absorbed through the intestine, is transported to and taken up by the liver. In part it is incorporated into newly synthesized ceruloplasmin that is excreted into the plasma. Besides incorporation of copper in copper-containing proteins, part is stored as metallothionein. The remaining copper is excreted into the bile (17). Thus, once ceruloplasmin is synthesized, there might be a decrease in liver copper content and an increase in plasma copper concentration. As a result of differential activity of the enzyme 3beta-hydroxysteroid dehydrogenase-delta5-delta4 isomerase more or less circulating glucocorticoids are produced resulting in more or less ceruloplasmin synthesis. This in turns leads to less or more copper in the liver. BC rats when compared with LEW rats have higher circulating concentrations of aldosterone and corticosterone (18). Thus, it could be anticipated that rats with a BC allele for *Hsd3b* have a lower hepatic copper content when compared with rats homozygous for the LEW allele (Table 3). We propose to the symbol *Hcuc3* (hepatic copper content 3) for the QTL on chromosome 2.

In summary, the present study confirms that chromosome 10 contains a QTL (*Hcuc1*) that plays a role in controlling the hepatic copper content in male rats and indicates that a QTL (tentatively indicated as *Hcuc3*) for liver copper content of female rats is located on chromosome 2. There is some evidence that the *Atox1* and *Hsd3b* genes are the candidate loci for *Hcuc1* (rat chromosome 10) and *Hcuc3* (rat chromosome 2), respectively. Furthermore, there was evidence that rat chromosomes 1, 8, 12, 14 and 19 also contain QTLs involved in hepatic copper content. Further

experiments including the development of congenic sublines of BC are necessary to confirm and precisely map the QTLs on rat chromosomes 2 and 10.

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Table 2: Summary of the QTLs for hepatic copper content in rats[1]

Phenotypic trait	Chromosome	Gender	Model[2]	Peak Lod[3]	Location[4]	% Variance[5]
Liver copper concentration						
(μg/g wet weight)	2	Females	Recessive	*2.44*	D2Rat234 *(41.8 cM)*	10.6
	10	Males	Dominant	*2.37*	D10Rat45 *(84.0 cM)*	11.4
(μg/g dry weight)	1	Females	Dominant	*2.23*	D1Rat185-D1Rat29 *(91.9 cM)*	10.3
	2	Females	Recessive	**3.87**	D2Rat185-D2Rat241 *(19.2 cM)*	21.2
	10	Males	Dominant	**4.15**	D10Rat27-D10Rat98 *(37.3 cM)*	20.3
	14	Males	Dominant	*2.59*	D14Rat1 *(75.6 cM)*	12.5
	19	Females	Additive	*2.36*	D19Rat98 *(51.6 cM)*	10.3
Liver copper store						
(μg/whole liver)	1	Males	Dominant	*2.02*	D1Rat185 *(90.4 cM)*	9.9
	8	Males	Recessive	*2.67*	D8Rat156 *(74.4 cM)*	13.3
	10	Males	Dominant	*2.30*	D10Rat27-D10Rat98 *(38.3 cM)*	11.3
	12	Males	Dominant	*2.59*	D12Rat2-D12Rat56 *(55.8 cM)*	15.5
(μg/whole liver/100 g body weight)	1	Females	Additive	*1.97*	D1Rat196-D1Rat19 *(116.7 cM)*	9.0
	2	Females	Recessive	*2.48*	D2Rat234 *(41.8 cM)*	10.9
	8	Males	Dominant	*2.19*	D8Rat71-D8Rat6 *(0.5 cM)*	10.6
	8	Males	Recessive	*2.35*	D8Rat156 *(74.4 cM)*	12.1
	12	Males	Dominant	*2.30*	D12Rat2-D12Rat56 *(56.8 cM)*	14.3
	14	Males	Dominant	*2.59*	D14Rat1 *(75.6 cM)*	12.7

[1] QTLs were investigated using the MapQTL software on data collected from 190 (LEW/OlaHsd x BC/CpbU) F_2-intercross rats.
[2] Additive or dominant or recessive was defined with respect to the LEW/OlaHsd parent's allele.
[3] Data were shown only when significant or suggestive results were found; thresholds for suggestive and significant linkage were those of Lander and Kruglyak (13); significant results are indicated in bold characters, suggestive results in italics.
[4] The location on the chromosome were the Lod score peaked is given in parentheses.
[5] Percentage of the genetic variance explained by the QTL.

Table 3: Co-segregation analysis results in F_2 progeny of LEW/OlaHsd and BC/CpbU rats.[1]

Marker	Gender	Phenotypic trait	Genotype[2] LL		Genotype[2] LB		Genotype[2] BB		Lod score[3]	*p* (one-way) ANOVA
Chromosome 2										
D2Rat185	Females	µg/g dry weight	15.8±3.2	(21)	13.6±2.6	(58)	13.3±2.3	(20)	2.60	0.0031
D2Rat241	Females	µg/g dry weight	15.6±2.6	(25)	13.5±2.7	(54)	13.3±2.5	(20)	2.69	0.0025
D2Rat234	Females	µg/g wet weight	6.4±1.3	(19)	5.7±1.0	(47)	5.5±0.8	(33)	2.44	0.0172[4]
	Females	µg/whole liver/100 g body weight	28.7±6.9	(19)	25.6±4.3	(47)	24.0±4.0	(33)	2.48	0.0292[4]
Chromosome 10										
D10Rat27	Males	µg/g dry weight	17.6±4.0	(13)	13.8±4.1	(43)	14.9±4.2	(32)	3.17	0.0198
	Males	µg/whole liver	72.2±16.2	(13)	67.4±16.8	(43)	78.6±21.5	(32)	1.82	0.0405
D10Rat98	Males	µg/g dry weight	17.9±4.5	(14)	13.7±3.7	(45)	14.8±4.4	(30)	4.02	0.0053
	Males	µg/whole liver	70.8±16.5	(14)	68.4±15.8	(45)	78.3±23.0	(30)	2.30	0.0821
D10Rat45	Males	µg/g wet weight	5.6±1.7	(24)	5.6±1.3	(43)	6.5±1.2	(22)	2.37	0.0547

[1] Values are means ± SD; number of rats is given in parentheses; some DNA samples failed to give a conclusive genotype, hence the number of rats typed varied slightly with each locus.
[2] L = LEW/OlaHsd allele, B = BC/CpbU allele.
[3] Lod scores reported are at the marker indicated; in some instances the Lod score between markers is higher (see Table 2).
[4] *P*-value after logarithmic transformation of the data.

Chapter 9

Cholesterol and copper in the liver of rabbit inbred strains with differences in dietary cholesterol response

I.D. de Wolf [1]
X.M. Fielmich-Bouman [1]
Æ. Lankhorst [1]
M. den Bieman [1]
B.A. van Oost [2, 3, 4]
A.C. Beynen [1, 5]
L.F.M. van Zutphen [1]
H.A. van Lith [1]

Departments of [1] Laboratory Animal Species, [2] Clinical Sciences of Companion Animals, [3] Equine Sciences, [4] Farm Animal Health and [5] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

submitted

Cholesterol and copper in the liver of rabbit inbred strains with differences in dietary cholesterol response

Abstract

In order to investigate whether cholesterol intake influences the hepatic copper content of rabbits, we compared the hepatic copper content of two rabbit inbred strains after feeding the animals a control or a cholesterol-rich diet. One strain was dietary cholesterol resistant (IIIVO/JU), whereas the other strain was susceptible to dietary cholesterol (AX/JU). Dietary cholesterol-susceptible rabbits when compared with their resistant counterparts had a higher hepatic copper content. The consumption of a hypercholesterolemic diet decreased liver copper concentration (expressed in μg/g dry weight) in both strains of rabbits. A decrease in the absolute hepatic copper content was found only in the dietary cholesterol-susceptible inbred strain. It is discussed that differences in glucocorticoid levels may be responsible for the strain difference in liver copper content. The cholesterol effect on the hepatic copper content in the hyperresponding strain might be caused by an increased bilirubin secretion.

Introduction

Copper is an essential trace element that is necessary for adequate functioning of various fundamental biochemical processes (1). Copper deficiency may have serious, and even lethal, consequences as is shown in Menkes' Disease (2). Too much copper may also be harmful as is evident from the liver and brain damage seen in the copper storage disorder Wilson's Disease (2). Maintaining copper homeostasis via a well-functioning copper metabolism is thus a very critical process. Copper metabolism, however, is affected by numerous internal and external factors, among which the pH in the gastrointestinal system, the hepatic and biliary function and the composition of the diet (3, 4). One of the nutrients associated with copper metabolism is cholesterol. For the rabbit, a dramatic decrease in liver copper concentration has been found after feeding a cholesterol-rich diet (5). In rats, a decrease in liver copper concentration has also been described after feeding a cholesterol-rich diet, while a reversed relationship has also been observed (6, 7). In previous research, we confirmed the decrease in liver copper concentration after feeding rats a hypercholesterolemic diet (8). However, dietary cholesterol did not reduce the absolute nor the relative copper store of the rats. We concluded that the decrease in liver copper

concentration in rats under influence of a hypercholesterolemic diet was not due to a decrease in the amount of hepatic copper, but due to dietary-induced hepatomegaly (8).

These previous findings and the apparent relationship between liver copper concentration and dietary cholesterol in rabbits prompted us to compare the hepatic copper content of dietary cholesterol resistant (hyporesponding) and dietary cholesterol susceptible (hyperresponding) rabbit inbred strains on a diet without added cholesterol and to test whether hyperresponding rabbits show a more pronounced decrease in hepatic copper content than hyporesponding rabbits when fed a diet with cholesterol.

Materials and methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals, housing, diets and preparation of samples

At the Department of Laboratory Animal Science (Utrecht, The Netherlands) two rabbit (*Oryctolagus cuniculus*) inbred strains are available: AX/JU, which is a dietary cholesterol susceptible (hyperresponding) strain and IIIVO/JU, which is a dietary cholesterol resistant (hyporesponding) strain (9, 10). The strains originated from the Jackson Laboratory colony, Bar Harbor, ME, USA (11). The two inbred strains are maintained by brother-sister mating. The rabbits were housed and studied in the Central Laboratory Animal Institute from the Utrecht University.

From weaning (i.e. at the age of 10 weeks) until the start of the experiment, the rabbits were fed a commercial, pelleted, natural-ingredient diet (LKK-20®, Hope Farms BV, Woerden, The Netherlands), containing 25.04 mg Cu/kg diet. The chemical composition of this commercial rabbit diet has been described previously (12). The rabbits were housed individually in stainless steel cages with wire mesh floors (Ruco BV, Waalre, The Netherlands) as previously described (13). The cages were located in rooms with controlled lighting (light from 07:00 to 19:00 hours), temperature (16-19^{o}C) and relative humidity (55-65%).

In the experiment we used adult AX/JU and IIIVO/JU rabbits from both sexes. The animals were fed daily the commercial pelleted diet with or without added cholesterol (0.3 g/100 g diet) for 42 days. Per strain the experimental groups had similar distributions of age (mean age: AX/JU, 79 weeks; IIIVO/JU 99 weeks).

The cholesterol (USP; Solvay Pharmaceuticals BV, Weesp, The Netherlands) was mixed into the test diets by the manufacturer (Hope Farms BV). During the experiment restricted amounts of

diet were given each day at 10:00 a.m. The daily amount of pellets was 100 g for each rabbit. Acidified tap water was provided *ad libitum*. The rabbits were allowed to practice caecotrophy. The cholesterol-rich diet was stored at 4°C until feeding. Body weight was measured at the beginning (day 0) and at the end (day 42) of the experimental period. Food intake was recorded once a week throughout the entire test period.

Blood samples were taken on days 0, 7, 14, 21, 35 and 42 in random order between 08.00 and 10.00 a.m. after a 16 hour fasting period. Samples of blood were taken from the lateral ear vein without anesthesia (days 0, 7, 14, 21 and 35) or via heart punction with anesthesia (day 42). Blood was collected in tubes without anticoagulant. To collect serum, the blood in the tubes was allowed to clot and serum was prepared by low-speed centrifugation. The serum samples were stored in the freezer until use.

At the end of the test period, the fasted rabbits were anesthetized in a random order by an intravenous injection of Hypnorm® (Janssen Pharmaceutica BV, Beerse, Belgium) sufficient to reach the surgical phase (approximately 0.3 ml/rabbit). Subsequently, the animals were killed by cardiac exsanguination and the liver was removed. The autopsy wet weight of the livers (without the gallbladder) was determined. From each animal aliquots of the liver (two pieces of 0.5 g from the quadrate liver lobe) were frozen immediately.

Chemical analyses

Lipids were extracted from liver homogenates according to a modification of the method of Abell *et al.* (14). The liver samples were homogenized on ice in ten volumes 12.5% (v/v) ethanol with a 180 s burst of an UltraTurrax tissue homogenizer (Janke and Kunkel, Staufen, Germany) at 20000 rev./min. The homogenates were frozen at -20°C, thawed and firmly stirred. From each homogenate 200 µl was taken and 2.0 ml of an ethanol-solution containing KOH (ethanolic alkali: 6 ml of 50%-KOH in a final volume of 100 ml absolute-ethanol) was added. The saponification was carried out in closed tubes overnight at 50°C. After this reaction the tubes were adjusted to room temperature and 2.0 ml distilled water plus 4.0 ml warm petroleum ether (40°C-60°C) was added. The tubes were closed and shaken for 10 minutes with a frequency of 500 movements/min. The liquids were allowed to separate for 10 minutes. Three ml of the petroleum-ether fraction was evaporated under nitrogen at 70°C. The residue was dissolved in 0.5 ml of absolute-ethanol and the cholesterol concentration was determined.

Total cholesterol in the liver lipid extracts and the serum was measured enzymatically according to Siedel *et al.* (15), using a kit (Monotest®) supplied by Boehringer Mannheim GmbH (Mannheim, Germany). Cholesterol analyses were performed on a Cobas-BIO automatic

micro-centrifugal analyser (Roche Diagnostics Systems, Hoffmann-La Roche, Basel, Switserland). In both experiments for each individual animal the area under the curve (AUC) for the total experimental period was derived from the measured concentrations by the trapezoidal rule.

Copper in the liver was determined by drying liver samples overnight at 105^{o}C, after which the dry weights were determined. Subsequently, the samples were ashed at 200^{o}C for one hour, 300^{o}C for two hours, 400^{o}C for three hours and 500^{o}C for ten hours. The remaining ash was dissolved in 1.0 ml concentrated $HClO_4$ which was then evaporated at 225^{o}C. This step was repeated until the ash was completely white. The ash was then dissolved in 1.0 ml 6 M HCl. Copper was measured by using flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia).

Statistical analyses

Since the rabbits were housed individually, each animal formed an experimental unit in itself. The Kolmogorov-Smirnov one-sample test was used to check normality of the data. All results within groups were normally distributed. The significance of the differences between groups was calculated by a three-way analysis of variance (ANOVA). Homogeneity of the variances was tested using Bartlett's test. When necessary, the variances were equalized by ranking-transformation (16) of the data. After transformation the variances were similar and the transformed within-group data were still normally distributed. Thus, application of an analysis of variance on the (transformed) data is then straightforward. If the analyses of variance showed significant effects the group means were further compared with the unpaired Student's *t* test. These tests were performed with pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was then tested using a F-test. To take into account the greater probability of a type I error due to multiple comparisons, the level of significance for the unpaired Student's *t* tests was pre-set at $P<0.05$/times a group is used for a comparison (i.e. $P<0.05/3 = 0.0167$) instead of $P<0.05$, according to Bonferroni's adaptation. In all other cases, the probability of a type I error <0.05 was taken as the criterion of significance. Between selected parameters, Spearman's coefficient of rank correlation (R) was calculated; significance was assessed by a two-tailed test. Two-side probabilities were estimated throughout. All statistical analyses were carried out according to Petrie and Watson (17) using a SPSS PC+ computer program (18).

Results

Growth performance

At the beginning of the test period IIIVO/JU rabbits were significantly heavier than AX/JU rabbits (results not shown) (two-way ANOVA, n=38: strain effect, p=0.001; gender effect, p<0.001; interaction effect, p=0.231). Compared to females rabbits, male rabbits had a slightly lower initial (results not shown) and final body weight (Table 1). Since the experiment was carried out with adult rabbits, this strain difference could not be explained by the difference in age (IIIVO/JU rabbits were on average 10 weeks older than AX/JU rabbits; see Materials and Methods). Furthermore, it is known for a long time that in the rabbit the female, when mature, is usually the larger one of the two sexes, which differs from most other mammals (19). During the course of the experiment, body weights of the rabbits on the high-cholesterol diet did not change, whereas body weights of the rabbits on the control diet were slightly, but significantly diminished. As a consequence, in the analysis of variance, a diet effect was detected (Table 1).

Serum cholesterol

Baseline serum cholesterol levels of the IIIVO/JU rabbits were significantly higher than those of AX/JU rabbits. Female rabbits when compared with male rabbits have higher initial serum cholesterol levels (IIIVO/JU: 28 ±6 mg/dL n=10, 43±8 mg/dL n=9; AX/JU: 16±3 mg/dL n=10, 29±3 mg/dL n=9; two-way ANOVA, n=38: strain effect, p<0.001; gender effect, p<0.001; interaction effect, p=0.704). This is in line with earlier observations (20). The cholesterol-rich diet dramatically increased the AUC in the two strains, the increment being significantly greater in the AX/JU rabbits. On the control diet female rabbits also have a higher AUC when compared with their male counterparts. In contrast, on the cholesterol-rich diet the AUCs for male and female rabbits were similar (Table 1).

Liver weight

Irrespective of the diet, IIIVO/JU male rabbits have statistically significantly higher absolute and relative liver (wet and dry) weights than AX/JU male rabbits (Table 1). In female rabbits this difference did not reach the level of statistical significance in the multiple comparison procedure. The consumption of cholesterol raised in both strains absolute and relative liver (wet and dry) weights, albeit in the female IIIVO/JU this cholesterol effect was not statistically significant in the multiple comparison procedure.

Liver cholesterol

The consumption of cholesterol raised liver cholesterol concentration in both strains and both sexes, although for female IIIVO/JU rabbits this increase was not statistically significant in the multiple comparison procedure (Table 2). The magnitude of the increase in the male IIIVO/JU and AX/JU is more than 5.3 and 9.5 times, respectively. In the female IIIVO/JU and AX/JU the increase is more than 2.4 and 8.2 times, respectively. This diet effect was also found for liver cholesterol content. On the diet without added cholesterol, liver cholesterol content (absolute and relative) of the two strains and two genders was similar. The consumption of cholesterol

Table 1: Body weight, serum cholesterol content and liver weight of hypo- and hyperresponsive rabbits fed diets with or without added cholesterol[1]

		Diet without added cholesterol		**Diet with added cholesterol**		
Measure	**Gender**	**IIIVO/JU (n=5M,5F)**	**AX/JU (n=5M,5F)**	**IIIVO/JU (n=5M,4F)**	**AX/JU (n=5M,4F)**	**Sign.[2]**
Final body weight	*Males:*	2718±71	2650± 82	2782±131	2692±121^{a}	D,G
(g)	*Females:*	2768±52	2691±129	2838±170	2903± 44^{a}	
Serum cholesterol level *(AUC, day 0 to day 42)*						
(mg.day/dL)	*Males:*	1311±114^{aeg}	692±90^{bfg}	7595±2008^{ah}	28556±4817^{bh}	
S,D,G,SxD,	*Females:*	1858±187^{cei}	1280±85^{dfi}	7514±1749^{cj}	28312±3173^{dj}	SxG,SxD, SxDxG[3]
Liver wet weight						
Absolute	*Males:*	64.78±3.50^{ac}	54.10±1.97^{ad}	107.78±8.21^{bc}	82.52±9.91^{bd}	S,D,SxG[3]
(g)	*Females:*	63.62±2.19	56.50±7.46^{e}	87.45±16.05	83.90±8.12^{e}	
Relative	*Males:*	23.82±0.79^{ac}	20.43±0.92^{ad}	38.74±2.08^{bcf}	30.62±3.15^{bd}	S,D,SxG,
(g/kg body wt.)	*Females:*	22.98±0.86	20.98±2.35^{e}	30.65±4.06^{f}	28.90±2.73^{e}	DxG[3]
Liver dry weight						
Absolute	*Males:*	19.16±1.22^{ac}	15.93±0.46^{ad}	32.71±2.67^{bc}	24.58±3.10^{bd}	S,D,SxG[3]
(g)	*Females:*	18.45±0.59	16.68±1.94^{e}	26.09±5.29	25.83±3.11^{e}	
Relative	*Males:*	7.04±0.30^{ac}	6.01±0.13^{ad}	11.75±0.60^{bcf}	9.12±0.98^{bd}	
S,D,G,SxG,						
(g/kg body wt.)	*Females:*	6.67±0.22	6.20±0.60^{e}	9.14±1.38^{f}	8.90±1.05^{e}	DxG[3]

1 Values are means ± SD; n is the number of male (M) and female (F) animals per group.

2 Significance (P<0.05) based on three-way ANOVA with main factors *strain*, *diet* and *gender*. S, effect of strain; D, effect of diet; G, effect of gender; SxG, interaction; DxG, interaction.

3 ANOVA after ranking of the data.

4 Contrast significance (Student's *t* test; P<0.0167). Within two rows (i.e. the males plus females row), values bearing the same superscript letter are significantly different.

drastically raised liver cholesterol content in both strains and both genders. However, the effect is most pronounced in the AX/JU strain and in males. The magnitude of the increase in liver cholesterol content in the male IIIVO/JU and AX/JU rabbit is about 9.0 and 14.5 times, respectively. In the female IIIVO/JU and AX/JU rabbit this increase is about 3.5 and 11.8 times, respectively. The increase in liver weight of test animals when compared with control animals (wet weight: 23.83 to 43.00 g; dry weight: 7.64 to 13.55 g) can only partly be attributed to the increase in hepatic cholesterol amount (mg/whole liver); the latter represented about 0.48 to 2.33 g.

Liver copper

Irrespective of the dietary composition, there was a statistically significant strain effect on the concentration and store of hepatic copper; the AX/JU rabbits having a much higher copper concentration and total store than the IIIVO/JU animals (Table 2). The diet with added cholesterol when compared with the control diet produced lower liver copper concentrations and stores in AX/JU, but not in the IIIVO/JU rabbits.

Liver copper concentration, but not liver copper store, was weakly correlated with serum cholesterol response. Both for µg Cu/g liver dry weight and µg Cu/g liver wet weight the R was -0.3739 (n=38, p=0.021). In rabbits, none of the parameters for liver copper store were significantly associated with the parameters for liver cholesterol content.

Discussion

In the literature substantial evidence for a relationship between cholesterol and copper has been described (5-7). The aim of the present work was to determine whether cholesterol in the diet influences liver copper content in rabbits. For this purpose we used two inbred rabbit strains which differ markedly in their cholesterolemic response to dietary cholesterol (9, 10).

The mean liver copper concentration of male IIIVO/JU rabbits fed a diet without added cholesterol was 18.06 µg/g dry weight; for female IIIVO/JU animals the value for this parameter was 15.88 (Table 2). The liver copper concentrations of male and female AX/JU rabbits on the control diet were remarkably higher than those of the IIIVO/JU, being 252.93 and 161.29 µg/g dry weight, respectively (Table 2). Klevay (5) reported that liver copper concentrations in New Zealand White rabbits fed a control diet ranged from 9.1 - 87.0 µg/g dry weight. Allain *et al.* (21) reported similar copper levels in the livers of Watanabe Heritable Hyperlipidemic (WHHL) rabbits and New Zealand White rabbits. Thus on a control diet, IIIVO/JU rabbits, which like WHHL rabbits originate from a New Zealand White stock (11), have liver copper concentrations

(range 14.5 - 21.7 µg/g dry weight) that fall in the range described in the literature. In contrast, control AX/JU rabbits with liver copper concentrations that vary between 144.4 and 343.2 µg/g dry weight have hepatic copper concentrations much higher than those reported thus far (5, 21).

The strain difference in hepatic copper content for these rabbits fed a normal diet might be explained by differences in the serum glucocorticoid levels. Glucocorticoids are able to stimulate the synthesis of metallothionein and ceruloplasmin in the liver (22). Dietary copper is absorbed from the diet through the intestine and is transported to and taken up by the liver. There, copper is partly incorporated into newly synthesized apoceruloplasmin, which is then excreted into the plasma. Besides incorporation of copper in copper-containing liver proteins, a part of the copper is stored as metallothionein, while the remaining copper is excreted into the bile. Thus, synthesis of metallothionein and ceruloplasmin may result in an increase in plasma and liver copper concentration (8). Recently we have reported that AX/JU when compared with IIIVO/JU rabbits have higher levels of circulating corticosterone (23). A difference in copper intake between the two strains could be excluded. During the experiment, the rabbits were fed restricted amounts of diet and the animals consumed all the administered food.

The cholesterol-rich diet resulted in a lower liver copper store in AX/JU rabbit, whereas no significant change was found in IIIVO/JU rabbits (Table 2). It is well-known that in rabbits a cholesterol-rich diet leads to an increased biliary excretion of cholesterol and its metabolites, (conjugated) bile acids. It is generally agreed that the bile is the main excretory route for copper and that copper is excreted as complexes of amino acids and/or (conjugated) bile acids. During biliary flow, the copper also becomes complexed with bilirubins; these complexes are unavailable for reabsorption. Keeping this in mind, Klevay (5) hypothesized that dietary cholesterol and (conjugated) bile acids, produce increased biliary loss of copper, which results in a decreased liver copper content. However, bile acid excretion has been reported to be consistently higher in the IIIVO/JU strain both on a control diet and on a cholesterol-rich diet (13).

Meijer *et al.* (24) showed that adding cholesterol to the diet caused significantly higher bilirubin concentrations in the serum of AX/JU rabbits, but not in IIIVO/JU animals. Dietary cholesterol induced hypercholesterolemia associated with hemolytic anemia has also been reported (25). Abnormally shaped erythrocytes are formed as a result of an increase in erythrocyte membrane cholesterol and these red cells are removed from the blood stream by the reticuloendothelial system. Within the phagocytic cells, hemoglobin and other heme proteins are catabolized to bilirubin. After its formation within phagocytic cells, bilirubin is released into the circulation. In this form the bilirubin is unconjugated and is cleared from the blood by the liver. In the liver

bilirubin is conjugated with glucuronic acid and excreted via the bile into the duodenum. Thus an increase in the serum concentration of bilirubin will be associated with an enhanced biliary secretion of bilirubins. As pointed out above, during biliary flow, copper becomes complexed with bilirubins, which cannot be absorbed. Therefore, it could be anticipated that on a cholesterol-rich diet the biliary excretion of bilirubin and thus of copper is enhanced in AX/JU when compared with IIIVO/JU rabbits.

In conclusion, the AX/JU and IIIVO/JU rabbit inbred strains show differences in liver copper content. These strain effects perhaps could be explained by differences in circulating glucocorticoid levels. In hyperresponding when compared with hyporesponding rabbits, cholesterol loading produced a marked decrease in liver copper store. We hypothesised that this cholesterol effect in rabbits is due to an enhanced hepatobiliary transport of copper via increased bilirubin production.

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Table 2: Liver cholesterol content of hypo- and hyperresponsive rabbits fed diets with or without added cholesterol[1]

Measure	Gender	Diet without added cholesterol		Diet with added cholesterol		Sign.[2]
		IIIVO/JU (n=5M,5F)	AX/JU (n=5M,5F)	IIIVO/JU (n=5M,4F)	AX/JU (n=5M,4F)	
Liver cholesterol concentration						
(mg/g wet weight)	*Males:*	2.71±0.12^{ac}	3.19±0.23^{ad}	14.96±4.57^{bc}	30.70±3.74^{bd}	S,D,SxG,
	Females:	2.90±0.11	2.96±0.21^{e}	7.21±3.61^{f}	25.43±2.92^{ef}	SxDxG[3]
(mg/g dry weight)	*Males:*	9.18±0.44^{ac}	10.83±0.73^{ad}	49.23±14.73^{bc}	103.17±13.25^{bd}	S,D,SxG,
	Females:	9.99±0.43	10.01±0.60^{e}	24.02±11.46^{f}	82.86±10.23^{ef}	DxG[3]
Liver cholesterol store						
(mg/whole liver)	*Males:*	175±6^{a}	172±7^{b}	1618±508^{a}	2504±78^{bc}	D,SxD,
	Females:	184±9	166±14^{d}	666±440^{e}	2120±154^{cde}	DxG[3]
(mg/100 g body wt.)	*Males:*	6.46±0.17^{a}	6.51±0.41^{c}	58.21±18.55^{ab}	93.10±2.62^{bcd}	D,G,SxD,
	Females:	6.66±0.35	6.18±0.32^{e}	22.92±14.11^{f}	73.09±5.71^{def}	SxG[3]

[1] Values are means ± SD; n is the number of male (M) and female (F) animals per group.

[2] Significance (P<0.05) based on three-way ANOVA with main factors *strain*, *diet* and *gender*. S, effect of strain; D, effect of diet; G, effect of gender; SxD, interaction; SxG, interaction; DxG, interaction.

[3] ANOVA after ranking of the data.

[4] Contrast significance (Student's *t* test; P<0.0167). Within two rows (i.e. the males plus females row), values bearing the same superscript letter are significantly different.

Table 3: Liver copper content of hypo- and hyperresponsive rabbits fed diets with or without added cholesterol[1]

Measure	Gender	Diet without added cholesterol		Diet with added cholesterol		Sign.[2]
		IIIVO/JU (n=5M,5F)	AX/JU (n=5M,5F)	IIIVO/JU (n=5M,4F)	AX/JU (n=5M,4F)	
Liver copper concentration						
(µg/g wet weight)	*Males:*	5.33±0.65[a]	74.23±17.84[ac]	4.19±0.86[b]	18.06±5.66[bc]	S,D,SxG[3]
	Females:	4.61±0.38[d]	47.63±3.65[d]	4.09±0.45	19.10±19.32	
(µg/g dry weight)	*Males:*	18.06±2.41[a]	252.93±64.03[ac]	13.81±2.76[b]	60.69±19.30[bc]	S,D,G[3]
	Females:	15.88±1.24[d]	161.29±15.06[d]	13.76±1.55	61.35±60.56	
Liver copper store						
(µg/whole liver)	*Males:*	344±25[ad]	4020±968[ac]	449±77[b]	1472±438[bc]	S,D,G[3]
	Females:	293±27[de]	2702±494[e]	356±72	1714±1917	
(µg/100 g body wt.)	*Males:*	12.67±1.22[ad]	152.50±40.37[ac]	16.19±3.11[b]	54.52±15.54[bc]	S,G,SxD[3]
	Females:	10.58±0.85[de]	100.47±17.52[e]	12.51±1.94	58.79±65.65	

[1] Values are means ± SD; n is the number of male (M) and female (F) animals per group.

[2] Significance ($P<0.05$) based on three-way ANOVA with main factors *strain*, *diet* and *gender*. S, effect of strain; D, effect of diet; G, effect of gender; SxD, interaction; SxG, interaction; DxG, interaction.

[3] ANOVA after ranking of the data.

[4] Contrast significance (Student's *t* test; $P<0.0167$). Within two rows (i.e. the males plus females row), values bearing the same superscript letter are significantly different.

Chapter 10

Mapping of QTLs for hepatic copper in rabbits

I.D. de Wolf [1]
M.G. den Bieman [1]
G.F. Gillissen [1]
X.M. Fielmich-Bouman [1]
Æ. Lankhorst [1]
B.A. van Oost [2, 3, 4]
A.C. Beynen [1, 5]
L.F.M. van Zutphen [1]
H.A. van Lith [1]

Departments of [1] Laboratory Animal Science, [2] Clinical Sciences of Companion Animals, [3] Equine Sciences, [4] Farm Animal Health and [5] Nutrition, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

submitted

Mapping of QTLs for hepatic copper in rabbits

Abstract

After feeding a cholesterol-rich diet, a significant difference in liver copper content has been observed between rabbits of the inbred strains AX/JU and IIIVO/JU. To define loci controlling this difference, the offspring (n=138) of an (IIIVO/JU x AX/JU) F_2-intercross has been analysed genetically. From each F_2-animal liver copper content was determined and tested for association with each of the 310 previously mapped autosomal markers. A quantitative trait locus (QTL) for liver copper content was found on linkage group (LG) U8 (Lod score = 3.68). This QTL accounted for about 16% of the genetic variance within each gender group. In addition, suggestive linkages for liver copper content were found on chromosomes 1, 7, 12 and 18 and on LGs U2, U5 and U6. These regions explained 8.1 to 20.2% of the genetic variance.

Introduction

The trace element copper is essential for the adequate functioning of various fundamental biochemical processes (1). Copper deficiency may have serious, or even lethal, consequences as in Menkes' Disease (2). As is evident from the disturbed copper metabolism in Wilson's Disease, increases in copper content may also be harmful (2). Copper homeostasis is maintained by numerous internal and external factors, among which hepatic and biliary function and diet composition (3, 4).

Differences in copper metabolism may be influenced by genetic factors. Previously, we described a strain difference in liver copper content, after feeding a cholesterol-rich diet, between inbred strains of both rabbits (5) and rats (6). Recently, we have looked for genetic factors associated with liver copper content in rats using a set of recombinant inbred strains (7) and an F_2-population (8). We found three chromosomal regions on rat chromosomes 2, 10 and 16 associated with liver copper content. Based on rat-human chromosomal homologies three candidate genes (Atox1, Cox15 and Hsd3b) were proposed to be involved in the control of the hepatic copper content in rats.

These results prompted us to perform a genome scan of a rabbit F_2-population in search for genetic factors controlling hepatic copper content after feeding a cholesterol-rich diet in this species. 138 F_2-intercross progeny from the AX/JU and IIIVO/JU inbred strains, previously used

for testing the genetic basis of differences in susceptibility for dietary cholesterol (9), was available for this study.

Materials and methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals and housing

At the Department of Laboratory Animal Science (Utrecht, The Netherlands) two rabbit inbred strains (IIIVO/JU and AX/JU) are available. These strains originate from The Jackson Laboratory colony, Bar Harbor, ME, USA (10). To produce F_1-hybrids, IIIVO/JU females were mated with one AX/JU male. The F_1-hybrids were intercrossed (brother x sister mating) producing F_2-progeny. From 61 F_2-males and 77 F_2-females the liver copper content was determined.

Experimental protocol

After weaning at the age of 10 weeks, all F_2-rabbits were fed a commercial diet (LKK-20®, Hope Farms B.V., Woerden, The Netherlands) and were housed individually. The chemical composition of the commercial rabbit diet has been described elsewhere (11). At 12-16 weeks of age, the commercial rabbit diet was replaced by a commercial diet, supplemented with 0.3% (w/w) cholesterol. This diet was fed for 12 weeks in restricted amounts (100 g/rabbit/day). At the end of this period, body weight was determined and the animals were anesthetized by an intravenous injection of Hypnorm® (Janssen Pharmaceutica B.V., Beerse, Belgium) sufficient to reach the surgical phase (approximately 0.3 ml/rabbit). After cardiac exsanguination, liver and spleen were removed and weighed. Spleen was used for DNA isolation. From each animal two liver samples (0.5 g) were frozen immediately frozen and used for copper determination (12).

Chemical analyses

The liver samples were dried overnight at 105°C and the dry weights were measured. Subsequently, the samples were ashed at 200°C for one hour, 300°C for two hours, 400°C for three hours and 500°C for ten hours. The remaining ash was dissolved in 1 ml concentrated $HClO_4$ which was then evaporated at 225°C. This step was repeated until the ash was completely white. The ash was then dissolved in 1 ml 6 M HCl. Copper was measured by using flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia). Liver

copper levels were expressed as µg Cu/g liver wet weight, µg Cu/g liver dry weight, µg Cu/whole liver and µg/whole liver/100 g body weight.

Genome scan

A total of 310 autosomal markers (6 biochemical, 44 microsatellite and 260 AFLP markers) polymorphic between the AX/JU and IIIVO/JU strains were genotyped in the F_2-progeny (13). In total, 1262 cM was covered by 24 linkage groups with four or more markers. Assuming a total length of 1500 cM this map covers about 84% of the total rabbit genome (14).

Statistical and QTL analyses

The Kolmogorov-Smirnov one-sample test was used to check normality of the liver copper traits within the male and female population. Within males all traits were normally distributed, but within females hepatic copper concentration (µg/g wet weight or µg/g dry weight) was not. Gender appeared to have a significant influence on liver copper concentration (µg/g wet weight or µg/g dry weight; Mann-Whitney *U* test, $P<0.005$), but not on liver copper store (µg/whole liver or µg/whole liver/100 g body weight; unpaired Student's *t* test, $p>0.05$).

In order to carry out a genetic analysis using both males and females together for hepatic copper concentrations, in each gender the traits were first normalized by logarithmic transformation. As would be expected gender still had a significant effect on the transformed liver copper concentrations (unpaired Student's *t* test, $p<0.005$). Therefore, for each gender the logarithmically transformed liver copper concentrations were subtracted by the mean transformed liver copper concentrations (of that gender) and then divided by the transformed standard deviation (of that gender). Within the combined male and female population these final transformed variables had a normal distribution according to the Kolmogorov-Smirnov one-sample test.

The location of the QTLs affecting the measured (transformed) quantitative traits and the variance explained by each locus were determined using the interval-mapping module of the MapQTL computer package (15, 16). Results were expressed as Lod scores. Lod score thresholds to achieve the genome-wide significance levels of 5% (significant linkage) were 4.3, as proposed by Lander and Kruglyak (17), when no model of gene action was specified ("free" genetics), 3.4 when gene action was restricted to recessive or dominant models, and 3.3 when gene action was restricted to an additive model. For suggestive linkage, Lod score values were 2.8 ("free" genetics), 2.0 (recessive or dominant) and 1.9 (additive), respectively.

Comparison of the liver copper traits of the F_2-animals after grouping by genotype of each marker was also performed. If a marker and the trait of interest are segregating independently, the values of the trait will be equally distributed among the homozygote and heterozygote genotypes. Again, the Kolmogorov-Smirnov one-sample test was used to check normality of these data. All traits within genotype groups were found to be normally distributed. For each population, co-segregation of phenotypes with alleles at marker loci was evaluated by comparing the values between different genotypes by one- or two-way analysis of variance (ANOVA). Homogeneity of the variances was tested using Bartlett's test. When necessary, the variances were equalized by logarithmic transformation of the data. After transformation, the variances were similar and the transformed within-genotype-group data were still normally distributed. Thus, application of an ANOVA on the (transformed) data is then straightforward. All statistical analyses were carried out according to Petrie and Watson (18) using a SPSS PC+ computer program (19).

Results and discussion

Liver copper content of F_2 rabbits was investigated on four quantitative traits, i.e. liver copper was expressed as µg/g wet weight, µg/g dry weight, µg/whole liver and µg/whole liver/100g body weight. The results of the QTL analysis using the MapQTL software are shown in Table 1. Whenever a (suggestive) QTL was found, a one-way ANOVA with post hoc Student's *t* test was performed for the markers flanking the peak of the QTL or at the peak of the QTL. The mode of inheritance was chosen as free, additive, dominant or recessive according to the significance of differences in the mean values of the traits between rabbits that were homozygous AX/JU, heterozygous AX/JU:IIIVO/JU and homozygous IIIVO/JU.

For the combined population of males and females we found only one significant QTL, located on the linkage group (LG) U8 and three suggestive QTLs on chromosome 7, LG U5 and U8. For females we found suggestive QTLs on rabbit chromosome 18 and LG U2, U6 and U8. For males suggestive QTLs were detected on chromosomes 1 and 12, and on LG U5 and U8. Some QTLs are recessive with respect to the AX/JU allele and other QTLs are additive. Both progenitor strains contain "plus" and "minus" genes for hepatic copper content (Table 1).

Figure 1 shows the Lod score curve across linkage group U8 for hepatic copper level (µg/g dry weight). As shown in Table 2, the liver copper level seems to be under intermediary inheritance of the G170 to P259 region from linkage group U8.

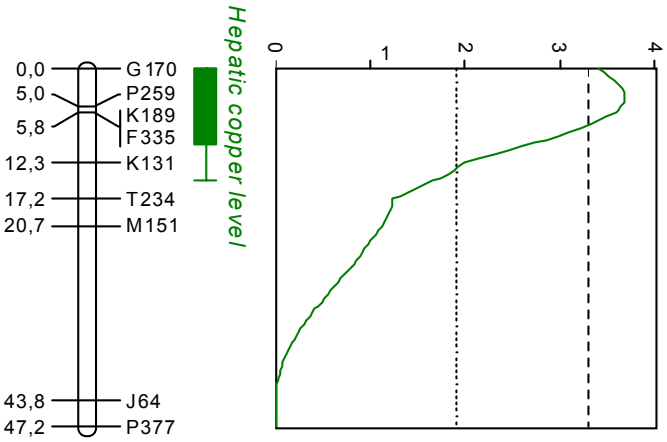

Figure 1: Lod score plot of linkage group U8 exhibiting significant linkage of hepatic copper concentration (µg/g dry weight) to microsatellite markers. The distances are indicated in cM. The positions of selected marker loci genotyped in the F_2 progeny are indicated on the x-axis of each panel. The most likely position for the QTL, determined by its 2.0-Lod support interval, is indicated by a solid bar under the plot. Dotted lines represent the threshold value of the Lod score considered as significant for linkage in the model of inheritance chosen according to the Student's *t* test analysis (additive model).

The two rabbit inbred strains used for this study differ for their susceptibility to dietary cholesterol (9, 11). It has been suggested that a relationship exists between cholesterol and copper metabolism (20-24). The region of LG U8 which contains the significant QTL for hepatic copper concentration also contains a suggestive QTL for liver cholesterol concentration (population: %% + &&; Lod score: 2.61; Van Lith *et al.* unpublished). It is, however, unlikely that these two QTLs are identical because the liver cholesterol concentration is affected by the AX/JU alleles in a recessive manner (Table 3), whereas the liver copper concentration is under intermediary inheritance (Tables 1 and 2) and, in corroboration with previous results (5), there was no correlation between liver copper concentration and liver cholesterol content. Also, the AX/JU progenitor strain contains a plus gene on LG U8 both for liver copper concentration and for hepatic cholesterol level. Thus, a dilution effect due to a fatty liver as the cause for the decreased copper levels can be rejected.

In conclusion, the present study showed that the linkage group U8 is significantly associated with liver copper concentration in rabbits after feeding a cholesterol-rich diet. Furthermore, there is evidence that other rabbit linkage groups (U2, U5, and U6) or chromosomes (1, 7, 12, and 18) also contain QTLs involved in hepatic copper content. In order to speculate about candidate genes it is necessary to assign the linkage groups to chromosomes. This can be realised by generating polymorphic markers (SSLPs) from microsatellite enriched chromosome-specific libraries (25). With the presently available BAC clones it is possible to orientate the linkage

maps (26). Once it is known to which rabbit chromosomal region a QTL belongs, candidate genes can be identified by using the results of the reciprocal chromosome painting between rabbit and human (27).

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Table 1: Summary of the QTLs for hepatic copper content in rabbits[1]

Phenotypic trait	Trans.[2]	Population	Model[3]	Peak Lod[4]	Chrom./LG	Location[5]	% Variance[6]
Liver copper concentration							
(µg/g wet weight)	yes	%% + &&	Recessive (-)[7]	*2.56*	7	J216 *(50.8 cM)*	8.2
	no	%%	Free (H)	*2.94*	12	E257 *(48.5 cM)*	20.2
	yes	&&	Recessive (+)	*2.60*	U2	M244 *(43.0 cM)*	14.4
	yes	&&	Additive (-)	*2.05*	U6	J162 to Sat8 *(6.6 cM)*	11.8
	yes	&&	Additive (+)	*2.82*	U8	P259 *(5.0 cM)*	15.5
	yes	%% + &&	Additive (+)	*2.68*	U8	G170 to P259 *(2.0 cM)*	8.8
(µg/g dry weight)	no	%%	Recessive (+)	*2.50*	1	C to D286 *(71.9 cM)*	17.2
	yes	%% + &&	Recessive (-)	*2.52*	7	J216 *(50.8 cM)*	8.1
	yes	&&	Free (H)	*2.20*	18	D476 to D57 *(10.5 cM)*	16.6
	no	%%	Additive (+)	*2.42*	U8	G170 to P259 *(0.5 cM)*	16.9
	yes	&&	Additive (+)	*2.93*	U8	P259 *(5.0 cM)*	16.1
	yes	%% + &&	Additive (+)	**3.68**	U8	G170 to P259 *(3.5 cM)*	12.0
Liver copper store							
(µg/whole liver)	no	%%	Free (H)	*2.43*	12	E257 *(48.5 cM)*	16.8
	no	%%	Additive (+)	*2.23*	U5	Q255 to R385 *(9.4 cM)*	16.7
	no	%% + &&	Additive (+)	*3.00*	U5	A194 to Q255 *(1.8 cM)*	9.9
(µg/whole liver/100 g	no	%%	Additive (+)	*2.49*	U5	A194 to Q255 *(3.3 cM)*	18.6
body weight)	no	%% + &&	Additive (+)	*2.72*	U5	A194 to Q255 *(2.3 cM)*	9.1

[1] QTLs were investigated using the MapQTL software on data collected from 138 (IIIVO/JU x AX/JU) F_2-intercross rabbits.

[2] In some cases the QTL analysis was performed on transformed (= yes) liver copper data, whereas in other cases this analysis was performed on the untransfomed (= no) data.

[3] Additive, dominant or recessive was defined with respect to the AX/JU grandparental allele.

[4] Data are shown only when significant or suggestive correlations were found. Thresholds for suggestive and significant linkage were those of Lander and Kruglyak (1995). Significant results are indicated in bold characters, suggestive results in italics.

[5] The location on the chromosome or linkage group (LG) where the Lod score peaked is given in parentheses.

[6] Percentage of the genetic variance explained by the QTL.

[7] In parentheses is indicated that the AX/JU progenitor strain contains in the chromosomal region a *"plus"* (+) or a *"minus"* (-) gene. For some regions the heterozygous genotype was higher than both homozygous genotypes (*heterosis-effect*, H). In these cases the two homozygous genotypes had an identical hepatic copper content.

Table 2: Co-segregation analysis of liver copper level (µg/g dry weight) in an F_2-progeny of IIIVO/JU and AX/JU rabbits[1]

Marker	Population	Genotype[2] AA		AI		II		Lod score[3]	(*p*-value) ANOVA[4]
Linkage Group U8									
G170	%%	11.9±2.6	(9)	10.4±1.3	(33)	9.8±0.8	(17)	2.42	G=0.002
	&&	13.1±4.1	(20)	11.3±2.0	(39)	10.3±1.6	(14)	2.25	S=0.034
									GxS=0.774
	%% + &&	12.7±3.7	(29)	10.9±1.8	(72)	10.0±1.2	(31)	3.40	G<0.001
P259	%%	11.8±2.4	(10)	10.3±1.3	(28)	9.9±1.0	(23)	2.05	G<0.001
	&&	13.3±4.1	(19)	11.2±1.9	(45)	10.1±1.6	(12)	2.93	S=0.039
									GxS=0.510
	%% + &&	12.8±3.7	(29)	10.9±1.8	(73)	10.0±1.2	(35)	3.64	G<0.001

[1] Values are means ± SD; number of rabbits is given in parentheses. Some DNA samples failed to give a conclusive genotype, hence the number of rabbits typed varied slightly with each locus.

[2] A = AX/JU allele, I = IIIVO/JU allele.

[3] Lod scores reported are at the marker indicated.

[4] Analysis of the (%% + &&)-population: one-way ANOVA with main factor *genotype*. Simultaneous analysis of %%- and &&-population: two-way ANOVA with main factors *genotype* and *gender*. G, effect of genotype at marker locus; S, effect of gender; GxS, interaction effect. The *p*-values are the values after logarithmic transformation of the data.

Table 3: Co-segregation analysis of liver cholesterol level (μmol/g wet weight) in an F_2-progeny of IIIVO/JU and AX/JU rabbits (based on Van Lith *et al.* unpublished)[1]

Marker	Population	Genotype[2] AA		Genotype[2] AI		Genotype[2] II		Lod score[3]	(*p*-value) ANOVA[4]
Linkage Group U8									
G170	%%	95.6±26.7	(9)	82.7±7.7	(33)	80.1±16.9	(17)	0.96	G=0.001
	&&	91.9±24.7	(20)	75.2±17.9	(39)	72.8±16.8	(14)	1.96	S=0.054 GxS=0.911
	%% + &&	93.1±24.9	(29)	78.7±18.1	(72)	76.8±17.0	(31)	2.59	G=0.002
P259	%%	96.4±25.3	(10)	84.2±16.8	(28)	78.0±16.7	(23)	1.53	G=0.002
	&&	90.9±26.3	(19)	75.8±17.2	(45)	77.5±24.8	(12)	1.53	S=0.100 GxS=0.650
	%% + &&	92.8±25.6	(29)	79.0±17.4	(73)	77.8±19.5	(35)	2.50	G=0.063[5]

[1] Values are means ± SD; number of rabbits is given in parentheses. Some DNA samples failed to give a conclusive genotype, hence the number of rabbits typed varied slightly with each locus.

[2] A = AX/JU allele, I = IIIVO/JU allele.

[3] Lod scores reported are at the marker indicated.

[4] Analysis of the (%% + &&)-population: one-way ANOVA with main factor *genotype*. Simultaneous analysis of %%- and &&-population: two-way ANOVA with main factors *genotype* and *gender*. G, effect of genotype at marker locus; S, effect of gender; GxS, interaction effect.

[5] *P*-value after logarithmic transformation of the data.

Chapter 11

Conclusions and summary

Conclusions and summary

Introduction

All research described in this thesis focuses on the role of copper in various biochemical processes. It appears that copper has various faces in laboratory animals. On the one hand, copper is an essential trace element, which implicates that a certain requirement for copper exists. On the other hand, copper may be involved in the formation of free radicals and reactive oxygen species (ROS), causing the development of oxidative stress. Oxidative stress has been associated with reduced lifespan and various diseases as a consequence of oxidative damage at the (sub)cellular level. Copper may not only affect biochemical processes in laboratory animals, it may also be affected itself by endogenous and exogenous factors, such as dietary cholesterol. Furthermore, strain differences in hepatic copper content and hepatic copper concentration have been found, which can be (partly) explained by genetic differences. All studies described in this thesis, except the review in chapter 3, were performed in laboratory animals keeping the 3 R's of laboratory animal science (reduction, refinement and replacement) in mind. Some of the studies performed are for the benefit of the animal (chapter 2), in some studies the animal is used as a model (chapter 6-10) and in some studies, the animal was used as a model, but the results of the study could be used for the benefit of the animal (chapter 4 and 5). In case the animal was used as a model, the purpose of the experiment was to study mechanisms *in vivo* rather than *in vitro*. The conclusions of all studies described in this thesis are briefly summarized.

Chapter 2

Although the mouse is the most commonly used laboratory animal, its copper requirement has not been well established, since specific studies that determine the copper requirement of the mouse during its various stages of life have not been published. We have attempted to estimate the copper requirement of the mouse by feeding groups of mice diets with 1, 2, 4 or 8 ppm Cu, respectively. Based on the effects of copper intake on reproductive outcome, growth performance and sustainment of maximum plasma and hepatic copper concentrations and of plasma ceruloplasmin, a copper allowance for the mouse is proposed. This study showed that NMRI mice fed a semipurified diet containing 1 ppm copper had a marked depression of reproductive performance. Plasma, hepatic and carcass copper concentrations were not indicative

as to the copper requirement of the mice. To take into account the various factors affecting copper requirement and the availability of dietary copper, it is suggested to set the general copper allowance of laboratory mice at 4 ppm.

Chapter 3

In chapter 3, the role of copper in the development of oxidative stress is reviewed. Copper appears to be involved in the generation of reactive oxygen species (ROS) and free radicals through the Haber-Weiss reaction. Evidence for the suggested relationship between copper and ROS and free radicals is obtained mainly through *in vitro* research or through experiments in which reducing agents such as paraquat were added as well. Copper seems to play an indirect, facilitating role in the generation of ROS and free radicals. Whether copper itself possesses the ability to generate ROS and free radicals without the presence of reducing agents remains unclear.

Chapter 4

To study whether high copper intakes can cause oxidative damage at the macromolecular level in intact animals, oxidative damage to DNA (8-oxodG), proteins (specific oxidised amino acids) and lipids (MDA) were measured as indicators of oxidative damage in mice fed diets containing 5, 25, 125 or 625 ppm Cu for 6 weeks. In addition, total antioxidant status was measured. Dietary copper increased the liver copper concentration in mice fed the diet with 625 ppm Cu, but did not significantly influence levels of 8-oxodG, MDA and specific oxidised amino acids. Likewise, the dietary copper level did not affect the total antioxidant status. We concluded that exposure to high copper levels do not result in oxidative damage under *in vivo* conditions.

Chapter 5

The parameters used in the previous study may have been inappropriate for detection of oxidative stress. Since longevity may be a more convincing parameter, we have studied whether high copper intake in mice results in reduced life span due to the induction of oxidative stress. No statistically significant decrease in survival was found in mice fed increasing dietary copper concentrations (5, 25 or 125 ppm Cu). Most likely, the body's antioxidant defence and repair system is able to compensate for oxidative stress caused by high copper intake.

Chapter 6

From previous experiments it could be concluded that in mice copper deficiency rather than an overload of copper has a negative effect on health and lifespan. The copper status of animals not only depends on the amount of copper in the food, but is also influenced by other nutrients and by genetic factors. In this thesis, the impact of these factors has been studied in rats and rabbits.

Cholesterol intake has been described in literature to affect the hepatic copper content and hepatic copper concentration in rats. Since not all rats are equally sensitive to dietary cholesterol, this triggered us to study (i) whether cholesterol intake influences the hepatic copper content of rats and (ii) whether hyperresponsive rats with regard to cholesterol show a larger decrease in hepatic copper than hyporesponsive rats. In order to answer both questions, the hepatic copper content of two rat inbred strains was compared after feeding the animals a control or a high fat, high cholesterol diet. One strain was dietary cholesterol resistant, whereas the other strain was susceptible to dietary cholesterol. Analysis revealed statistically significant strain differences for hepatic copper content. On the control diet, the dietary cholesterol-susceptible rats have a lower hepatic copper content than their resistant counterparts. Furthermore, the consumption of a hypercholesterolemic diet decreased liver copper concentration in both strains but this was probably due to dietary-induced hepatomegaly, since dietary cholesterol did not reduce the absolute and relative copper store of rats.

Chapter 7

In order to study the strain specific differences in liver copper content described in chapter 6 and gain more insight in the genes that are involved in copper regulation, thirty recombinant inbred (RI) strains were used in order to search for quantitative trait loci (QTLs) that are responsible for these differences. The heritability of liver copper concentration and liver copper store was estimated to be 57% and 46%, respectively. In a total genome scan of the RI strains, a suggestive association was found between liver copper store (□g/whole liver) and the *D16Wox9* marker on chromosome 16 (lod score = 2.8), and between liver copper concentration (µg/g dry weight) and the *D10Cebrp1016s2* marker on chromosome 10 (lod score = 3.0). These putative QTLs are responsible for nearly 34% and 40% of the additive genetic variability for these liver copper content parameters.

Chapter 8

Because the previous QTL analysis, described in chapter 7, were performed with male rats only, and because of the limited power of recombinant inbred strains for detecting QTLs, a total

genome scan of a (LEW/OlaHsd x BC/CpbU) F_2-intercross was performed to search for additional genetic factors controlling liver copper content. A major QTL for liver copper content was found for females on chromosome 2 and for males on chromosome 10. Both QTLs accounted for approximately 20% of the genetic variance. In addition, suggestive linkage for liver copper content was found on rat chromosomes 1, 8, 12, 14 and 19. The regions on these chromosomes contain genes that are responsible for 9.0 to 15.5% of the genetic variance of liver copper content.

Chapter 9

The results of the experiment described in chapter 6 encouraged us to investigate whether differences in hepatic copper content also occur between cholesterol-resistant and cholesterol-susceptible rabbits. The hepatic copper content of two rabbit inbred strains was compared after feeding the animals a control or a cholesterol-rich diet. One strain was dietary cholesterol resistant, whereas the other strain was susceptible to dietary cholesterol. Again, analysis revealed statistically significant strain differences for hepatic copper content, dietary cholesterol-susceptible rabbits this time having a higher hepatic copper content when compared with their resistant counterparts. Furthermore, the consumption of a hypercholesterolemic diet decreased liver copper concentration in both strains of rabbits. A decrease in the hepatic copper store was found only in the dietary cholesterol-susceptible inbred strain. Increased bilirubin secretion might play a role in the effect of cholesterol on the hepatic copper content in the hyperresponding strain.

Chapter 10

In chapter 9, a significant difference in liver copper content between the AX/JU and IIIVO/JU inbred strain of rabbits was shown. To define loci controlling this trait, the offspring from an F_2-intercross of these strains has been genetically analysed. A QTL for liver copper content was found on linkage group (LG) U8 (Lod score = 3.68). This QTL accounted for about 16% of the genetic variance within each gender. In addition, suggestive linkage for liver copper content was found on chromosomes 1, 7, 12 and 18 and on LGs U2, U5 and U6. The regions on these chromosomes and linkage groups explained 8.1 to 20.2% of the genetic variance for liver copper content in these two rabbit inbred strains. In order to identify genes that may be involved in copper regulation, the linkage groups need to be assigned to chromosomes first.

Overall conclusions

I. Copper is an essential trace element, implicating that a certain copper requirement exists to compensate for endogenous losses. The dietary copper allowance for the NMRI outbred laboratory mouse, as determined in the study described in chapter 2, is 4 ppm Cu, which is lower than the NRC's estimated allowance of 6 ppm Cu for maintenance and 8 ppm for growing and lactating mice, but in line with results described by other authors. The difference in dietary copper allowance probably stems from the fact that the recommendation of the NRC is based on rats and on four studies in mice, of which three were not designed to study copper requirement. We feel that this study contributes to a soundly based estimated copper allowance for mice.

II. Evidence for the involvement of copper in the formation of free radicals and ROS comes mainly from *in vitro* research (chapter 3). No evidence was found for copper-mediated oxidative damage at the (sub)cellular level (chapter 4) nor a reduced lifespan was found (chapter 5) in mice fed diets with increasing copper concentrations. These results raise serious questions about the likelihood of developing oxidative stress in other rodents or in human *in vivo* after high copper intakes.

III. In literature, dietary cholesterol has been associated with reduced liver copper concentrations and/or liver copper content. Feeding a cholesterol-rich diet to rats did not affect the liver copper content. In rabbits, a decrease in the hepatic copper store was found only in the dietary cholesterol-susceptible inbred strain. The idea that cholesterol-susceptible animals will show a greater decrease in hepatic copper content after being fed a cholesterol-rich diet thus could not be consistently confirmed. In both rabbits and rats a decrease in liver copper concentration was found, but the decrease in rats was probably due to diet-induced hepatomegaly. The reduced liver copper concentrations in rats fed a cholesterol-rich diet as described in literature may also be the result of dietary-induced hepatomegaly.

IV. Strain differences in liver copper store and liver copper concentrations can (partly) be explained by genetic differences between the strains. QTL analysis can be helpful in identifying genes that are involved in such quantitative traits. Some of the QTLs found in rats may give a clue as to what genes are involved in copper regulation. In the rabbit, however, more research on the structure of the genome is needed before candidate genes for QTLs can be identified.

Nederlandse samenvatting
- voor niet-vakgenoten -

Nederlandse samenvatting voor niet-vakgenoten

In dit proefschrift staat het metaal koper centraal. Koper komt vooral via de voeding in het lichaam van mensen en dieren terecht. Daar wordt het in de lever opgeslagen, uitgescheiden via het gal of in eiwitten ingebouwd. Distributie van koper vanuit de lever over de rest van het lichaam vindt plaats door uitscheiding van deze eiwitten door de lever. Via allerlei mechanismen komt koper uiteindelijk weer terug in de lever en wordt het via de in de feces aanwezige gal uitgescheiden. Het koper dat door het lichaam wordt gebruikt en uitgescheiden, moet worden aangevuld met koper uit de voeding. Dat betekent dat er een minimale hoeveelheid koper in de voeding moet zitten die deze verliezen kan compenseren. Dit geldt overigens niet alleen voor koper, maar voor een heleboel voedingsstoffen.
De Amerikaanse National Research Council heeft voor veel verschillende dieren schattingen gemaakt van deze minimale hoeveelheden voedingsstoffen. Zij heeft ook een schatting gemaakt van het koperniveau dat in de voeding van muizen zou moeten zitten. Als je de onderbouwing van deze schatting bestudeert, blijkt dat eigenlijk nooit goed is onderzocht hoeveel koper in de voeding van muizen zou moeten zitten om de verliezen weer aan te vullen. Wij hebben daarom een onderzoek gedaan waarbij we muizen hebben onderverdeeld in verschillende groepen. Elke groep kreeg een voer met een bepaald koperniveau. Door biochemische parameters (bijvoorbeeld het koperniveau van de lever) en proefdierkundige parameters (bijvoorbeeld groei of reproductie) te meten, hebben we een schatting kunnen maken van het benodigde koperniveau in het voer van muizen. Onze schatting van dit koperniveau ligt lager dan dat van de National Research Council, maar is wel in lijn met de resultaten van andere onderzoekers.

Veel van de muizen die in zogenaamde dierenlaboratoria worden gehouden voor onderzoek krijgen een commercieel verkrijgbaar voer. De koperniveaus in deze voeders blijken hoger te zijn dan onze schatting van het benodigde koperniveau (soms wel een factor 10 zo hoog). Hoewel de muizen niet direct ziek worden van wat extra koper in het voer, is het wel de vraag of het wel goed is voor de muizen om ze (aanzienlijk) meer te voeren dan ze eigenlijk nodig hebben. Koper lijkt namelijk betrokken te zijn bij het genereren van vrij radicalen en reactieve zuurstofdeeltjes. Dit zijn moleculen die heel reactief zijn en met allerlei andere moleculen in het lichaam kunnen reageren. Tijdens die reacties veroorzaken ze nogal wat schade in het

lichaam. Opname van grotere hoeveelheden koper zou er toe kunnen leiden dat er meer vrije radicalen en reactieve zuurstofdeeltjes worden gegenereerd en dat kan resulteren in schade aan DNA, eiwitten en vetten.
Om dit idee te onderzoeken hebben wij muizen verschillende hoeveelheden koper gevoerd en schade aan DNA, eiwitten en vetten gemeten. De hoeveelheid schade bleek niet verschillend te zijn tussen de verschillende dieetgroepen. We weten uit de literatuur dat het lichaam een mechanisme heeft om zich te beschermen tegen de effecten van deze reactieve moleculen. Het lijkt er op dat dit mechanisme zo goed werkt dat het de productie van grotere hoeveelheden reactieve moleculen in de aanwezigheid van koper kan opvangen.
Naast schade op moleculair niveau door vrije radicalen en reactieve zuurstofdeeltjes, hebben onderzoekers ook een verband gelegd tussen deze reactieve moleculen en ouderdoms-processen. Ook zouden deze moleculen betrokken zijn in de ontwikkeling van ziekten zoals kanker, Parkinson en Alzheimer. Als een hogere koperinname er toe kan leiden dat er meer vrije radicalen en reactieve zuurstofdeeltjes worden gevormd, zou dit betekenen dat muizen die voer krijgen met een hogere concentratie koper korter leven dan muizen die voer krijgen met een lager koperniveau. Om dit te onderzoeken hebben wij muizen verdeeld over drie dieetgroepen met oplopende koperconcentraties en hun levensduur bepaald. Wij hebben echter geen statistisch significante verschillen in levensduur tussen de verschillende dieetgroepen gevonden. Dit bevestigt het vermoeden dat het mechanisme van het lichaam om zich te weren tegen de schade die reactieve moleculen kunnen veroorzaken uiterst effectief is en dat hogere koperinnames niet resulteren in een verstoring van dit mechanisme.

Zoals reeds kort beschreven heeft het lichaam koper nodig voor een tal van biochemische processen. Het is daarom belangrijk dat het koperniveau in het lichaam niet te laag wordt. Er zijn tal van factoren die het koperniveau in het lichaam beïnvloeden. Een voorbeeld is cholesterol. Uit de vakliteratuur blijkt dat verhoogde opname van cholesterol via de voeding kan leiden tot een daling van het koperniveau. Omgekeerd is ook bekend dat een kopertekort kan leiden tot verhoogde cholesterolniveaus in het lichaam.
Niet iedereen is even gevoelig voor cholesterol en ook binnen diersoorten bestaan er verschillen in de gevoeligheid voor cholesterol. Zo bestaan er ratten en konijnen die heel gevoelig zijn voor cholesterol en sterk reageren op cholesterolrijk voer (zogenaamde hyperresponders) en ratten en konijnen die veel minder gevoelig zijn voor cholesterol (zogenaamde hyporesponders). Wij verwachtten dat het koperniveau in de lever van hyperresponderende ratten en konijnen sterker zal dalen als gevolg van een cholesterolrijk

dieet dan in ratten en konijnen die niet zo gevoelig zijn voor cholesterol. Om dit te toetsen hebben we twee soorten (stammen) konijnen (een hyper- en een hyporesponderende stam) en twee rattenstammen (een hyper- en een hyporesponderende stam) een cholesterolrijk of een controle dieet gevoerd en het koperniveau in de lever gemeten. In de rat werd geen effect van cholesterol op de totale hoeveelheid koper in de lever gevonden. Wel werd een daling van de koperconcentratie gevonden, maar dit is het gevolg van het zwaarder worden van de lever door leververvetting. In de beide konijnenstammen werd ook een daling in de koperconcentratie van de lever gevonden. Alleen in de hyperresponderende stam nam ook de totale hoeveelheid koper in de lever af. Uit dit onderzoek blijkt dus dat het koperniveau in de lever niet zondermeer daalt onder invloed van een cholesterolrijk dieet. Wel bleken er zowel voor de konijnen als voor de ratten significante stamverschillen in het koperniveau van de lever te bestaan en waren deze verschillen onafhankelijk van het dieet dat de dieren kregen.
Omdat we vermoedden dat de stamverschillen in het koperniveau van de lever deels erfelijk bepaald zijn, hebben we het DNA van verschillende ratten- en konijnenstammen onderzocht met behulp van een zogenaamde QTL (quantitative trait loci)-analyse. De QTL-analyse maakt gebruik van het feit dat het DNA van deze diersoorten zo goed in kaart is gebracht dat we bepaalde stukjes DNA kunnen herkennen. Het doel van ons onderzoek was het vinden van stukjes DNA die betrokken lijken te zijn bij de koperhuishouding. Naarmate we meer weten van de stukjes DNA die we met behulp van de QTL-analyse gevonden hebben, wordt het mogelijk om hypothesen te formuleren over mogelijke manieren waarop het koper in het lichaam gereguleerd worden. Zowel voor de rat als voor het konijn hebben we een aantal van zulke stukjes DNA gevonden.

Dankwoord

Dankwoord

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Lieve Marc, bedankt voor jouw geduld, steun en vertrouwen tijdens de afronding van mijn proefschrift en scriptie. Het waren zeker niet de gezelligste maanden door de vele uren die ik vaak verhit en geïrriteerd achter een dwarse computer met een eigen willetje heb doorgebracht. Maar we gaan nu eindelijk echt leuke dingen doen (alvast inlopen voor Denali of Kenai?)!

...and though few believed any of his tailes,
he remained very happy to the end of his days,
and those were extraordinarily long.

J.R.R. TOLKIEN
THE HOBBIT

Curriculum vitae

Curriculum Vitae

Ingeborg de Wolf is geboren te Leimuiden op 30 augustus 1973. Het Voorbereidend Wetenschappelijk Onderwijs werd grotendeels gevolgd aan het Eindhovens Protestants Lyceum (1996-1991). Aan de Rijksuniversiteit Limburg werd de doctoraalstudie Milieugezondheidkunde voltooid (1992-1996), na eerst de propedeuse Gezondheids-wetenschappen te hebben behaald (1991-1992). Na een aantal maanden uitzendwerk, waaronder het herschrijven van een wiskundecursus voor machinisten bij het opleidingsinstituut van de Nederlandse Spoorwegen, volgde in 1997 een aanstelling bij de vakgroep Proefdierkunde, Faculteit der Diergeneeskunde, Universiteit Utrecht als assistent-in-opleiding. Het onderzoek dat in deze periode is verricht wordt in dit proefschrift beschreven. In 1998 begon Ingeborg daarnaast aan het Parttime Doctoraal Programma Bedrijfskunde van de Universiteit Nyenrode, hetgeen in september 2001 werd afgesloten met het cum laude behalen van de doctorandustitel in de Bedrijfskunde. Momenteel is Ingeborg werkzaam als medewerker strategie bij Essent.

Stellingen

behorende bij het proefschrift 'The various faces of copper in laboratory animals' door Ingeborg de Wolf

1. De muis heeft een dusdanig efficiënt antioxidant en defensie systeem, dat verhoogde opname van koper uit het voer niet zal resulteren in radicaalschade. *(dit proefschrift)*

2. Koper speelt een rol in de ontwikkeling van de ziekte van Alzheimer door inductie van zuurstofradicalen als gevolg van binding van koper aan het amyloid ß-4 eiwit.

3. Koper speelt een directe dan wel indirecte rol bij de verstoring van de oxidant – antioxidant balans, hetgeen resulteert in oxidatieve stress. *(dit proefschrift)*

4. Vanuit sociaal-wetenschappelijk oogpunt is huisvesting van mannelijke muizen een probleem: het is onwenselijk om de muizen individueel te huisvesten, maar huisvesting in groepjes resulteert eveneens in niet-gewenste situaties.

5. "Het is heel goed om meer vrouwen in de exacte wetenschappen te hebben. Ze zijn namelijk beter. Door jarenlang te knokken hebben ze uithoudingsvermogen gekweekt. Ze hebben eelt op de ziel en presteren daardoor beter dan mannen." *(F.W. Saris, Natuur & Techniek, 10 (1997))*

6. Het uitvoeren van een promotieonderzoek anno 2001 is niet zozeer een proeve van wetenschappelijke bekwaamheid als wel een proeve van analytische bekwaamheid.

7. De kenmerkende arrogantie van veel randstedelingen ten opzichte van 'de rest van Nederland' is buitengewoon betreurenswaardig daar Amsterdam gerekend wordt tot de regio Eindhoven. *(Philips Communicatiegids 1999: Eindhoven en omstreken (incl. Amsterdam))*

8. Vanuit het oogpunt van verkeersveiligheid dienen ligfietsen, naar analogie van kinderfietsjes, voorzien te zijn van een vlaggetje dat ca. 1.50 m boven het wegdek uitsteekt.

9. De weersgesteldheid is omgekeerd evenredig met het aantal e-mails dat men op een reguliere werkdag ontvangt.

10. "Alle dingen worden overvloediger, gemakkelijker en kwantitatief beter voortgebracht als men datgene doet, dat bij zijn aanleg past, dit op het juiste moment doet en de overige zaken aan anderen overlaat." *(Plato, ca. 387 v. Chr.)*