

# **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 ( $\mu\text{g}/\text{whole liver}$ ) and the *D16Wox9* marker on chromosome 16 (lod score = 2.8), and between liver copper concentration ( $\mu\text{g}/\text{g dry weight}$ ) 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<sub>2</sub>-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<sub>2</sub>-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.