# Aspirin Reduces Blood Cholesterol in Copper-Deficient Rats: A Potential Antioxidant Agent?

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**The purpose of this study was to examine whether the hypocholesterolemic effect of aspirin is to due to its antioxidant properties. Oxidative stress was induced in rats by feeding them a copper-deficient diet. Copper deficiency reduced the activity of the enzyme superoxide dismutase (SOD) and lowered liver copper concentration but elevated liver iron. The combination of reduced SOD activity, high liver iron, and low liver copper resulted in an oxidative stress assessed by increased liver lipid peroxidation compared with copper-adequate controls. In addition, copper-deficient rats exhibited elevation of blood cholesterol. The administration of aspirin lowered both liver lipid peroxidation and blood cholesterol. It is suggested that the hypocholesterolemic properties of aspirin could be due to its ability to reduce oxidative stress.**


STUDIES CONDUCTED in our laboratory consistently showed that oxidative stress produced by the combination of dietary copper deficiency and excess liver iron retention was an inducer of hypercholesterolemia in rats.[1-3]

Copper is a component of the enzyme superoxide dismutase (SOD), which plays a major role in the defense against oxygen radicals.[4] In dietary copper deficiency, activity of this enzyme is suppressed, leading to inadequate protection against reactive oxygen species and to peroxidative damage to tissues.[5-7] Copper deficiency is also accompanied by a spontaneous increased accumulation of liver iron.[8] Iron, a strong oxidant, has the ability to generate free radicals under certain redox conditions.[9] When the diet is adequate in copper, the rats are protected against iron insult because their antioxidant defense system is adequate. However, excess liver iron coupled with inadequate antioxidant protection results in generation of free radicals and oxidative stress.[10]

The hypothesis that links oxidative stress with elevation of blood cholesterol is also supported by studies conducted in other independent laboratories. These studies report that hypercholesterolemia is induced by a combination of an oxidant or a free radical generator under conditions of inadequate antioxidant protection. Oxidative stress occurred when either selenium deficiency or absence of $\beta$-carotene and $\alpha$-tocopherol were challenged by oxidants such as iron[11] and doxorubicin.[12]

If oxidative stress is a potential inducer of hypercholesterolemia, then prevention of oxidative stress should suppress elevation of blood cholesterol. Indeed, preventing oxidative stress by reducing liver iron retention in copper deficiency eliminated free radical formation and reduced hypercholesterolemia.[2,10,13] Is it possible to prevent hypercholesterolemia by antioxidant agents?

Aspirin, acetylsalicylic acid, is widely used as an analgesic-antipyretic agent, to reduce and prevent the risk of cardiovascular disease due to its antithrombotic potential.[14-16] Recently, it has been suggested that the protective properties of aspirin are due to its antioxidant capabilities.[17] Aspirin protected cultured endothelial cells against oxidative stress elicited by exposure to iron-induced oxygen radicals.[17]

It was instrumental in elevating ferritin[17] since ferritin binds iron in a manner that inhibits its ability to participate in oxidant injury. Ferritin, too, can be viewed as an antioxidant.[17]

Copper deficiency in rats is associated with hypercholesterolemia.[18,19] Klevay[20] reported that the administration of aspirin to copper-deficient rats reduced their plasma cholesterol levels. Is the reduction of plasma cholesterol in copper-deficient rats treated with aspirin due to its antioxidant properties? This study was conducted to answer this question.

## MATERIALS AND METHODS

Weanling male Sprague-Dawley rats (n = 34) were randomly divided into 4 dietary groups: group 1, copper-adequate (n = 8); group 2, copper-adequate + aspirin (n = 8); group 3, copper-deficient (n = 10); and group 4, copper-deficient + aspirin (n = 8). All diets contained (g/kg) carbohydrate as fructose, 627; salt mix,[21] 35, prepared in our laboratory and formulated to omit copper; vitamin mix,[22] 10; biotin, 0.002 (mg/kg); choline bitartrate, 2.7 (mg/kg); egg white, 200; fat (corn oil), 95; and fiber, 30.

The copper-adequate diet was prepared by adding copper carbonate to the copper-deficient diets. The diets contained either 0.8 mg Cu/kg (deficient) or 6.0 mg Cu/kg (adequate). Aspirin (acetylsalicylic acid) USP grade, was purchased from Sigma, St Louis, MO, and was added to diets (2.84 g/kg).[20] Rats were fed their respective diets for 5 weeks. They were allowed free access to food and deionized, distilled water. Rats were killed after an overnight fast. Livers and hearts were removed and weighed. Portions of livers were taken for copper and iron analyses. Other portions of livers were immediately frozen in liquid nitrogen and kept frozen at -70°C until analyzed. Blood was collected into heparinized capillary tubes to measure hematocrit. Blood was also collected into heparinized test tubes for the measurements of cholesterol and ferritin. Plasma cholesterol was measured by the enzymatic automated procedure of the Centrifichem (Baker Instruments, Pleasantville, NY) using Trace Reagents from Trace America (Miami, FL). Ferritin was measured in plasma with rat ferritin kit. (Cat. No. RF 69; Ramco Labs, Houston, TX) a sandwich solid-phase enzyme radioimmunoassay. The activities of SOD and lipid peroxidation were measured in liver. Livers were homogenized in 20 mmol/L Tris-HCl, pH 7.4. They were then centrifuged and the upper phase removed. SOD was measured using a Bioxytech SOD-525 kit (Oxis International,

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Portland, OR). Lipid peroxidation was measured using a Lipid Hydroperoxide Assay Kit, Cat. 705002 (Cayman Chemical, Ann Arbor, MI) following extraction of lipid hydroperoxides into chloroform. Copper and iron concentrations in tissue homogenates and diets were determined according to the procedure of Hill et al,[23] using a flame atomic absorption spectrophotometer (model 5000, Perkin-Elmer, Norwalk, CT). Iron and copper concentrations in the sample were determined using standard curves of each mineral. Standard Reference Materials, bovine liver (SRM #1577b) and wheat flour (SRM #1567a) of the National Institute of Standards and Technology (Gaithersburg, MD) were used to verify accuracy. These materials were analyzed concurrently with the tissue samples, beginning at the digestion phase.

All data were expressed as the mean ± SEM and analyzed by analysis of variance (ANOVA) with 2 concentrations of copper and 2 of aspirin. The independent effects of copper and aspirin and the interactions between them were examined. Differences at $P < .05$ were considered statistically significant.

## RESULTS

This study had to be terminated prematurely after 5 weeks due to untimely death of 1 copper-deficient rat fed aspirin. Postmortem examination revealed an enlarged heart that had ruptured in the area of the apex.

Body mass, relative liver and heart sizes, and hematocrit are summarized in Table 1. Body mass was reduced by copper deficiency. The consumption of aspirin was responsible for a reduced growth rate. Liver weight was reduced by copper deficiency. When expressed in relation to body weight, relative liver size was increased by copper deficiency and by aspirin. Relative heart size was enlarged by copper deficiency. Aspirin had no effect on heart size. All rats became anemic when they consumed the copper-deficient diet. The consumption of aspirin increased hematocrit.

Hepatic lipid hydroperoxide, SOD activity, and plasma cholesterol are summarized in Table 2. Hepatic lipid peroxidation assessed by hydroperoxide was elevated by copper deficiency. Aspirin significantly reduced lipid peroxidation in copper-deficient rats. Plasma cholesterol was elevated by copper deficiency. Regardless of the levels of dietary copper, plasma cholesterol was reduced by aspirin administration. The activity of SOD whether expressed per gram of liver or per total liver was significantly reduced by copper deficiency. Aspirin was responsible for an increase in SOD, however, only in copper-adequate rats.

Liver copper and iron concentrations and plasma ferritin are summarized in Table 3. The consumption of a copper-deficient diet resulted in a reduced liver copper compared with copper-adequate controls. Aspirin was responsible for elevating liver copper in copper-deficient and -adequate rats. Liver iron was increased by copper deficiency. Aspirin reduced liver iron in copper-adequate controls but had no effect on liver iron in copper-deficient rats. Plasma ferritin was lowered by copper deficiency.

**Table 1. Body Mass, Relative Liver and Heart Sizes, and Hematocrit**

| | Copper-Adequate | | Copper-Deficient | |
|---|---|---|---|---|
| | −Aspirin | +Aspirin | −Aspirin | +Aspirin |
| Body mass (g) | 230 ± 10 | 214 ± 3 | 154 ± 5 | 140 ± 4 |
| Liver wt (g) | 8.1 ± 0.4 | 8.1 ± 0.1 | 7.4 ± 0.3 | 7.0 ± 0.3 |
| Liver size (g/100 g) | 3.5 ± 0.05 | 3.8 ± 0.07 | 4.8 ± 0.09 | 5.1 ± 0.11 |
| Heart wt (g) | 1.0 ± 0.03 | 0.9 ± 0.03 | 1.2 ± 0.06 | 1.0 ± 0.04 |
| Heart size (g/100 g) | 0.44 ± 0.009 | 0.42 ± 0.01 | 0.75 ± 0.03 | 0.69 ± 0.02 |
| Hematocrit (%) | 43 ± 1.0 | 46 ± 0.5 | 18 ± 0.9 | 21 ± 2.0 |

| | ANOVA | | |
|---|---|---|---|
| ANOVA | Cu | Aspirin | Cu × Aspirin |
| Body mass | .0001 | .0187 | NS |
| Liver wt | .0068 | NS | NS |
| Liver size | .0001 | .0012 | NS |
| Heart wt | .0336 | .0058 | NS |
| Heart size | .0001 | NS | NS |
| Hematocrit | .0001 | .0187 | NS |

NOTE. Values are means ± SEM. The independent effects of copper (Cu) and aspirin and the interaction between them are reported. Differences at $P < .05$ were considered statistically significant.

Abbreviation: NS, not significant.

**Table 2. Hepatic Lipid Hydroperoxide and SOD Activity and Plasma Cholesterol**

| | Copper-Adequate | | Copper-Deficient | |
|---|---|---|---|---|
| | −Aspirin | +Aspirin | −Aspirin | +Aspirin |
| LPO μmol/g liver | 29.5 ± 4.9 | 37.2 ± 6.7 | 97.8 ± 12.3 | 53.9 ± 15.7 |
| LPO μmol/liver | 236 ± 42 | 303 ± 52 | 714 ± 90 | 383 ± 111 |
| SOD U/g liver | 796 ± 46 | 1042 ± 65 | 156 ± 12 | 140 ± 19 |
| SOD U/liver | 6,442 ± 476 | 8,480 ± 545 | 1,151 ± 98 | 983 ± 137 |
| Cholesterol (mg/dL) | 127 ± 4 | 110 ± 4 | 173 ± 4 | 149 ± 6 |

| | ANOVA | | |
|---|---|---|---|
| | Cu | Aspirin | Cu × Aspirin |
| LPO/g liver | .0005 | NS | .0247 |
| LPO/liver | .0016 | NS | .0189 |
| SOD U/g liver | .0001 | .0078 | .0029 |
| SOD U/liver | .0001 | .0153 | .0050 |
| Cholesterol | .0001 | .0001 | NS |

NOTE. Values are means ± SEM.

Abbreviations: LPO, lipid hydroperoxide; SOD, superoxide dismutase.

**Table 3. Liver Copper and Iron Concentrations and Plasma Ferritin**

| | Copper-Adequate | | Copper-Deficient | |
|---|---|---|---|---|
| | −Aspirin | +Aspirin | −Aspirin | +Aspirin |
| Liver copper (μg/g wet wt) | 3.5 ± 0.4 | 4.2 ± 0.2 | 1.1 ± 0.1 | 1.6 ± 0.3 |
| Liver iron (μg/g wet wt) | 119 ± 7 | 86 ± 4 | 135 ± 4 | 130 ± 6 |
| Plasma ferritin (μg/L) | 675 ± 88 | 447 ± 64 | 448 ± 36 | 346 ± 30 |

| | ANOVA | | |
|---|---|---|---|
| | Cu | Aspirin | Cu × Aspirin |
| Liver copper | .0001 | .0348 | NS |
| Liver iron | .0001 | .0010 | .0131 |
| Plasma ferritin | .0111 | .0105 | NS |

NOTE. Values are means ± SEM.

Similarly, aspirin administration was responsible for reducing plasma ferritin in copper-deficient and -adequate rats.

## DISCUSSION

In agreement with past studies[1-3] and other investigators,[18,19] all copper-deficient rats of the present study developed hypercholesterolemia. The administration of aspirin decreased plasma cholesterol in both copper-deficient and copper-adequate rats.

The hypocholesterolemic effect of aspirin has been previously reported by Klevay.[20] This effect of aspirin was explained by the increase of liver copper.[20] However, we propose that hypocholesterolemic effect of aspirin is due to its antioxidant capabilities.

We hypothesized that oxidative stress was an inducer of hypercholesterolemia.[3] Past studies supported this hypothesis.[1-3,10,13] In order to induce oxidative stress, we fed rats a copper-deficient diet. The consumption of this diet was responsible for a depressed activity of SOD accompanied by an increased retention of liver iron. In the past, this combination of a depressed antioxidant defense system with excess liver iron resulted in oxidative stress, generation of free radicals,[10,13] and lipid peroxidation.[7] In agreement with previously reported data and data reported here, rats fed the copper-deficient diet exhibited oxidative stress. This was measured by increased hepatic lipid hydroperoxide content. The condition of oxidative stress could be responsible for hypercholesterolemia.

If we assume that oxidative stress is a potential inducer of hypercholesterolemia, then preventing oxidative stress should eliminate hypercholesterolemia. Prevention of oxidative stress can be achieved by either reducing or scavenging free radicals. In our past studies, preventing generation of free radicals by lowering liver iron or by chelation therapy was instrumental in reducing oxidative stress and lowering levels of blood cholesterol.[2,10,13] Other investigators reported that blood cholesterol was reduced by altering tissue levels of the antioxidant glutathione.[24,25]

A logical extension of the hypothesis that oxidative stress is responsible for hypercholesterolemia would be to test the potential that antioxidants have in reducing oxidative stress. Reduction of oxidative stress should be able to lower blood cholesterol. This study was conducted to test this hypothesis. Aspirin was chosen as an antioxidant agent since it has been reported that it had the capacity to protect endothelial cells from the deleterious toxic effects of hydrogen peroxide and, in particular, from iron-dependent oxygen radical formation.[17]

In the present study, aspirin was instrumental in lowering hepatic lipid peroxidation in copper-deficient rats. It also lowered blood cholesterol. Aspirin, an analgesic-antipyretic drug, is also an antithrombotic agent.[14-16] Recently, aspirin has been reported to possess antioxidant functions.[17] It has been suggested that antioxidant properties of salicylate are due to gentisic acid, the metabolite of salicylate.[26] Gentisic acid could have strong radical scavenging abilities due to its chemical nature.[26] Other salicylic acid derivatives were ineffective as free radical scavengers.[26] Gentisic acid may form a stable phenoxyl radical and thus may not initiate a radical chain reaction with polyunsaturated fatty acids.[26] Regardless, whether it is salicylate per se or one of its metabolites, the consumption of aspirin by copper-deficient rats proved beneficial in reducing both lipid peroxidation and blood cholesterol.

As reported by Klevay,[20] aspirin administration caused an elevation of liver copper. These data are in agreement with the present study. However, this increase in liver copper was accompanied by a rise in SOD activity only in copper-adequate rats. Therefore, it seems likely to assume that the reduced lipid peroxidation in copper-deficient rats treated by aspirin may be due to aspirin per se, and not to SOD. In addition, SOD activity was elevated by aspirin only in copper-adequate rats. However, lipid peroxidation was not affected. Blood cholesterol was lowered. Under the experimental conditions of the present study where copper deficiency induces inadequate antioxidant protection, aspirin, but not SOD, was instrumental in reducing lipid peroxidation and blood cholesterol.

It is well established that the availability of iron enhances the injury-producing effects of peroxides through generating hydroxyl radical-like species.[9,27] It is also established that ferritin is a sensitive indicator of available iron stores.[28] However, in certain instances ferritin cannot be used in diagnosis of anemias of chronic diseases, infections, inflammation, liver disease, and malignancies because ferritin is a positive acute-phase reactant protein that is increased in inflammation.[29] Indeed, plasma ferritin did not reflect magnitude of liver iron in copper-deficient rats.[30] The most severe anemia of copper deficiency was induced by the consumption of a copper-deficient diet. These rats also exhibited the highest levels of liver iron.[28]

The consumption of aspirin lowered plasma ferritin in both copper-adequate and copper-deficient rats. Aspirin also lowered liver iron. Reduction of liver iron should be beneficial in view of the fact that iron has the potential to induce reactive oxygen species.[9,27] It has been suggested that increased body iron, as measured by serum ferritin, is a risk factor for the development of atherosclerotic complications.[31] This hypothesis is in agreement with the data reported herein. Rats that exhibited the highest levels of liver iron also exhibited the highest levels of blood cholesterol. They also showed the highest magnitude of lipid peroxidation. Plasma ferritin, however, did not reflect these changes in liver iron. Rats fed the copper-deficient diet had the highest levels of liver iron but did not exhibit the highest levels of plasma ferritin. These data are in agreement with our previous publication which reported that ferritin was not an indicator of available hepatic iron stores in copper deficiency.[30]

It has been suggested that ferritin protects endothelial cells from oxidized low-density lipoproteins in vitro.[32] Similarly, it has been shown that aspirin increased ferritin synthesis in endothelial cells.[17] These results suggest that induction of ferritin may prevent endothelial injury. The authors also suggest that aspirin may be considered as an antioxidant because of its induction of ferritin. Ferritin may be regarded as an antioxidant in endothelial cells. However, it may not be regarded as an antioxidant in copper deficiency.

Taken together, data of the present study suggest that, in addition to its role as an antipyretic and antithrombotic agent, aspirin may be considered as an hypocholesterolemic agent. This suggestion is derived from 2 independent laboratories that

clearly demonstrated that when antioxidant defense system is compromised, aspirin has the ability to reduce both lipid peroxidation and blood cholesterol. This potential quality of aspirin deserves further attention, particularly in view of the fact that aspirin is one of the most frequently used analgesic in medical care[33] and a high percentage of all analgesic agents contain aspirin.[33] In addition, median intakes of copper from food were lower than Estimated Safe and Adequate Daily Dietary Intake values for most age, gender, and racial/ethnic subgroups.[34] Furthermore, copper was classified as potential public health issue because epidemiological and clinical studies suggested that inadequacy of copper intake is associated with ventricular arrhythmias.[34] If the same type of an effect of copper deficiency seen in the rat occurs in humans, then the inadequate intake of copper has the potential to induce oxidative stress, which in turn increases blood cholesterol. Can antioxidants be used to reduce blood cholesterol? Further studies are needed to test this hypothesis.

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