

# The Effect of Acetaminophen on Oxidative Modification of Low-Density Lipoproteins in Hypercholesterolemic Rabbits

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**Summary** Oxidative modification of low-density lipoproteins (LDL) contributes to the pathology of atherosclerosis. Antioxidants may protect LDL against oxidative modification. Acetaminophen, a widely used analgesic and antipyretic agent, has significant antioxidant properties. However, there is little evidence to suggest that acetaminophen acts as an antioxidant for LDL oxidation *in vivo*. In this study, we investigated the *in vivo* effect of acetaminophen on LDL oxidation in hypercholesterolemic rabbits. The oxidative modification of LDL was identified by conjugated dienes and thiobarbituric acid-reactive substances (TBARS). In the cholesterol group which rabbits were fed a diet contained 1% g cholesterol for 8 weeks, TBARS contents and conjugated diene levels in the plasma and isolated LDL samples significantly increased compared with the control rabbits ( $p<0.05$ ). However, in the cholesterol + acetaminophen group, the TBARS contents and conjugated diene levels were significantly lower than that of the cholesterol group ( $p<0.05$ ). The results from *in vitro* studies also demonstrated that the LDL isolated from serum was oxidized by  $\text{Cu}^{++}$  ions and this oxidation reduced in the presence of acetaminophen. The reduced oxidative modification of LDL by acetaminophen may be of therapeutic value in preventing the development and progression of atherosclerosis.

**Key Words:** Oxidation of LDL, atherosclerosis, acetaminophen, hypercholesterolemia, rabbit

## Introduction

Atherosclerosis and related cardiovascular diseases remain a major health problem in developed countries. High plasma total cholesterol and low density lipoproteins (LDL) levels show significant positive correlation to the development of atherosclerosis and cardiovascular diseases [1, 2]. It was reported that the cholesterol content of erythrocytes, platelets, polymorphonuclear leukocytes and endothelial cells increased in hypercholesterolemia and this increase activate these cells and cause the enhanced production of

oxygen free radicals [3, 4].

LDL, the major carrier of blood cholesterol, is thought to become atherogenic after undergoing oxidative modifications. It was known that LDL oxidation is accompanied by alteration in its biological properties, such as endothelial cell damage, uptake via scavenger receptors in macrophages, formation of foam cells and fatty streak and increased amounts of mediators of cell proliferation and platelet aggregation [1, 2, 5–8].

Many studies have been focused on mechanisms of LDL oxidation. It was shown that *in vitro* LDL oxidation can be induced by transition metal ions, hypochlorite, superoxide/nitric oxide, organic peroxy radicals, tocopheryl radicals and peroxidases, the *in vivo* mechanisms for LDL oxidative modification has not yet identified [1, 2, 5–10].

A number of drugs with antioxidant capacity have been

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shown to protect LDL against oxidative modification [11–13]. Such drugs may have beneficial therapeutic values in the prevention of atherosclerosis. Acetaminophen, widely used analgesic and antipyretic drug, is an example which possesses significant antioxidant effects under certain experimental conditions [14–17]. However, literature survey has indicated the paradoxical results for the effect of acetaminophen on LDL oxidation. Some *in vitro* studies demonstrated that oxidative modification of LDL with  $\text{Cu}^{2+}$ -ions, azo compounds and peripheral blood mononuclear cells are significantly reduced by acetaminophen [16, 18], others contrarily claimed that acetaminophen may also act as a catalyst of LDL oxidation by myeloperoxidase [19]. Moreover, unique *in vivo* data available for the effects of acetaminophen on LDL oxidation in the literature is an abstract reporting limited experimental details [20].

Those conflicting results encourage us to investigate the *in vivo* effect of acetaminophen on LDL oxidation in hypercholesterolemic rabbits in order to clarify the paradox.

## Materials and Methods

### Reagents and chemicals

Trisodium citrate anhydrous, 2-thiobarbituric acid, 1,1,3,3-tetramethoxy propane and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Heparin was obtained from Liquemine 25000 IU/ml, Roche. Paracetamol (4-hydroxyacetanilide, 4-acetamidophenol) was obtained from Fluka. Cholesterol (95%) was obtained from ABCR GmbH & Co. Cholesterol, high-density lipoprotein (HDL)-cholesterol and triglyceride enzymatic kits were from Randox Laboratories Ltd., Antrim, UK. Agarose gels (Titan gel lipoprotein electrophoresis system) were purchased from Helena Laboratories, Beaumont, TX USA. Other chemicals used were of analytical grade.

### Animals and diets

Male New Zealand white rabbits weighed from 1.4 to 2 kg (mean  $\pm$  SD =  $1.6 \pm 0.16$ ) were studied. They were housed in individual cages in a room maintained at a constant temperature and kept on a 12:12 h light-dark cycle. After a 1-month period of adaptation of the animals, the experiment was started. Food and water were given daily and any unused food discarded. The control group ( $n = 10$ ) was fed with the standard rabbit diet (Flaran, Izmir, Turkey). The cholesterol group ( $n = 10$ ) was fed with standard rabbit diet containing 1% cholesterol (w/w). The acetaminophen group ( $n = 10$ ) were fed with the standard diet containing 1% cholesterol and treated with acetaminophen 200 mg/day for 8 weeks. The acetaminophen dose (200 mg/daily) is reported to relevant for analgesic effect to rabbits by oral route [21]. Acetaminophen solution in water was prepared daily and was administered to rabbits by gavage.

All animal protocols were approved by the Ege University, Faculty of Pharmacy Animal Care and Use Committee.

### Blood sample collection

For biochemical determinations, blood was taken from the central ear vein and collected in the test tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant at the beginning and at the end of the 8-weeks period. Plasma was recovered by centrifugation at  $4^{\circ}\text{C}$ , 1500 g for 15 min. At the end of the study period, 10 ml venous blood from an ear vein of each animal was drawn into a tube to obtain serum for LDL isolation.

### Preparation of LDL

LDL was isolated by buffered heparin as described in the literatures [22, 23]. The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5N HCl, and contained 50.000 IU/l heparin. Before precipitation of LDL, serum samples (to which 1 mg/ml of EDTA were added) and precipitation reagents were allowed to equilibrate to room temperature. 1 ml of the sample was added to 7 ml of the heparin-citrate buffer. After mixing with a vortex, the suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1000 g for 10 min. The pellet was resuspended in 1 ml of 0.1 M Na-phosphate buffer, pH 7.4, containing 0.9% of NaCl. The concentration of LDL protein was measured by the Lowry method using bovine serum albumin as standard [24].

### $\text{Cu}^{++}$ -mediated oxidation of LDL

The isolated LDL from normal human serum was incubated with  $10 \mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  for 6 and 24 hours in  $37^{\circ}\text{C}$  in the absence and presence of acetaminophen. The oxidation of LDL was identified by measurement of thiobarbituric acid reactive substances (TBARS) content and agarose gel electrophoresis.

### Plasma lipids analysis

Plasma total cholesterol (TC), triglyceride (TG) and HDL-cholesterol were determined enzymatically using a commercial kit (Randox Lab. Ltd. Crumlin, UK). LDL-cholesterol was calculated by Friedewald formula [25].

### Identity of oxidized LDL

Oxidation of LDL was identified by measuring two parameters: the formation of conjugated dienes, the production of TBARS.

TBARS content was used to represent lipid peroxide levels and measured by spectrophotometric assay using 1,1,3,3-tetramethoxy propane as a standard, as described by Yagi and Satoh [26, 27]. The results were expressed as nmoles of malondialdehyde equivalents.

The conjugated dienes in LDL lipids were determined directly by measuring the absorbance at 234 nm [22, 28]. Lipids were extracted from LDL samples by chloroform-methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm. Absorbance units were converted to molar units using the molar extinction coefficient  $2.95 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . The results were expressed as  $\mu\text{mol/l}$  to have an estimation of actual level of oxidized LDL in circulation.

#### Statistical analysis

All determinations were carried out in duplicate and median values  $\pm$  SD were calculated. Differences between groups were assessed by Kruskal-Wallis and Mann-Witney non-parametric statistical tests. A difference was considered significant when  $p < 0.05$ .

### Results

Table 1 shows the plasma levels of TC, TG, HDL-cholesterol, LDL-cholesterol and TBARS at the beginning (0 week) and at the end of the experiment (8 weeks). At the beginning of the experiment, plasma lipid levels of three groups were similar. However, feeding a cholesterol diet to rabbits

for 8 weeks significantly increased the plasma TC, LDL-cholesterol and TG levels, in comparison to controls ( $p < 0.05$ ).

No significant differences in rabbit weights were observed at the end of the study [ $2.49 \pm 0.14$  (0 week) –  $2.48 \pm 0.12$  (8 weeks),  $2.498 \pm 0.08$  –  $2.451 \pm 0.086$ ,  $2.5 \pm 0.08$  –  $2.42 \pm 0.11$  kg for control, cholesterol and cholesterol + acetaminophen groups, respectively].

As shown in Table 1, the plasma TBARS levels in the cholesterol group were significantly higher than that of the controls ( $p < 0.05$ ). However, in the cholesterol + acetaminophen group, the plasma TBARS levels were significantly lower than that of the cholesterol group ( $p < 0.05$ ).

Table 2 shows TBARS contents and conjugated diene levels in isolated LDL samples from experimental groups serum. The result indicated that TBARS and conjugated diene levels in isolated LDL from the cholesterol group were significantly higher than that of the control group ( $p < 0.05$ ). However, TBARS and conjugated diene levels in cholesterol + acetaminophen group has lowered when compared to the cholesterol group levels (Table 2) ( $p < 0.05$ ).

The results from *in vitro* studies demonstrated that the LDL samples isolated from normal human serum was oxidized by  $\text{Cu}^{++}$  ions and this oxidation reduced in the presence of acetaminophen (Table 3).

Table 1. The effect of high cholesterol diet on plasma lipid levels and TBARS content in rabbits

Experimental period (week)	Experimental group	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	TBARS (nmol/ml)
0	Control	$39.73 \pm 9.27$	$77.85 \pm 23.98$	$13.71 \pm 2.75$	$8.85 \pm 4.02$	$0.701 \pm 0.05$
	Cholesterol	$42.71 \pm 7.14$	$143.28 \pm 30.96$	$16.26 \pm 6.52$	$10.75 \pm 3.05$	$0.720 \pm 0.05$
	Cholesterol + Acetaminophen	$44.60 \pm 8.99$	$74.24 \pm 29.60$	$14.13 \pm 1.60$	$7.90 \pm 1.25$	$0.740 \pm 0.07$
8	Control	$28.98 \pm 14.25$	$60.35 \pm 19.03$	$7.56 \pm 2.57$	$7.36 \pm 5.10$	$0.725 \pm 0.05$
	Cholesterol	$745.89 \pm 280.36^*$	$182.35 \pm 101.14$	$9.73 \pm 2.54$	$588.28 \pm 219.88^*$	$0.923 \pm 0.16^*$
	Cholesterol + Acetaminophen	$534.73 \pm 264.25^*$	$57.75 \pm 18.82$	$8.16 \pm 3.60$	$510.25 \pm 253.47^*$	$0.717 \pm 0.05^{**}$

The results are expressed as means  $\pm$  SD of ten animals per group.

\* $p < 0.05$ , significant difference to the control group.

\*\* $p < 0.05$ , significant difference to the cholesterol group.

Table 2. Conjugated diene levels and TBARS content in LDL isolated from rabbits serum

Experimental groups	TBARS (nmol/mg LDL protein)	Conjugated diene levels ( $\mu\text{M/mg}$ LDL protein)
Control	$0.07 \pm 0.02$	$6.40 \pm 1.33$
Cholesterol	$0.15 \pm 0.06^a$	$17.26 \pm 6.63^a$
Cholesterol + Acetaminophen	$0.10 \pm 0.04^{a,b}$	$11.61 \pm 2.22^{a,b}$

The results are expressed as Means  $\pm$  SD of ten animals per group.

<sup>a</sup>  $p < 0.05$ , significant difference to the control group.

<sup>b</sup>  $p < 0.05$ , significant difference to the cholesterol group.

Table 3. TBARS levels of LDL samples after incubation of Cu<sup>++</sup> ions for 6 and 24 hours

	TBARS levels (nmol/mg protein) (Mean ± SD) (n = 5)	
	Incubation time	
	6 hours	24 hours
Control (LDL + PBS)	2.12 ± 0.19	2.27 ± 0.23
LDL + Cu <sup>++</sup> (Oxidized LDL)	7.54 ± 0.83 <sup>a</sup>	7.78 ± 0.45 <sup>a</sup>
LDL + Cu <sup>++</sup> + A (0.1 mM)	5.64 ± 0.30 <sup>a,b</sup>	5.79 ± 0.34 <sup>a,b</sup>
LDL + Cu <sup>++</sup> + A (0.5 mM)	5.43 ± 0.28 <sup>a,b</sup>	5.38 ± 0.33 <sup>a,b</sup>
LDL + Cu <sup>++</sup> + A (1 mM)	5.09 ± 0.17 <sup>a,b</sup>	5.09 ± 0.15 <sup>a,b</sup>
LDL + Cu <sup>++</sup> + A (2 mM)	3.62 ± 0.25 <sup>a,b</sup>	3.88 ± 0.74 <sup>a,b</sup>

A: Acetaminophen

<sup>a</sup> p<0.05 significant difference to control LDL.

<sup>b</sup> p<0.05 significant difference to oxidized LDL.

## Discussion

There is a contradiction about the effect of acetaminophen on LDL oxidation as prooxidant or antioxidant. It has been shown that acetaminophen is a potent inhibitor for Cu<sup>++</sup>-induced and cell-mediated LDL oxidation *in vitro* [16]. In addition, Chou *et al.* [18] demonstrated that myeloperoxidase(MPO)-H<sub>2</sub>O<sub>2</sub>-nitrite mediated oxidation of LDL was inhibited by acetaminophen. However, Kapiotis *et al.* [19] found that acetaminophen increased LDL oxidation by MPO that generate phenoxyl radical and act as a catalyst of LDL oxidation in MPO-induced reaction systems. These conflicting results may be attributed to the different experimental conditions employed in the studies.

On the other hand, Merrill *et al.* [14, 15] have reported that acetaminophen causes an important cardioprotective effect on isolated heart tissue from experimental animals. Similarly, Taylor *et al.* [20] have examined the effect of acetaminophen on the formation of atherosclerotic plaques in the cholesterol fed rabbit model and this study was published as abstract without detailed experimental data. They reported that formation of atherosclerotic plaques was significantly decreased at the end of the study and acetaminophen has capable to inhibit the formation of oxysterols in LDL.

Those conflicted results encourage us to investigate the *in vivo* effect of acetaminophen on LDL oxidation in hypercholesterolemic rabbits.

Table 1 indicates that the cholesterol-supplemented diet caused marked alterations in the plasma lipoproteins, particularly an increase of LDL and a decrease of HDL in comparison with the control group. These findings indicated that hypercholesterolemia was occurred.

The most widely used methods for monitoring LDL oxidation are the measurement of conjugated dienes and

TBARS levels [30–33]. Beside the plasma TBARS levels, conjugated diene levels and TBARS contents in the isolated LDL samples from the cholesterol group were also significantly higher than that of the control group (p<0.05) (Table 2). It is known that high cholesterol diet induce production of oxygen free radicals leading to lipid peroxidation. The significant increase of plasma lipid peroxidation in cholesterol-fed rabbits agrees with previous reports [32, 33].

The addition of acetaminophen to daily diet (200 mg/kg a day) was clearly reduced TBARS and conjugated diene levels when compared to the cholesterol group levels (see the cholesterol + acetaminophen group in Table 2). The reductive effect of acetaminophen on LDL oxidation may well be related with its antioxidant capacity which is attributed to its phenolic structure and are believed to work out via scavenging free radicals [16, 17, 34, 35].

As a result, our study demonstrate that; (1) after 8 weeks of feeding 1% g cholesterol diet, rabbits developed significant elevations of plasma lipid levels; (2) cholesterol-enriched diet increased in lipid peroxidation; (3) the administration of 200 mg/daily acetaminophen to cholesterol feeding rabbits reduced lipid peroxidation. On the otherhand, the results from *in vitro* studies demonstrated that the LDL samples isolated from serum was oxidized by Cu<sup>++</sup> ions and this oxidation reduced in the presence of acetaminophen (Table 3).

At this point, a suggestion can be made that acetaminophen might have a therapeutic value as an alternative drug for prevention of atherosclerosis which is one of the main causes of human death in industrialized countries.

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