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Screening of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Inhibitors *In Vitro* and Its Application to Pullets

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HMG-CoA Reductase의 저해제 탐색과 가금의 콜레스테롤 저하 효과

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Abstract

The primary objective of these studies was to screen the materials showing inhibitions of HMG-CoA reductase *in vitro*. The secondary objective was to determine the effect of garlic, lovastatin and copper on cholesterol concentrations in plasma, liver and breast tissues in pullets. The degree of inhibition of the selective samples on HMG-CoA reductase activity was determined *in vitro*. The inhibition ratios of water soluble garlic extracts, lovastatin (methanol extracts) and copper to HMG-CoA reductase activity were 51.3%, 87.5%, and 82.0%, respectively. Control diet (basal diet) and experimental diets, garlic powder (3% in diet), lovastatin (300mg/Kg of diet) and copper (200mg/Kg of diet) were fed to pullets in order to investigate the changes of cholesterol concentration in plasma and tissues. Total cholesterol, HDL- and LDL-cholesterol in blood plasma were significantly reduced in pullets fed diet containing 3% garlic powder. However, copper significantly increased total cholesterol compared to control and lovastatin did not affect plasma cholesterol concentration. Total cholesterol and triglyceride of liver and breast tissues in pullets were not affected by adding the cholesterol-lowering materials to diets. The data suggests that it is not easy for HMG-CoA reductase inhibitors to reduce cholesterol levels in body due to complication of cholesterol metabolism. However, garlic administration can lower the levels of plasma cholesterol in pullets.

Key words : HMG-CoA reductase, garlic, lovastatin, copper, cholesterol.

Introduction

The evidence of correlating plasma cholesterol levels with coronary heart disease was established from the early observations that cholesterol was the major component of atherosclerotic plague[1]. Recently, much attention has been drawn to the effects of dietary components on hypercholesterolemia and atherosclerosis, and the concern has led to publication of a number of reports resulting in changes in human diet. The 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase is the rate-limiting enzyme in cholesterol biosynthesis. Studies with microsomal HMG-CoA reductase have demonstrated *in vitro* that the enzyme requires thiols for

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activation, however low concentrations of disulfides inactivate the enzyme[2].

Recent studies have shown that garlic contains various active components which are lowering lipid levels in humans[3] and animals[4]. This effect has been attributed to the aliphatic disulfides of allicin(a disulfide oxide) existing in garlic[4]. However, the information about the effect of garlic on cholesterol metabolism is quite limited presently and the mechanism of the hypocholesterolemic effect of garlic is unclear.

Dietary copper has been demonstrated to alter the lipid metabolism[5]. Metabolic changes by dietary copper include those of the rate of cholesterol biosynthesis and hepatic glutathione concentrations. Glutathione is known to stimulate the enzyme HMG-CoA reductase in cholesterol biosynthesis. Copper deficiency appears specifically to increase hepatic and plasma reduced glutathione(GSH) while oxidized glutathione (GSSG) concentrations remain unaffected[2].

Lovastatin is a competitive inhibitor of HMG-CoA reductase and thus inhibits cholesterol synthesis. In humans with heterozygous familial or nonfamilial hypercholesterolemia, lovastatin treatment usually reduces plasma cholesterol and low density lipoprotein (LDL) cholesterol concentrations by 25~40%[6].

The objective of these studies was to screen the materials showing the inhibitions of HMG-CoA reductase *in vitro*. The secondary objective was to determine the effects of garlic, lovastatin and copper on cholesterol concentrations in plasma, liver and breast tissues in pullets.

Materials and Methods

1. Screening of HMG-CoA Reductase Inhibitors *in vitro*

Samples were extracted with either methanol or water, and stored at 4℃ before enzyme assay using Lee *et al.*'s method[7]. Selected materials are shown in Table 1.

First of all, yeasts (*Saccharomyces cerevisiae* ATCC 96519) were incubated in the medium including glucose 1%, polypeptone 0.5%, yeast extract 1% in 30℃ semianaerobic condition for 24 h. Then the 1% incubation extracts were incubated in new medium including glucose 3%, polypeptone 0.5%, yeast extract 0.5%, KH_2PO_4 0.5% in 30℃, for 15h. For the enzyme assay, yeast extracts were collected, centrifuged at 4,000rpm for 15 min at 4℃ and washed with distilled water, 4℃. The pellet was suspended in 0.1M Triethanolamine buffer, pH 7.4 (5~15% W/V) including 20mM EDTA (ethylene diamine tetraacetate). The supernatant was homogenized with hand homogenizer and centrifuged at 8,000rpm for 15 min at 4℃ to exclude mitochondria. Then the supernatant was ultracentrifuged at 34,000rpm for 90 min at 4℃ to isolate microsome. Microsomal protein was washed with 0.1M Triethanolamine buffer, pH 7.4 in-

Table 1. Screening materials of HMG-CoA reductase inhibitors

Botanical name	English name	Korean name	Used part
Phellodendron amurens[1]	Amurcork tree	HwangBaek	bark
Eugenia caryophyllus[1]	Clove	JeonHyang	seeds
Paulownia corena[1]	Paulownia	OdongNamu	leaves
Pinus densiflora[1]	Pine tree	JeokSong	leaves
Lonicera japonica Thunb	Honey suckle	KumEunHwa	flower
Allium satium var. *pekinense*[2]	Garlic	MaNul	bulb
Platycodom glavcum[1]	Platycodon	DoRaGee	root
Bombyx mori L.[1]	Bombycidae	BaekKangZam	body
Scrophvlaria buergeriana Miguel[1]	Radix scrophulariae	HyunSam	root
Aloe arborescens[1]	Aloe	Aloe	leaves
Copper[2]	Copper	Guri	
Lovastatin[1]	Lovastatin	Lovastatin	

[1] Methanol extracts
[2] Water extracts

cluding 2mM DTT (Dithiothreitol) and stored at −70℃ prior to assay for enzymatic activity. Protein concentration was determined by the method of Lowly *et al.*[8]. Inhibition activitiy against HMG-CoA reductase was measured using a modified method of Hulcher *et al.*[9]. Microsomal protein 1mg, HMG-CoA 150nM, NADP 2μM, Glucose-6-phosphate 3μM, Glucose-6-phosphate dehydrogenase 2 units and Test samples (100mg/ml) 100μl were mixed, reacted at 37℃ for 30 min and added 10mM, sodium arsenite 20μl. Then the solution was stayed for 1 min and incubated at 37℃ for 10 min by adding 0.1ml 2mM, citrate buffer (pH 3.5) including 3% sodium tungstate to stop the reaction. The 1ml supernatant centrifuged at 15,000rpm was added 2M Tris buffer (pH 10.6) 0.2ml and 2M Tris buffer (pH 8.0) 0.1ml. In this soultion, 0.4M sodium arsenite 50ml incubated for 5 min and the reaction mixture 1ml was accomplished by adding 20μl 3mM DTNB (5, 5`-Dithio-bis(2-nitrobenzoic acid)) and CoA-SH concentration was measured by spectrophotometer at 420nm.

2. Animals, Management and Diets

Field study was conducted with commercial 13-wk-old ISA Brown pullets. The hens were alloted to individual wire-floored metabolism cages (one hen per cage) and subjected to four dietary treatments : 1) control 2) 3% garlic 3) 300mg lovastatin/Kg 4) 200mg copper/Kg. A commercial pullet diet was used as control treatment and garlic, lovastatin and copper were added to the control diet. Garlic powder and lovastatin were provided from Ottogi Co., and Jongkeundang Co., respectively. Copper was supplemented as feed grade cupric sulphate pentahydrate. Feed and water were provided ad libitum throughout the 30 day experimental periods. The experiment was conducted at environmentally controlled house. Ten pullets were involved for the each experimental work, including control. Three times were repeated for analysis, therefore totally one hundred twenty pullets were employed for this work.

3. Sample Preparation and Analyses

At the start and end of experiment, all pullets were fasted for 15 hour and individual blood samples were drawn from wing veins using heparinized syringes. Then, pullets were killed by decapitation. Plasma was separated by centrifugation at 3,000 rpm for 10 min at 4℃ and stored at −70℃. Liver and breast muscle samples were frozen at −70℃ until analysis. After thawing, tissue samples were thoroughly homogenized and extracted by the method of Folch *et al.*[10]. Total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride concentrations of plasma, liver and breast tissues samples were estimated using the chemical automatic analyzer, Hitachi-7150 (Hitachi medical Co., Japan). The methods and units were shown in Table 2.

4. Statistical Analysis

Data obtained from blood were subjected to analysis of covariance using the General Linear Model procedure of SAS®[11] and comparisons of means between treatment were conducted by Student Newman Keuls test[12] when significant difference ($P<.05$) was found. Data obtained from liver and breast were analysed by one-way analysis of variance test. Statistical significances among treatment means were determined by the method of new multiple range test of Duncan[13] when the F value was significant at 5% level.

Results and Discussion

The results of the screening of HMG-CoA reductase inhibitor from several materials *in vitro* are shown in Table 3. Methanol extracts of lovastatin had the highest inhibiton ratio 87.5% against HMG-CoA reductase, the

Table 2 . Method of clinical chemistry

Items	Unit	Method	Corporation
Cholesterol	mg/dl	Enzymatic Colorimetry	BM(Germany)
HDL-cholesterol	mg/dl	Enzymatic Colorimetry	Daichi(Japan)
LDL-cholesterol	mg/dl	Enzymatic Colorimetry	Daichi(Japan)
Triglyeride	mg/dl	Lipase. GK, GPO, POD + Glycerol blank	BM(Germany)

Table 3. Inhibition rate of HMG-CoA reductase activity by samples *in vitro*

Treatment*	Specific activity (CoA-SH pmoles/min/mg protein)	Degree of Inhibition (%)
Phellodendron amurense	65.9	6.9
Eugenia caryophyllus	61.0	13.8
Paulownia corena	59.3	16.3
Pinus densiflora	41.6	41.3
Lonicera japonica Thunb	58.4	17.5
Allium satium var. *pekinense*	34.5	51.3
Platycodom glavcum	37.6	46.9
Muscadomestica	50.0	29.4
Scrophvlaria buergeriana Miguel	57.1	19.4
Aloe arborescens	49.1	30.0
Copper	12.7	82.0
Lovastatin	8.8	87.5
Control	70.8	0

*100ppm.

inhibiton of water extracts of copper was 82.0% and water-soluble garlic extract also had the degree of inhibition of 51.3%. Methanol extracts of platycodon and pine tree showed the degree of inhibition of 46.9% and 41.3%, respectively.

HMG-CoA reductase is responsible for the conversion of HMG-CoA to mevalonate and thus plays a key enzyme in cholesterol biosysthesis[14]. Therefore, many attempts have been conducted to reduce cholesterol levels by inhibiting the activity of HMG-CoA reductase. Alternatively, the activation of low density lipoprotein (LDL) receptor can suppress the enzyme and thus lower cholesterol levels in blood[6].

Qureshi *et al.*[15] reported that the activity of HMG-CoA reductase was decreased in pullets fed solvent extracts of garlic and consequently reduced plasma cholesterol levels. The activity of HMG-CoA reductase was decreased by 3% dietary garlic[16] and significantly reduced by supplementing 50 μg/ml of garlic extracts. The results of these experiments are in agreement with those of our experiments, in ways which water-soluble garlic extracts lowered the activity of HMG-CoA reductase (Table 3).

Anomalously, the activity of HMG-CoA reductase was increased, however cholesterol levels in blood were reduced by increasing activity of cholesterol-7α-hydroxylase.

It is reported that lovastatin has the pharmacological characteristics in reducing cholesterol levels by inhibiting the activtiy of HMG-CoA reductase and stimulating receptor-mediated uptake and degradation of LDL cholesterol in the liver[6]. The results of the present study show that lovastatin inhibits the activity of HMG-CoA reductase *in vitro* (Table 3).

Copper also depressed the activity of HMG-CoA reductase *in vitro* (Table 3), however no inbibition was reported in hens fed copper[16]. Klevay *et al.*[5] reported that dietary copper deficiency caused hypercholesterolemia and increased hepatic HMG-CoA reductase activity and hepatic glutathione in rats.

Garlic, lovastatin and copper, which showed significant inhibition of HMG-CoA reductase *in vitro* were added to the pullets' diet in order to determine their effect on cholesterol levels in pullets.

Supplementation of 3% garlic powder, 300mg lovastatin/Kg or 200mg copper/Kg did not affect feed consumption (Table 4). Plasma total cholesterol and LDL cholesterol were significantly decreased in pullets fed garlic powder ($P<0.05$). Plasma triglyceride concentrations were shown to be lowered by 3% garlic and 300㎎ lovastatin/Kg. Dietary copper did not influence on levels of cholesterol and triglyceride in blood, and none of treatments affected cholesterol and triglyceride concentrations in livers and breast tissues in pullets (Table 5, Table 6).

Table 4. Influence of dietary garlic, lovastatin and copper on feed intake, and total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride in blood

	Treatment			
	Control	3% garlic	300mg lovastatin/Kg	200mg Cu/Kg
Feed intake (g/hen/day)	62.7± 3.3	67.2± 7.5	70.3±11.7	64.5± 7.2
Total cholesterol (mg/100ml)	119.4±19.2^{b}	99.7±19.6^{c}	114.4±11.7^{b}	138.4±15.4^{a}
HDL-cholesterol (mg/100ml)	87.7±16.7^{a}	62.6±21.9^{b}	94.5±10.2^{a}	99.0±11.3^{a}
LDL-cholesterol (mg/100ml)	55.1±10.5^{a}	30.1±15.2^{b}	44.5±15.2^{ab}	51.1±11.3^{a}
Triglyceride (mg/100ml)	29.7± 4.7	27.0± 8.0	25.4± 5.8	35.0±11.9

^{a,b}Means having same superscripts do not significantly differ ($P<0.05$).
Values are mean±SD.

Table 5. Influence of dietary garlic, lovastatin and copper on total cholesterol, HDL-cholesterol and triglyceride in liver

	Treatment			
	Control	3% garlic	300mg lovastatin/Kg	200mg Cu/Kg
Total cholesterol (mg/100g)	50± 5.4	56± 6.2	52± 5.9	52± 2.1
HDL cholesterol (mg/100g)	33± 7.8	39±10.4	39± 7.3	35± 5.4
Triglyceride (mg/100g)	46±19.4	39± 0.5	47±15.3	58±16.3

Values are mean±SD.

Table 6. Influence of dietary garlic, lovastatin and copper on total cholesterol, HDL cholesterol, and triglyceride in breast muscle

	Treatment			
	Control	3% garlic	300mg lovastatin/Kg	200mg Cu/Kg
Total cholesterol mg/100g	9.8±0.5	9.3± 1.5	11.5±1.4	11.3± 1.0
HDL cholesterol mg/100g	10.5±0.6	9.5± 1.0	11.8±3.1	11.0± 1.2
Triglyceride mg/100g	22.0±6.5	36.0±19.2	24.3±6.9	26.0±10.6

Values are mean±SD.

Konjufca *et al.*[16]reported that garlic affected lipid and cholesterol metabolism without any side effects and cholesterol levels in broilers fed 3% garlic were significantly reduced, which are in agreement with our results. Garlic lowered serum cholesterol by 20~25%, LDL cholesterol by 28~41%, and triglyceride by 10~26% in White Leghon pullets[15]. Reduced activities of HMG-CoA reductase and fatty acid synthetase were also reported in birds and rats fed garlic[15,16]. However, Kang *et al.*[17]reported that cholesterol and triglyceride in plasma and liver were decreased only in the rats fed cholesterol-rich diets containing garlic while no changes in rats fed normal diet containing garlic were found.

Although there were no significant differences in plasma, cholesterol and triglyceride were tended to reduce in groups fed lovastatin compared to the control group (Table 4). HDL cholesterol was numerically increased in groups fed lovastatin and copper without having statistical significances among treatments. Supplementing lovastatin with laying hen's diet decreased plasma cholesterol and egg yolk cholesterol[18], however the doses may need to be greater than those found to be effective for humans[19]. Luhman *et al.*[19] found that low amounts of lovastatin did not reduce cholesterol in plasma, liver, and muscle because laying hens must synthesize much more cholesterol per kilogram of metabolic body weight for

deposition in the egg yolk when consuming a diet containing little cholesterol.

The copper requirement for the hen is not known, however it has been known that copper deficiency in diet may cause hypercholesterolemia in poultry[20]. Feeding 200mg/Kg copper to pullets did not reduce total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride in blood (Table 4). Konjufca *et al.*[16]reported that supplementation of 180mg copper/Kg as cupric sulphate pentahydrate could reduce the levels of plasma and breast muscle cholesterol in young broiler chickens. Pesti *et al.*[20]observed that plasma and egg yolk cholesterol concentrations were decreased by feeding 125mg copper/Kg to laying hens.

Plasma and liver cholesterol belong to the "fast turnover cholesterol pool" while the changes of cholesterol in muscle and egg yolk are not sensitive to dietary treatment so they may be affected by longer feeding period[16].

The results of our experiments demonstrated that under normal dietary conditions, pullet may be capable of synthesizing cholesterol in excess of its needs for preparing yolk deposition.

요 약

체내 cholesterol 수치를 낮추기 위하여 HMG-CoA reductase 활성을 저해하는 물질들을 닭에게 급여하여 in vitro상에서 검토한 결과, HMG-CoA reductase 활성에 대한 마늘, lovastatin과 copper의 순으로 저해능은 51.3%, 87.5%, 82.0%이었다. 또한 혈장, 가슴조직, 간장의 cholesterol 함량에 대한 마늘(3% in diet), lovastatin(300mg/Kg of diet), copper(200mg/Kg of diet)의 효과를 검토하였다. 혈장 중의 cholesterol 함량과 HDL, LDL cholesterol 함량은 3% 마늘의 투여로 감소되어졌으나 copper의 투여는 오히려 증가하였고, lovastatin 투여에 의한 차이는 나타나지 않았다. 그리고 간장과 가슴조직의 cholesterol과 triglyceride는 사료에 cholestrol의 수치를 저하시키는 물질을 첨가하였으나 별다른 차이가 없었다.

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