

Effects of monacolin K-containing nutraceutical on cholesterol homeostasis re-establishment and CVD risk reduction in hypercholesterolemic subjects

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Abstract. – **OBJECTIVE:** Hypercholesterolemia is caused by cholesterol homeostasis (CH) disruption, and it contributes to cardiovascular diseases pathogenesis and progression. Status of CH can be assessed by measuring serum concentrations of non-cholesterol sterols (NCS) which serve as cholesterol synthesis and absorption surrogate markers. Monacolin K, isolated from red yeast rice, influences cholesterol synthesis by inhibiting HMG-CoA reductase activity and reduces serum total cholesterol (TC) concentration.

PATIENTS AND METHODS: This longitudinal study included 30 hypercholesterolemic patients, with systematic coronary risk estimation (SCORE) values <10%, who received 3-months-long supplementation with nutraceutical mixture containing monacolin K, and vitamins C, B1 and K2. Serum NCS were quantified by HPLC-MS/MS method. Atherogenic indexes were calculated from lipid status parameters concentrations. Albumin degradation inhibition test was conducted to estimate *in vitro* anti-inflammatory activity of the nutraceutical mixture, whereas *in vitro* antioxidant activity was measured in serum enriched with prooxidants and antioxidants.

RESULTS: TC, LDL-cholesterol (LDL-C), and triglycerides (TG) concentrations ($p<0.001$), as well as atherogenic indexes and SCORE values ($p<0.001$, $p<0.01$, respectively) were lowered following the supplementation. Concentrations of cholesterol synthesis markers were decreased ($p<0.001$), whereas levels of cholesterol absorption markers remained unchanged after the supplementation. Reduction in cholesterol synthesis went alongside reductions in lipid status parameters and atherogenic indexes. *In vitro* analyses showed certain anti-inflammatory and antioxidant activity of the nutraceutical.

CONCLUSIONS: These results suggest that supplementation with monacolin K containing nutraceutical favorably influences lipid status parameters and atherogenic indexes by acting on cholesterol synthesis. Anti-inflammatory and antioxidant effects of this unique nutraceutical mixture may exhibit beneficial pleiotropic effects.

Key Words:

Red rice yeast, Monacolin K, Cholesterol synthesis, Cholesterol absorption, Atherogenic index, Pleiotropic effects.

Introduction

Cardiovascular diseases (CVD) represent the leading cause of morbidity, comorbidity and mortality worldwide¹. Underlying origin of the majority of CVD is atherosclerosis, which is directly related to the lipid status parameters (LSP) disturbance¹. Various novel atherogenic indexes, such as lipoprotein combine index (LCI) and atherogenic index of plasma (API), are used for the assessment of dyslipidemia². Current guidelines for CVD prevention and treatment are based on the systematic coronary risk estimation (SCORE) scoring system, which can assess the 10-year risk of fatal CVD³. SCORE value is estimated according to age, gender, systolic blood pressure, smoking status and total cholesterol concentration (TC). Blood TC concentration mainly depends on cholesterol synthesis and absorption capacity. Balance between these processes is responsible

for maintaining of cholesterol homeostasis (CH) and its disturbance is seen in various diseases and during pharmacological treatment⁴⁻⁶. Quantification of cholesterol synthesis and absorption markers (non-cholesterol sterols – NCS) allow direct monitoring of CH and its re-establishment after the therapy^{1,7}. Serum concentrations of desmosterol (Bloch pathway precursor) and lathosterol (Kandutsch-Russell pathway precursor) are used for estimation of cholesterol synthesis level. Moreover, phytosterols (campesterol and β -sitosterol) serve as surrogate markers of cholesterol absorption^{5,7,8}.

Nowadays, statins are widely used in dyslipidemia treatment and CVD prevention^{4,5}. Their pleiotropic effects, anti-inflammatory and antioxidant, are well-established⁹. However, almost 10% of patients do not exhibit an adequate response and nearly 20% discontinue the therapy due to well-known side effects (myopathy, liver injury, new-onset diabetes)¹⁰. Monacolin K is statin-like nutraceutical compound, isolated from red yeast rice (RYR)¹¹⁻¹³. According to the latest ESC/EAS guideline for dyslipidemias management and treatment, monacolin K use may be considered in patients with elevated TC levels who do not qualify for statin treatment based on estimated global CVD risk³. Although studies have shown comparable effects of monacolin K and statins on LSP^{12,13}, lower concentration of monacolin K and other active components in nutraceuticals reduces the likelihood of adverse events related to a single component and may have beneficial effects in statin intolerant patients^{11,13}.

The aim of this study was to investigate potential effects of nutraceutical containing monacolin K 10 mg, vitamin C 40 mg, vitamin K2 45 μ g and vitamin B1 0.18 mg (ARTEROPROTECT[®] FORTE – APF, AbelaPharm, Belgrade, Serbia) on LSP, atherogenic indexes and markers of CH after 3-months-long use. Additionally, we were interested in exploring the associations between these parameters. Also, the aim of the study was to investigate possible pleiotropic effects of this nutraceutical, by measuring its antioxidative and anti-inflammatory *in vitro* activity.

Patients and Methods

Study Design and Patients

In this study we enrolled 30 statin- and ezetimibe-untreated patients with confirmed dyslipidemia [3]. Patients were 57.0 (51.0-65.0) years old,

20% of subjects were male, 47% were smokers, and 73% were treated by antihypertensive therapy. Median body mass index was 25.8 (23.2-28.0) kg/m² and waist-to-hip ratio was 0.84 (0.79-0.90). Median systolic and diastolic blood pressures were 120 (110-130) and 77 (70-81), respectively. All patients had SCORE values below 10% and the physician implemented diet, physical activity and nutraceutical supplementation for lowering cholesterol concentration³. All patients consumed one tablet of APF daily for 3 months. The study participants were not treated with products enriched with plant sterols and/or stanols. None of the participants was vegetarian or vegan.

This study was designed as a longitudinal study in accordance with the principles laid down in Declaration of Helsinki and was approved by Ethical Committee of the Health Center Vozdovac, Belgrade (11.7.2018.; No 3515). All participants have signed informed consent before enrolment.

Laboratory Analyses

EDTA plasma and serum were collected after 12-h fasting period and separated by centrifugation at 1500 \times g for 10 min at 4°C. Concentrations of TC, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and high-sensitivity C reactive protein (hs-CRP) were measured by routine methods on an OLYMPUS AU400 analyzer (Olympus, Tokyo, Japan) using reagents from BioSystems (Barcelona, Spain). Low-density lipoprotein cholesterol (LDL-C) levels were calculated with the Friedewald equation. LCI was calculated by TC, TG, LDL/HDL-C formula and AIP by log (TG/HDL-C)². SCORE values were estimated using a chart for European populations at high cardiovascular disease risk³.

Cholesterol Synthesis and Absorption Markers Determination

Analytical HPLC grade standards were used for quantification of the NCS (Sigma-Aldrich, St. Louis, MO, USA). Deuterated internal standard cholesterol-26,26,26,27,27,27-d₆,25(OH)D₃-d₆ was obtained from Medical Isotopes (Pelham, NH, USA). KOH was purchased from POCH (Center Valley, PA, USA), while ethanol, methanol, n-hexane and acetonitrile (HPLC grade) were obtained from Fisher (Pittsburgh, PA, USA). NCS were quantified by previously reported LC-MS/MS method⁸. Cholesterol synthesis level (CSL) represents the sum of desmosterol and lathosterol concentration, whereas cholesterol absorption level (CAL) represents the sum of campesterol

and β -sitosterol concentration. To assess overall CH, the ratios between cholesterol synthesis and absorption markers were calculated.

In Vitro Test of Anti-Inflammatory and Antioxidant Activity

In vitro anti-inflammatory effects were investigated by inhibition of albumin denaturation technique¹⁴. Albumin and acetyl-salicylic acid used for this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA). Briefly, the reaction mixture containing APF (100 μ mol/L) and 1% albumin solution was incubated at 37°C for 20 min and then heated to 51°C for 20 min. Acetyl-salicylic acid (100 μ mol/L) mixed with albumin solution (1%) was used as a control and it was also incubated under the same conditions. After cooling the turbidity was measured by UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at 660nm. The experiment was done in triplicate. The percentage of inhibition of APF sample was calculated as follows: $((Abs_{Control} - Abs_{Sample}) / Abs_{Control}) \times 100$.

The *in vitro* antioxidant activity measurement was performed according to the already published method¹⁴. A Trolox (Sigma-Aldrich, St. Louis, MO, USA) solution (2 mmol/L), was used as a control. The test substances were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and following solutions were prepared: APF (APFS), APF and terc-butyl-hydroperoxide-TBH (APF+TBH), TBH (TBHS), and vitamin E solution (VES). 50 μ L of each of these solutions were added to 450 μ L of serum. Blank serum solution was prepared by mixing 50 μ L DMSO and 450 μ L of serum. Antioxidant activity was tested by measuring total antioxidative status (TAS), total oxidative status (TOS) and prooxidative-antioxidant balance (PAB) in triplicate, after 2h incubation on 37°C. All methods were optimized by Kotur-Stevuljevic et al¹⁵.

Statistical Analysis

All data were analyzed using IBM® SPSS® Statistics version 22 software (IBM, Armonk, NY, USA). Data distributions were tested using the Shapiro-Wilk test. Categorical variables were presented as relative frequencies. All continuous variables were asymmetrically distributed and expressed as median (interquartile range – IQR). The Wilcoxon signed-rank test was used to compare two related samples. Spearman's correlation analysis was used to assess associations between the percentage changes of all tested parameters

before and after supplementation. A $p < 0.05$ was considered statistically significant. ANOVA was used for comparison in *in vitro* experiments.

Results

The study included patients whose SCORE values were below 10%. Although there was no change in SCORE median and IQR before and after supplementation [1 (1-5) %], SCORE was decreased in subjects with previously highest values (Figure 1). This decrease in SCORE values caused a statistically significant difference before and after treatment ($p < 0.01$).

Concentrations of LSP, hsCRP, and calculated atherogenic indexes values before and after the supplementation are presented in Table I. After APF supplementation significant changes in LSP were observed. Concentrations of TC, LDL-C, and TG were significantly reduced. During the study period, LDL-C decreased by 28.6% (17.9-34.8%) suggesting beneficial APF supplementation effects. In contrast, HDL-C concentration did not significantly change after the supplementation. Also, significant changes in atherogenic indexes values have been demonstrated. A trend towards lower hsCRP values was observed after the supplementation.

Table II shows the changes in NCS concentrations after APF supplementation. Expectedly, desmosterol and lathosterol concentration, and CSL values were significantly decreased. β -sitosterol and campesterol concentrations, and CAL showed trend towards higher values after supplementation. A significant reduction in every CH ratio except desmosterol/ β -sitosterol was observed.

Further on, the association between all tested parameters were analyzed. Following supplementation, desmosterol and lathosterol concentration percentage changes correlated positively with concentration changes of TC ($p = 0.487$, $p = 0.010$; $p = 0.670$, $p = 0.001$, respectively), LDL-C ($p = 0.520$, $p = 0.005$; $p = 0.538$, $p = 0.004$, respectively), TG ($p = 0.525$, $p = 0.005$; $p = 0.549$, $p = 0.003$, respectively), non-HDL-C ($p = 0.553$, $p = 0.003$; $p = 0.660$, $p = 0.001$, respectively). Also, desmosterol and lathosterol concentration changes positively correlated with percentage changes of TC/HDL-C ($p = 0.446$, $p = 0.020$; $p = 0.563$, $p = 0.002$, respectively), LDL-C/HDL-C ($p = 0.454$, $p = 0.017$; $p = 0.432$, $p = 0.025$, respectively), non-HDL-C/HDL-C ($p = 0.410$, $p = 0.031$; $p = 0.489$, $p = 0.010$, re-

Table 1. Concentration of basic lipid status parameters, calculated atherogenic indexes values and hsCRP concentrations before and after treatment.

Parameter	Before supplementation	After supplementation	<i>p</i>
TG, mmol/L	1.72 (1.19-2.03)	1.23 (0.87-1.67)	<0.001
TC, mmol/L	7.24 (6.90-7.74)	5.65 (4.87-6.51)	<0.001
HDL-C, mmol/L	1.62 (1.39-1.85)	1.52 (1.30-1.85)	0.274
LDL-C, mmol/L	4.87 (4.23-5.27)	3.38 (2.97-4.01)	<0.001
non-HDL-C, mmol/L	5.72 (5.21-6.02)	3.90 (3.43-4.84)	<0.001
TC/HDL-C	4.60 (3.77-5.17)	3.58 (3.12-4.09)	<0.001
LDL-C/HDL-C	3.02 (2.35-3.49)	2.18 (1.88-2.69)	<0.001
nonHDL-C/HDL-C	3.60 (2.77-4.17)	2.58 (2.12-3.09)	<0.001
LCI	34.3 (22.1-54.3)	13.85 (8.75-24.97)	<0.001
AIP	0.029 (-0.158-0.139)	-0.133 (-0.317-0.038)	<0.001
hsCRP, mg/L	1.20 (0.90-1.80)	1.15 (0.70-1.67)	0.068

TG-triglyceride; TC-total cholesterol; LDL-C-low-density lipoprotein cholesterol; HDL-C-high-density lipoprotein cholesterol; LCI-lipoprotein combine index; AIP-atherogenic index of plasma; hs-CRP, high sensitivity C-reactive protein. Data are expressed as median values (25% - 75%). Continuous variables were compared using Wilcoxon signed-rank test. Bold values indicate statistical significance.

spectively), and LCI ($p=0.527$, $p=0.002$; $p=0.327$, $p=0.001$, respectively). Changes in HDL-C concentration and AIP did not show any significant correlations with NCS changes. The cholesterol absorption markers changes did not show any correlation with changes in lipid parameters before and after supplementation.

The results of the *in vitro* analysis showed that when APFS was added to serum or serum + TBH, the concentrations of prooxidant markers (TOS and PAB) remained lower compared to TBHS. At the same time, TAS level was higher in the presence of APF, and it nearly corresponded to levels observed in the sample +VES mixture. Additionally, TAS/TOS ratio showed that APF had an equal ability to prevent oxidative damage, compared to VES (Figure 2). Finally, APF exhibited 18% of the anti-inflammatory activity observed with acetylsalicylic acid (100%).

Discussion

According to the SCORE system, subjects with low to moderate risk level (calculated SCORE <5%) are considered as candidates for diet and life-style changes, whereas in subjects with high-risk level (SCORE $\geq 5\%$ and <10%) pharmacological therapy administration should be considered, in addition to dietary and life-style changes³. Our patients had borderline dyslipidemia, and were treated for 3-months by diet, physical activity regime, and APF³. Our study was conducted according to the ESC/EAS guidelines, which rec-

ommended the use of 5-10 mg/day of monacolin K³. Also, this is in concordance with European Food Safety Authority (EFSA) regulations¹⁶. We demonstrated favorable changes in cholesterol metabolism after supplementation.

In a study conducted by Lu et al¹⁷, patients with a documented previous myocardial infarction (MI) received RYR extract over a period of 4.5 years and experienced a significant improvement in the LSP (reduction of TC, LDL-C and TG concentrations, and increase in HDL-C levels), and

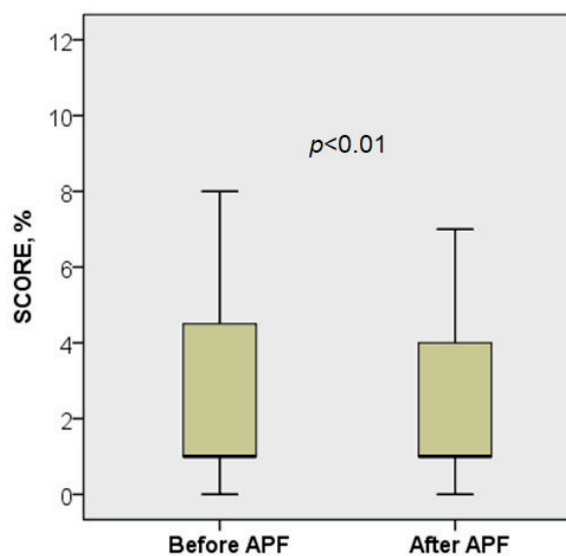


Figure 1. SCORE values before and after APF supplementation. SCORE – Systematic coronary risk estimation; APF – ARTEROprotect® FORTE.

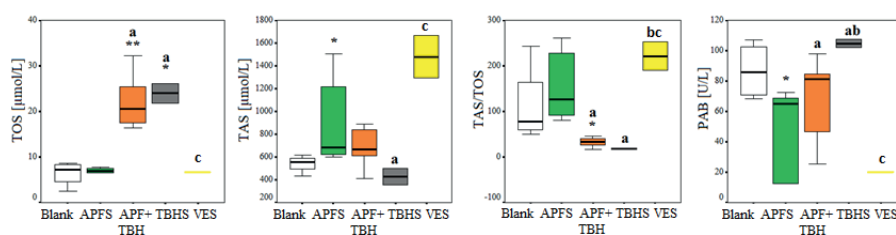


Figure 2. Examination of *in vitro* antioxidant activity of APF. APF – ARTEROprotect® FORTE; TOS-total oxidative status; TAS-total antioxidant status; PAB-protoxidative-antioxidant balance; APFS-ARTEROprotect® FORTE solution in serum; TBH-terc-butylhydroperoxide; VES-vitamin E analogue Trolox solution in serum; (*,**- $p < 0.05, 0.01$ resp. vs. Blank (pure serum pool), a- $p < 0.05$ vs. APFS, b- $p < 0.05$ vs. APF+TBHS, c- $p < 0.05$ vs. TBHS).

reduction of recurrent cardiovascular events and CVD mortality. The results of our study indicated that after 3-months-long APF supplementation there was a significant decrease in TC, LDL-C, and TG concentrations (Table I). We have demonstrated that there were no significant changes in HDL-C concentrations after supplementation. These findings are in agreement with the study of Mazza et al¹¹ in patients with metabolic syndrome. In these patients, 2-months-long administration of nutraceutical (10 mg of monacolin K and 30 mg of coenzyme Q10) resulted in reduction of TC, LDL-C and TG concentrations, without significant changes of HDL-C concentration. They credited the absence of HDL-C concentration changes to higher HDL-C values at the treatment beginning, the intrinsic characteristics of study participants and duration of treatment¹¹. Similar explanation could be proposed for our results, because average HDL-C concentration was higher than 1.5 mmol/L and these values persisted after the supplementation period (Table I).

A large number of comprehensive lipid indexes calculated from LSP concentrations, have shown

high sensitivity in CVD prediction^{3,18}. Cai et al² have validated the predictive sensitivity of AIP and LCI and proved their superiority in relation to LSP as CVD predictors. The results of our study have shown that, after supplementation with monacolin K containing product, the values of all lipid indexes were significantly reduced (Table I).

As monacolin K produces a direct effect on cholesterol synthesis by HMG-CoA reductase inhibition¹¹⁻¹³, examination of overall CH was necessary. Matthan et al⁴ showed that 6-months statin therapy treatment significantly reduced cholesterol synthesis and increased cholesterol absorption marker concentrations. This finding suggests that statins are able to re-establish CH. Additionally, another study showed a trend towards higher values of cholesterol absorption markers in CVD patients on statin therapy compared to patients without therapy⁵. Ruscica et al²⁰ reported a reduction in cholesterol synthesis after a 12-week supplementation by the nutraceutical combination (monacolin K, niacin, coenzyme Q10 and probiotic culture), while cholesterol absorption did not change. Expectedly, we obtained a lower concen-

Table II. Concentration of cholesterol synthesis and absorption markers before and after treatment.

Parameter	Before supplementation	After supplementation	<i>p</i>
Desmosterol, µmol/L	3.02 (2.49-3.96)	2.23 (1.38-2.70)	<0.001
Lathosterol, µmol/L	3.95 (3.15-4.72)	2.78 (2.43-3.50)	<0.001
CSL, µmol/L	7.02 (5.63-8.03)	4.70 (3.88-6.27)	<0.001
Campesterol, µmol/L	8.33 (6.70-11.91)	9.36 (6.52-12.00)	0.414
β-sithosterol, µmol/L	0.61 (0.35-1.10)	0.82 (0.23-1.04)	0.517
CAL, µmol/L	9.21 (6.94-12.92)	10.16 (6.63-13.03)	0.501
Desmosterol/campesterol	0.315 (0.230-0.524)	0.243 (0.118-0.373)	<0.01
Desmosterol/β-sitosterol	5.30 (2.75-10.52)	2.90 (1.50-8.89)	0.112
Lathosterol/campesterol	0.471 (0.247-0.624)	0.315 (0.223-0.417)	<0.01
Lathosterol/β-sitosterol	6.12 (2.58-13.00)	4.10 (2.45-12.63)	<0.05
CSL/CAL	0.775 (0.421-1.100)	0.518 (0.307-0.710)	<0.01

CLS-cholesterol synthesis level; CAL-cholesterol absorption level. Data are expressed as median values (25% - 75%). Bold values indicate statistical significance.

tration of cholesterol synthesis markers and a trend towards higher values of absorption markers after supplementation. Additionally, we observed a significant decrease of cholesterol synthesis to absorption ratios following APF supplementation. Analogically to statin therapy, APF supplementation may also contribute to CH re-establishment, which is in concordance with the abovementioned ESC/EAS guidelines³. Strandberg et al²¹ showed the association of elevated values of desmosterol/ β -sitosterol ratio with an increased incidence of CVD mortality. In our study trend towards lower values of this ratio was observed after supplementation.

Furthermore, we observed positive associations between changes in cholesterol synthesis markers and changes in TC, LDL-C, TG levels and atherogenic indexes values. This is in concordance with the results of Framingham Offspring study of statin therapy effects on LSP⁶. Huang et al²² observed that monacolin K induces the expression of adipose triglyceride lipase and reduces the amount of fatty acid synthase and sterol regulatory element-binding protein 1 (SREBP1) in HepG2 cell line. Decrease in TG concentrations, as well as cholesterol synthesis rate reduction can be explained by the action of monacolin K on key genes expression in SIRT1/AMPK metabolic pathways and promotion of lipid catabolism²².

Finally, in order to examine the pleiotropic effects of APF, we conducted an *in vitro* analysis. Cicero et al²³ showed that monacolin K, in combination with a mixture of antioxidants, has a beneficial effect on endothelial function, matrix metalloproteinase 2 and 9 activities, and hsCRP concentrations. A significant decrease in hsCRP (independent inflammatory predictive marker of MI, stroke and peripheral artery disease) was recorded after 4 weeks of treatment²³. In our study, the trend towards lower values of hsCRP after supplementation with the APF was observed (Table I). Our *in vitro* analysis has demonstrated that APF exhibits a mild anti-inflammatory activity (18% of that achieved by the control drug, i.e., acetylsalicylic acid) (Figure 2). There is insufficient knowledge about the pleiotropic effects of monacolin K. Since oxidation of LDL particles represents the central event in the progression of atherosclerotic plaque⁹, the antioxidant ability of antihyperlipidemic drugs is one of their most significant pleiotropic effects. Various statins and their metabolites have shown to exhibit a significant antioxidant role^{24,25}. The results of our *in vitro* experiment showed that APF, by increasing TAS, TAS/TOS

ratio, and decreasing TOS and PAB levels, exhibits a promising antioxidant capacity (Figure 2). APF, in addition to monacolin K, contains a unique mixture of vitamins and antioxidants, so the beneficial antioxidant and anti-inflammatory effect of this composition can be attributed to the synergistic action of its compounds. Although the results of our study showed positive effects of this unique nutriment mixture on lipid status, the study should be conducted on a larger number of subjects and over a longer period of supplementation, in order to confirm these results. To the best of our knowledge, this was the first study which examined the effects of 3-month-long supplementation with such unique nutraceutical combination on inflammation, oxidative stress and CVD risk reduction. Additionally, for the first time monacolin K effect on desmosterol concentration and consequently Bloch cholesterol synthesis pathway was presented herein.

Conclusions

In conclusion, our study has shown that 3-month-long supplementation with the nutraceutical containing monacolin K, vitamin C, vitamin K2 and vitamin B1 leads to reduction in cholesterol synthesis, as well as beneficial effects on TC, LDL-C and TG concentrations. Although we have demonstrated *in vitro* anti-inflammatory and antioxidant activity of APF nutraceutical, it is also necessary to examine its pleiotropic effects *in vivo*.

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