THE EFFECT OF EFFORT TEST ON THE LEVELS OF ISCHEMIA MODIFIED ALBUMIN, 7-KETOCHOLESTEROL AND CHOLESTAN-3β, 5α, 6β-TRIOL AND THEIR ROLE IN THE DIAGNOSIS OF CORONARY ARTERY DISEASE

Efekat testa napora na nivele ischemije modifikovanog albumina, 7-ketoholesterola i holestan-3β, 5α, 6β-triola i njihova uloga u dijagnozi koronarnog arterijskog oboljenja

Muhammed Emin Keles¹, Afshin Samadi¹, Selen Yılmaz Isikhan³, Yusuf Ziya Sener², Ali Sezgin², Emine Keles⁴, Incilay Lay¹, Uğur Canpolat²

¹Hacettepe University, Faculty of Medicine, Department of Medical Biochemistry, Ankara/Turkey, ²Hacettepe University, Faculty of Medicine, Department of Cardiology, Ankara/Turkey, ³Hacettepe University, Faculty of Medicine, Department of Biostatistics, Ankara/Turkey, ⁴Hacettepe University, Faculty of Medicine, Department of Chest Diseases, Ankara/Turkey

Summary

Background: Oxysterols have been shown to play a role in plaque formation while ischemia modified albumin (IMA) is widely accepted as an acute marker for ischemia. The effort test is one of the methods used to identify the presence of coronary artery disease. Thus, there may be a relationship between effort test result and the levels of IMA, 7-ketocholesterol (7-KC) and cholestane-3β,5α,6β-triol (C-triol).

Methods: Thirty patients who underwent effort test and 30 healthy subjects were included in the study. IMA levels were determined with the albumin-cobalt binding test, 7-KC and C-triol levels were determined with LC-MS/MS. Among the patients, two subgroups were identified according to the results of the effort test, group 1 consisted of patients with a positive effort test (n = 12), and group 2 consisted of patients who had a negative effort test (n = 18).

Results: 7-KC levels of patients were significantly higher compared to healthy subjects (39.87 ± 2.13 ng/mL, 20.26 ± 1.35 ng/mL; p = 0.001). In patients, post-test 7-KC levels were significantly lower than pre-test levels (post-test vs. pre-test: 37.73 ± 2.44 ng/mL vs. 41.07 ± 2.18 ng/mL; p < 0.001). There was a significant difference in

Kratak sadržaj

Uvod: Pokazano je da oksisteroli imaju ulogu u formiranju plaka, dok je ishemija modifikovani albumin (IMA) široko prihvaćen kao akutni marker ischemije. Test napora je jedna od metoda koja se koristi za identifikaciju prisustva koronarnog arterijskog oboljenja. To znači, da mora postojati odnos između rezultata testa napora i nivoa IMA, 7-ketoholesterola (7-KC) i holestan-3β,5α,6β-triola (C-triol).

Metode: Trideset pacijenata podvrgnuto je testu napora i 30 zdravih osoba je bilo uključeno u ovo istraživanje. IMA nivoi su određivani primenom albumin-kobalt vezujućim testom, nivoi 7-KC i C-triola su određivani primenom LC-MS/MS. Među pacijentima, identifikovane su dve podgrupe, prema rezultatima test napora. U grupi 1 bili su pacijenti sa pozitivnim testom napora (n = 12), a u grupi 2 su bili pacijenti sa negativnim testom napora (N = 18).

Rezultati: Nivoi 7-KC bili su značajno viši kod pacijenata u poređenju sa zdravim osobama (39,87 ± 2,13 ng/mL, 20,26 ± 1,35 ng/mL; p = 0,001). U pacijentima, post-test nivoi 7-KC su bili značajno niži nego pre-test nivoi (post-test vs. pre-test: 37,73 ± 2,44 ng/mL vs. 41,07 ± 2,18 ng/mL; p <0,001). Postojeći je značajna razlika post-test nivoa 7-KC
post-test 7-KC levels among all study groups (negative, positive, and healthy: 37.73 ± 2.44 ng/mL, 39.87 ± 2.13 ng/mL, and 20.26 ± 1.35 ng/mL, respectively). There was no significant difference in IMA levels.

Conclusions: Patients with positive effort test had significantly higher levels of 7-KC. Additionally, after the effort test, the 7-KC value was reduced. 7-KC is a biomarker of oxidative damage and its value or changes before and after the effort test may be used as a biomarker in the diagnosis and follow-up of coronary artery disease.

Keywords: effort test, oxysterols, ischemia modified albumin, coronary artery disease

Introduction

Coronary artery disease (CAD) is the most common cause of death worldwide and it manifests with chest pain (1). Chest pain has many other causes besides CAD. In order to identify if chest pain is due to CAD, tests such as exercise stress test, CT angiography, myocardial perfusion scintigraphy and direct conventional angiography are utilized (2, 3). Exercise stress test (effort test) is a noninvasive test, this provides a great advantage, but it is necessary to increase the accuracy of the test because the specificity of the test is low and its evaluation is subjective (4, 5). Exercise stress test (effort test) is a noninvasive test, this provides a great advantage; however, it is necessary to increase the accuracy of the test because the specificity of the test is low and its evaluation is subjective (4, 5).

Oxysterols have been implicated in the formation and progression of atherosclerotic plaques (6, 7). 7-ketocholesterol (7-KC), 7ß-hydroxycholesterol (7ß-OHC), beta-isomers of epoxide, 27-hydroxycholesterol (27-OHC) and cholestane-3ß,5α,6ß-triol (C-triol) have been shown to increase in plasma and/or atherosclerotic plaque in various studies (8–12). 7-KC occurs via the reaction of peroxyl and alkoxyl radicals and the Russell mechanism, and also can be converted from 7ß-OHC by the enzyme 11β-hydroxysteroid dehydrogenase (13, 14). Epoxy-cholesterol, which is formed by peroxyl radicals via the reaction of lipid hydroperoxides with cholesterol, are transformed to C-triol. Thus, the measurement of 7-KC together with C-triol should be sufficient in showing oxidative stress and could be assumed to be the best biomarkers among oxysterols (15, 16).

Ischemia modified albumin, as the name suggests, is albumin, which has a modified N-terminal due to the effects of ischemia (17). The last amino terminal of the albumin structure is the region to which transition metals such as cobalt, copper and nickel are bound (18). Hypoxia, acidosis, free radical damage and membrane breakdown in the case of ischemia reduces the binding of these transition metals to the N-terminal of albumin. The resulting albumin is called ischemia-modified albumin (IMA), which can be measured with the albumin-cobalt binding test (19). Although the production of IMA is not specific to myocardial injury, IMA concentration is accepted to be an early marker for myocardial ischemia and is used to assess patients with acute coronary syndrome (20). To our knowledge, there are no studies which investigated the levels of oxysterols and IMA in patients who underwent effort test. In this study, we aimed to determine and investigate the pre-test and post-test levels of 7-KC, C-triol and IMA in patients who underwent exercise stress test, to determine their relationship with ECG findings during the test (positive/negative), and to compare results with healthy controls.

Materials and Methods

Patients who were admitted to the cardiology clinic with chest pain and underwent elective exercise stress tests at our center were included in the study. The inclusion criteria were: being over the age of 18, accepting to participate in the study and providing informed consent, and having no chronic disease including diabetes, thyroid dysfunctions and hypoalbuminemia. Exclusion criteria were: having a chronic disease, undergoing stress test due to any other reason than the suspicion of CAD due to newly emerging chest pain, and having an effort test result which was inconclusive. Healthy volunteers adjusted for age and sex were chosen as controls. A total of 30 patients were included of which 12 had positive effort test, and 18 had negative effort test. IMA levels were determined with the albumin-cobalt binding test, 7-KC and C-triol levels were determined with LC-MS/MS. Measurements of these parameters were done twice in patients (termed as pre-test and post-test values). The number of healthy controls was also 30. Blood was drawn immediately before and half hour after the test to observe the effects of the effort test on the parameters to be measured. These data are grouped as ‘pre-test’ and ‘post-test’ values. All of the individuals in our study were selected from persons aged 18-65 years who had no comorbidities. The study protocol adhered to the Declaration of Helsinki Guidelines and was approved by the Ethics Committee of Hacettepe University. Informed consent was obtained from each study participant.

Serum IMA Level Measurement

Serum IMA levels were measured using the colorimetric method described by Bar-Or et al. (19).
this method, 200 µL of serum is added to 50 µL of cobalt chloride solution of 0.1% (w/v) and it is expected that the reaction of albumin cobalt binding will be sufficient by gentle mixing for 10 minutes. Then 50 µL of dithiothreitol (DTT) (1.5 mg/mL H2O) is added as the coloring agent. After a 2-minute incubation, 1.0 mL of 0.9% NaCl is added to terminate the reaction. The color change is then measured by spectrophotometry (Shimadzu UV-1600) at 470 nm. The measurement results are reported as absorbance unit (AbsU).

**LC-MS/MS Analysis**

Oxysterol analysis was performed by LC-MS/MS (Schimadzu Scientific Instruments, 8040) based on the method of Jiang et al. (21). Saponification of plasma samples was not required and only free and unesterified oxysterol species were measured. Plasma 7-KC and were derivatized into N,N-dimethylglycine esters. This step enhanced the ionization and fragmentation of 7-KC for mass detection of the oxysterol esters. This step enhanced the ionization and fragmentation of 7-KC for mass detection of the oxysterol species in the human plasma. 3β,5α,6β-trihydroxycholestanate D7 (Toronto) and 3β-hydroxy-5-cholestone-7-one D7 (Avanti) were used as internal standards. Eight point calibrators (3.12–400 ng/mL) were prepared for quantification. Plasma quality control samples were prepared by spiking known amounts of standards of 7-KC and to yield an endogenous level 40/40 and 150/150 ng/mL, respectively. The chromatographic separation was performed on a symmetry C18 column (100 mm×2.1 mm, 5 µm) (Thermo Fisher Scientific) using a linear gradient of water and acetonitrile (pH 3; 1 mmol/L ammonium formate). Mass spectrometry analysis was performed in the positive ionization mode using electrospray ionization (ESI). 7-KC and C-triol were determined in 50 µL of plasma. Sample preparation consisted of three phases: Phase one included protein precipitation, separation and drying; phase two was the derivatization phase and phase three was sample cleaning by LC.

All oxysterol studies regarding coronary artery disease were performed using GC–MS method. Although GC–MS is excellent in its selectivity, its sensitivity has not proven sufficient compared to liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) technology. Moreover, the analytical procedure for GC–MS includes the extraction of cholesterol oxides, which is a complicated and time consuming procedure; furthermore, artifactual oxidation may occur throughout the entire procedure. However, to date, there have been no randomized controlled studies assessing the levels of oxysterols in IMA patients by LC–MS/MS. This method, with its excellent sensitivity and specificity, has many advantages and is suitable for routine oxysterol analysis in laboratories.

**Statistical Analysis**

The descriptive statistics of numerical variables are summarized as means, standard deviations, minimum and maximum values and the demographic and clinical characteristics of the patients are expressed as frequencies and percentages. Normality of distribution was tested with the Shapiro-Wilk’s test. Welch’s analysis of variance (ANOVA) and Kruskal-Wallis tests was used to examine differences among groups in Plasma oxysterols 7-KC, C-Triol, and IMA. The mean parameter comparisons between the patient and control groups before the effort test was implemented with an independent-sample t-test. Error chart was utilized to show differences between groups.

The Repeated Measures Variance Analysis was used to evaluate the change in oxysterol measurements (7-KC and C-triol) according to time, patient groups (negative, positive, and healthy), and the interaction between time and patient groups. The results that met the parametric assumptions were taken into account in the analysis. Tukey and Games-Howell tests were used in pairwise comparisons for group difference, depending on the homogeneity of variances. All statistical analyses were performed using IBM SPSS Statistics version 20. The level of significance was accepted as p<0.05.

**Results**

Thirty patients and 30 healthy subjects (controls) were included in the study. In the patient group, two subgroups were identified according to the results of the effort test, group 1 consisted of patients with a positive effort test (n=12) and group 2 consisted of patients who had a negative effort test (n=18). The patients and controls were formed to be homogeneous in terms of age and gender. The distribution of age within groups were as follows: positive patient group range was 35–60 years, mean was 42.7±4.9 years; negative patient group range was 38–55 years, mean was 43.7±4.9 years; healthy control group range was 30–45 years, mean was 37.5±3.8 years (independent- samples t test, P=0.31). The gender distribution was (F/M ratio 6/6 in positive group, 10/8 in the negative group and 17/13 in the control groups; χ² test, P=0.407). There were no significant differences in terms of lipid status (total cholesterol, HDL, LDL and triglyceride), hypertension, BMI and other clinical parameters in patients and controls. Individuals did not receive any medication due to a chronic illness.

Mean post-test plasma oxysterol levels of the patient groups are shown in Table 1. The 7-KC levels of patients having the effort test were significantly higher compared to healthy subjects (59.87 ± 2.13 ng/mL, 20.26 ± 1.55 ng/mL, p<0.001). According to the pairwise comparison, the 7-KC level of the healthy group was significantly lower than both the
positive and negative patient groups. The C-triol was also significantly different between at least two of the patient groups (p<0.001). C-triol level was significantly higher in the negative group (16.08 ± 1.96 ng/mL) than in the healthy group (13.82 ± 1.72 ng/mL). There was no significant difference in terms of IMA between the study groups (p>0.05).

Table I Post test results and comparison of study groups.

| Parameter      | Positive Mean±S.D. (range) n=12 | Negative Mean±S.D. (range) n=18 | Healthy Mean±S.D. (range) n=30 | p-value |
|----------------|----------------------------------|----------------------------------|---------------------------------|---------|
| 7-KC (post-test) | 39.87±2.13(35–42)                | 37.73±2.44(35–42)               | 20.26±1.35*(18–24)             | <0.001  |
| C-triol (post-test) | 15.09±2.27(12–20)               | 16.08±1.96**(10–19)             | 13.82±1.72**(10–16)            | <0.001  |
| IMA (post-test)   | .09±.07(0.05–0.34)              | .08±.02(0.04–0.14)              | –                              | 0.735   |

*significantly different from the other two groups; **significantly different from each other; ***all three groups differ significantly.

Table II Comparison of patient and healthy groups based on the pre-tests.

| Parameter      | Patient Mean±S.D. n=30 | Healthy Mean±S.D. n=30 | t    | p-value |
|----------------|-----------------------|------------------------|------|---------|
| 7-KC (pre-test) | 40.90±2.33            | 20.26±1.35             | 41.78| <0.001  |
| C-triol (pre-test) | 16.15±2.19            | 13.82±1.72             | 4.57 | <0.001  |

Table III Change in plasma oxysterols: Repeated Measures of ANOVA results for 7-KC, C-Triol, and IMA levels.

| Parameter | Time       | Mean ±SD | Group | Effect Time | Time×group |
|-----------|------------|----------|-------|-------------|------------|
| 7-KC      | Pre-test   | 41.07±2.18 | Negative | 20.26±1.35* | 1566 <0.001 | 13.41 0.001 | 8.70 0.001 |
|           | Post-test  | 37.73±2.44*** | Positive | 20.26±1.35*** | 13.09 <0.001 | 1.34 0.251 | 0.46 0.632 |
| C-triol   | Pre-test   | 16.45±2.08 | Negative | 13.82±1.72* | 13.09 <0.001 | 1.34 0.251 | 0.46 0.632 |
|           | Post-test  | 16.08±1.96** | Positive | 13.82±1.72** | 13.09 <0.001 | 1.34 0.251 | 0.46 0.632 |
| IMA       | Pre-test   | .07±.050  | –      | 1.55        | 0.22       | 0.22 0.637 | 0.04 0.834 |
|           | Post-test  | .08±.028  | –      | –           | –          | –     –     | –     –     |

*significantly different from the other two groups; **significantly different from each other; ***all three groups differ significantly.
Table II provides the comparison results between the patient and healthy groups for mean 7-KC and C-Triol levels of pre-tests. Both 7-KC and C-Triol means differed significantly between patient and healthy groups (p<0.001). Patients had higher plasma oxysterol levels than healthy controls (Figure 1).

There were significant differences in terms of group, time, and time-group interaction effect for 7-KC level (Table III). Decreased 7-KC levels were found after the effort test (post-test vs. pre-test: 37.73 ± 2.44 ng/mL vs. 41.07 ± 2.18 ng/mL; p<0.001). There was a significant difference in 7-KC levels among the all study groups (negative, positive and healthy) after the effort test (37.73 ± 2.44 ng/mL, 39.87 ± 2.13 ng/mL, 20.26 ± 1.35 ng/mL, respectively). According to the time-group interaction, no change was observed in the healthy group, while the 7-KC level of the negative group was very close to that of positive group in the pre-test, it fell below the positive group’s level significantly in the post-test. There was no time and interaction effect for C-triol level, but significant difference only in terms of group (Figure 2B). According to this result, the pre-test C-triol level of healthy group was significantly lower than those of negative and positive groups. After the effort test, there was only significant difference between negative (16.08 ± 1.96 ng/mL) and healthy groups (15.82 ± 1.72 ng/mL). The C-triol level of the negative group was significantly higher than the healthy group. However, there was no significant difference in terms of group, time or group-time interaction for IMA levels (Table III, Figure 2C).

Discussion

The role of oxysterols in various pathologies including atherosclerosis, neurodegenerative disease, inflammatory bowel disease, retinal degeneration, diabetes, and fatty liver disease have been documented (22). Oxysterols exert their effects, mostly through their pro-inflammatory effects which result in an increase of inflammatory cytokines in circulation and
In our study, we found that 7-KC levels were in patients suspected to have CAD (regardless of the result of the effort test) had higher 7-KC and C-triol levels compared to controls. C-triol levels were significantly higher in patients, regardless of effort test result. However, there were no differences in regard to time and interaction effects for C-triol levels. There were no statistically significant differences in IMA values.

In a study by Rimner et al. (10), the levels of oxysterols in patients with stable CAD were found to be twice the value found in controls. They reported that this increase was primarily due to increases in 7-KC, epoxide beta isomers, and 7β-OHC levels. They also found that oxysterol increase was unaffected by the patients’ LDL cholesterol levels. Another study, focused on human aortic endothelial cells (25), reported that 7-KC increased mitochondrial oxidative stress, reduced NO bioavailability, and thus endothelial relaxation; which supports the evidence that 7-KC levels are an ideal marker for coronary artery disease. In our study, we found that 7-KC levels were increased in patients who were suspected to have CAD regardless of their effort test result. Furthermore, we found that C-triol levels also increased in patients versus controls. We believe that this elevation is caused by the role of 7-KC (and possibly C-triol) in the formation of atherosclerotic plaque. Song et al. (12) investigated 7-KC levels in 1016 patients and they noted that high 7-KC levels caused increased risk of cardiovascular disease, total mortality and increased morbidity of coronary artery disease. In our study, the post-test 7-KC levels were highest (39.87 ± 2.13 ng/mL) in the positive group and the lowest in the healthy group (20.26 ± 1.35 ng/mL).

To our knowledge, no studies have compared the pre-test and post-test oxysterol levels of patients in regard to their effort test results. We found that the post-test 7-KC levels of patients were reduced compared to their pre-test values. Although this reduction was not statistically significant, it is an interesting finding and may point to the effects of exercise on oxysterol level. Furthermore, when analysis of pre- and post-test 7-KC levels were performed in regard to effort test results (positive/negative), we found that patients with negative effort tests had greater 7-KC reduction (Figure 2A). This may suggest that, although exercise can reduce oxysterol levels, patients with CAD identified by a positive effort test may not benefit from this reduction as much as patients with a negative test result. Thus, the reduction in 7-KC level (or rather the absence of reduction) after stress test may be helpful in the diagnosis of CAD.

In our study, C-triol levels were found to be higher in patients entering the effort test (16.15 ± 2.19 ng/mL) than in healthy subjects (13.82 ± 1.72 ng/mL). Although this difference was not statistically significant, there are only a few studies which have reported C-triol levels in CAD patients, thus the this data may be useful. Future studies may have more insight into the etiopathogenesis of C-triol and coronary artery disease. Both 7-KC and C-triol levels were measured lower after the effort test than before the test. Although this reduction was not statistically significant, we believe that oxysterol levels may be positively influenced by physical exertion.

In the current study, the effort test had no effect on IMA levels, both pre-test and post-test values were similar (Figure 2C). This finding is in contrast with the majority of data in the literature (26–29). However, there are also studies in which no significant difference was found for IMA in similarly arranged groups, which is in parallel with our results (30, 31). We believe that these differences may suggest that IMA levels could vary with factors such as, the duration of the effort test, the amount of vascular occlusion and the severity of coronary artery disease. However, IMA is an important early marker in the diagnosis of ischemia, and thus coronary artery disease. Thus further studies are required to determine if effort tests have any effect on IMA levels.

**Conclusion**

These results indicate that high 7-KC may be closely associated with the progression of coronary atherosclerosis and inflammation. Similar studies in the literature also point to the importance of 7-KC levels in atherosclerosis; thus 7-KC (and various other oxysterols) may have important implications in the diagnosis and evaluation of CAD and may also demonstrate the risk for cardiovascular events in select patients.

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**Conflict of interest statement**

The authors stated that they have no conflicts of interest regarding the publication of this article.
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