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UČINKOVITOST SUPLEMENTACIJE ALFA LIPOIČNOM KISELINOM KOD PREMALIGNIH PROMJENA VRATA MATERNICE

DOKTORSKI RAD

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THE EFFICIENCY OF SUPPLEMENTATION WITH ALPHA LIPOIC ACID IN PATIENTS WITH PRECANCEROUS CERVICAL LESIONS

DOCTORAL THESIS

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SAŽETAK:

Premaligne lezije vrata maternice etiološki su vezane uz infekciju humanim papiloma virusom (HPV) i posljedičan razvoj upale i oksidacijskog stresa zbog čega bi se antioksidansi mogli smatrati učinkovitim protiv progresije skvamozne intraepitelne lezije niskog stupnja (LSIL). Recentna istraživanja pokazuju da je alfa lipoična kiselina (ALA) jedan od najpotentnijih prirodnih antioksidansa s (još uvijek) ograničenom terapijskom primjenom u liječenju bolesti povezanih s upalom i oksidacijskim stresom. Cilj ove randomizirane, dvostruko slijepe placebom kontrolirane studije je istražiti učinkovitost suplementacije ALA-om (600 mg/dan) na regresiju LSIL-a na uzorku od 100 pacijentica te dobiti širi uvid u terapijski potencijal i mehanizme djelovanja ALA-e na oksidativni stres, upalne biljege i parametre lipidnog statusa. Prisutnost LSIL-a utvrđena je nakon citološkog probira, kolposkopskog pregleda i ciljane biopsije te histološke potvrde citološko-kolposkopske dijagnoze. Parametri oksidativnog stresa, upale, lipidnog statusa i prisustvo HPV-a su utvrđeni standardnim laboratorijskim metodama. Prehrambene i životne navike istražene su korištenjem standardiziranog i validiranog semikvantitativnog upitnika o konzumiranju hrane i pića (FFQ). Dobijeni rezultati pokazali su da je suplementacija ALA-om znatno reducirala broj pacijentica s citološkim abnormalnostima niskog stupnja u odnosu na pacijentice koje su uzimale placebo. Uzimajući u obzir dobijenu razinu značajnosti ($p<0,001$), prezentirani rezultati upućuju da kratkoročna suplementacija ALA-om pokazuje klinički značajan učinak na cervikalnu citologiju. Intervencija nije imala značajan utjecaj na parametre antioksidativnog statusa iako su određeni pokazatelji antioksidativnog statusa povećani nakon suplementacije kod određenih podskupina pacijentica u ovisnosti o njihovom unosu antioksidanasa hranom. Utjecaj suplementacije ALA-om na lipemiju ovisio je o inicijalnom lipidnom statusu pacijentica. Buduće studije bi trebale biti usmjerene na istraživanje učinkovitosti dugoročnije terapije (>3 mjeseca) te uključiti i pacijentice s visokim stupnjem SIL-a; istraživanje inovativnih formulacija ALA-e povećane bioraspoloživosti te na istraživanje sinergističkih učinaka modifikacija životnog stila i suplementacije ALA-om.

Ključne riječi: skvamozna intraepitelna lezija niskog stupnja (LSIL), humani papiloma virus (HPV), alfa lipoična kiselina (ALA), parametri antioksidativnog statusa, parametri upale, parametri lipidnog statusa, indeks kvalitete prehrane

SUMMARY

Introduction. Alpha lipoic acid (ALA) is potent antioxidant that performs pleiotropic actions on biological pathways linked to numerous diseases. Among other effects, it shows direct antioxidant activity, regulates the status of gluthatione and modulates different signalling transduction pathways (such as insulin and nuclear factor kappa B (NFkB) and regulates glucose homeostasis and inflammatory response. Therefore, its potential as therapeutic agent in diabetic neuropathy, brain disease and cognitive dysfunction, cardiovascular diseases, endothelial dysfunction, hemorrhoidal illness, obesity and cancer is being intensively investigated. The majority of known therapeutic effects of ALA can be contributed to its direct antioxidant activity. ALA has specific structural properties characterized by a dithiolane ring, enabling the existence of both the oxidized and reduced form that together create a potent redox couple that has a standard reduction potential of −0,32 V. This makes ALA a potent direct antioxidant capable of scavenging a variety of reactive oxygen species. Furthermore, it regenerates other antioxidants, chelates redox-active transition metals, and induces the uptake (or enhances the synthesis) of endogenous low molecular weight antioxidants or antioxidant enzymes, particularly glutathione (GSH) (by activating Nrf2 pathway). ALA functions as the cofactor of oxidative decarboxylation reactions in glucose metabolism; the function that requires the disulfide group of the lipoic acid to be reduced to its dithiol form, dihydrolipoic acid, DHLA. It exerts the wide range of anti-inflammatory activities, including the reduction in the lipopolysaccharide (LPS)-stimulated release of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α); interleukin-1 beta (IL-1β); IL-6; LPS-induced expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Recent investigation shows that through the combination of antioxidative and anti-inflammatory mechanisms, and through regulation of glucose homeostasis, ALA could also ameliorate lipid abnormalities in the hyper-lipemic and atherosclerotic environment *in vivo*. Low-grade squamous intraepithelial lesion (LSIL) is a diagnostic term used when cytology reveals either permissive HPV infection or cervical intraepithelial neoplasia I (CIN I). This classification is also applied in histopathology, where LSIL and low-grade CIN are used interchangeably. LSILs constitute most abnormal findings in cervical cancer screenings, and typically resolve spontaneously within a year of diagnosis. The exact rate of spontaneous regression is difficult to ascertain due to variations in study methodologies, with reported regression percentages ranging between 7% and 95%. Progression to a high-grade squamous intraepithelial lesion (HSIL) is more likely if

LSIL is caused by high-risk, oncogenic human papillomavirus (HPV) genotypes. HPV infection triggers the release of inflammation mediators, leading to the generation of reactive oxygen species (ROS) and a decrease in antioxidant levels. HPV integration, coupled with oxidative stress, induces genomic damage and epigenetic changes that hinder apoptosis and alter cellular proliferation. Dietary components with antiviral, anti-inflammatory, and antioxidant properties may offer protection against SIL progression and cervical cancer development. Observational studies suggest that Western dietary patterns increase the risk of persistent HPV infection, whereas adherence to the Mediterranean diet reduces this risk. Consumption of a diverse range of whole foods rich in vitamins, minerals, and bioactive compounds, particularly those with antioxidant and antiviral properties, appears effective in preventing LSIL progression to HSIL or cervical cancer. Therefore, a balanced diet rich in mentioned health-promoting components should be recommended to patients upon LSIL diagnosis as a preventive strategy. The primarily goal of this study is to investigate the impact of supplementing patients with diagnosed LSIL with alpha lipoic acid (ALA) and observe the effects on the progression/regression of LSIL after a 3-month supplementation. Additionally, we aim to investigate if a 3-month supplementation with 600 mg of ALA shows significant effect on the parameters of antioxidant - or lipid status. The secondary goal of this investigation is to find out if the observed responses to supplementation depend on the diet characteristics of the study participants, having in mind that the human antioxidant defense system resists modulation by dietary antioxidants and might depend on their nutritional intake.

Methods. This study was designed as a double-blind, randomized, placebo-controlled trial that recruited 100 female patients with the diagnosis of LSIL, which was determined after cytological screening, colposcopy, and a targeted biopsy. Exclusion criteria were diabetes, malignant diseases, chronic inflammatory diseases, hysterectomy, abortion, destructive therapy of the cervix, HPV vaccination and menopause. Patients who reported regular use of antioxidant dietary supplements and lipid-lowering pharmacotherapy were also not eligible for inclusion into the study. The sample size was calculated by using a randomized clinical trial sample size formula. Block randomization was used to distribute participants to either the placebo or the intervention group in a 1:1 ratio. Patients were supplemented with either 600 mg per day of ALA (oral capsules containing all-rac ALA) or a placebo (provided as oral capsules containing rice starch, visually identical to ALA capsules) for 3 months. The capsules of both ALA and placebo were provided by Zada Pharmaceuticals (Lukavac, Bosnia and Herzegovina). At the initial appointment patients filled a standardized and validated semi-quantitative food

frequency questionnaire to provide information on diet characteristics, use of dietary supplements, smoking, and physical activity. FFQ was designed as a 192-item questionnaire with one month as the reference period of intake. There were 100 dietary items listed, and the remaining items were questions about supplementation use and eating habits**.** The primary outcome of the study was LSIL, which was determined after the performed cytological screening, colposcopic examination of the cervix, targeted biopsy and histological confirmation of cytological–colposcopic diagnosis at the study baseline and after the 3 months intervention. Uterine tissue samples obtained by targeted colposcopic biopsy were processed by a standard histological method. Cervical smears were also tested for the presence of high-risk HPV strains. Assessment of the pathological diagnosis was done as blindness by a single experienced pathologist at baseline and after the intervention. At the initial and the follow-up appointment, blood samples were taken from the patient's cubital vein, using the standard procedure. The serum was separated by centrifugation and multiple aliquots of each sample were either analyzed immediately (biochemical parameters) or stored at −80 °C for future analysis (oxidative stress- and inflammation parameters). Biochemical parameters (total cholesterol (CHO), LDL, high density lipoproteins (HDL), triglycerides (TG) and antioxidant status indicators (oxygen radical absorbance capacity (ORAC); Trolox equivalent antioxidant activity (TEAC); Folin–Ciocalteu reducing capacity (FC); ferric reducing activity (FRAP); superoxide dismutase (SOD) activity; reduced glutathione (GSH); and malondialdehyde (MDA) levels) and inflammation indicators (high-sensitive C-reactive protein (hsCRP), fibrinogen (FI) and sedimentation (SE)) were determined from the collected blood samples.

Results. All patients recruited for the study had a diagnosis of LSIL at the initial visit; by the end of the study, this number of patients was reduced in both groups - in the placebo group 44 patients still had an LSIL diagnosis; in the treated group of patients only 2 of them still had an LSIL diagnosis ($p < 0,0001$). The percentage of patients with positive HPV findings remained the same during the 3 months of study; 37,5% in the placebo and 46,3% in the treated group of patients were HPV-positive and observed differences were not statistically significant. The number of LSIL-positive patients decreased from 48 to 44 in the placebo group (8,33% of patients recovered), but the observed change was found to be statistically insignificant ($p = 0,1171$). On the other hand, in the ALA-treated group as much as 95,12% of patients recovered ($p < 0,0001$). None of the patients in either of the investigated groups progressed to the higher grade of SIL. The percentage of HPV-infected patients remained the same in both groups.

At the initial visit, hsCRP and FI were significantly lower in the placebo group (p = 0,0344 and p = 0,0130, respectively) but still within the normal range, while SE rates were comparable (p = 0,0785). At the 3-month follow-up visit, the situation was reversed: inflammation parameters were higher in the placebo group and observed differences were statistically significant for FI and SE rates (p = 0,0437 and 0,0046, respectively). All inflammation parameters increased during 3 months of supplementation in the placebo group and observed changes were statistically significant (p < 0,001). On the contrary, in the ALA-treated group all observed inflammation parameters decreased moderately but significantly (p < 0,0001). Investigation on the biomarkers of antioxidant status showed that at the initial measurement there were no significant differences between medians of MDA levels (p = 0,151), FRAP (p = 0,381), SOD (p = 0,180), ORAC (p = 0,488), or GSH (p = 0,389) in the placebo and the intervention groups; TEAC was significantly higher (p = 0,048) and FC reducing capacity was significantly lower in the intervention group (p = 0,005). After the 3 months of supplementation the situation remained unchanged except for the GSH levels that were now significantly lower in the intervention group compared to the placebo (p = 0,050). However, the apparent decrease of GSH values that was observed in the ALA-supplemented group after 3 months of supplementation was found to be statistically insignificant (p = 0,411). Moreover, none of the monitored antioxidant status parameters were significantly changed, neither in the placebo nor in the intervention groups. Since diet characteristics can play a significant role in modulating the overall effectiveness of antioxidant supplements, a subgroup analysis was conducted to compare the ALA effectiveness in subgroups of patients with high (>11) and low (<7) Med-DQI (indicating low and high degree of adherence to Mediterranean dietary patterns). The Med-DQI was chosen because it quantifies the levels of adherence to the Mediterranean diet and dietary diversity, which are known to contribute to lower antioxidant and inflammation biomarkers. Subgroup analysis showed that the effects of antioxidant supplementation on antioxidant status indicators might be significantly affected by the patient's dietary characteristics. In the subgroup of patients with higher levels of compatibility with Mediterranean dietary patterns (Med-DQI < 7) supplementation with ALA showed positive effects on SOD activity (it prevented the decrease in SOD activity that was observed in the placebo group). This was not observed in the remaining patients (Med-DQI > 7). Lipid parameters didn't differ significantly between the placebo and intervention groups, initially. However, after 3 months, the supplementation LDL levels in the intervention group were significantly higher compared to the placebo group (p = 0,033). During the 3-month supplementation period, CHO and LDL levels of the placebo group remained unchanged, while

in the intervention group they increased from 5,19 to 5,69 (p = 0,001) and from 2,89 to 3,46 (p = 0,006). The obtained results are not consistent with the results of other authors who showed that ALA either decreased LDL or showed no significant effect; however, those data were mostly obtained in studies focusing on patients with metabolic diseases and hyperlipidemia. Based on the assumption that ALA could have different effects on LDL levels depending on the initial lipid status, subgroup analysis was conducted focusing specifically on participants with hypercholesterolemia (LDL > 3 mmol L^{-1}) and specifically on normolipemic patients (LDL ≤ 3,00 mmol L^{-1}). In patients with hyperlipidemia supplementation with ALA resulted in a very small but statistically significant decrease in LDL values (the median decreased from 3,95 to 3,89 mmol L−1; p = 0,049); however, a more pronounced effect has been observed in the placebo group (with a decrease from 3,76 to 3,54 mmol L^{-1}; p = 0,022). This might be explained by a possible positive modification of dietary and lifestyle habits of the patients upon recruitment into the dietary clinical study (despite clear instruction that dietary habits and lifestyle should not be changed during the study), which has been noted in the literature. In the subgroup of patients with normal LDL levels, a significant increase in LDL levels was observed in the intervention but not in the placebo group (median increased from 4,01 to 4,07 mmol L^{-1}; p = 0,003), which confirmed our hypothesis that the effect of ALA supplementation on lipid status might be affected by the initial lipid status of the patient.

Conclusions. The results of the conducted trial demonstrate that 3 months of supplementation with 600 mg of ALA significantly promotes regression of LSIL but does not affect HPV infection rates. Positive effects are probably assciated with observed antiinflammatory effects of ALA. ALA showed modest effect on the parameters of antioxidant status that were partially affected by diet characteristics – primarilly by the degree of compliance to a Mediterranean dietary pattern. ALA supplementation resulted with a small but statistically significant increase in LDL and CHO levels, indicating that the lipid-lowering effect of ALA observed in some studies might depend on the initial lipid status of the participants (which has been confirmed by the post-hoc subgroup analysis).