Consumption of nutritionally enriched hen eggs enhances endothelium-dependent vasodilation via cyclooygenases’ metabolites in healthy young persons - A randomized study

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Research Article

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Abstract

Background

Present study aimed to evaluate the effects of enriched hen eggs consumption on the endothelium-dependent vasodilation (EDV) and the role of cyclooxygenases in EDV in microcirculation of young healthy individuals.

Methods

Participants consumed 3 eggs per day for 3 weeks: Control group (n = 14) consumed regular hen eggs (approximately 0.330 mg of lutein, 1.785 mg of vitamin E, 0.054 mg of selenium and 438 mg of n-3 PUFAs daily) and Nutri4 group (n = 20) consumed enriched eggs (approximately 1.85 mg of lutein, 0.06 mg of selenium, 3.29 mg of vitamin E, and 1026 mg of n-3 PUFAs daily). Skin microvascular blood flow in response to EDV (post-occlusive reactive hyperemia (PORH) and iontophoresis of acetylcholine (AChID)) and sodium nitroprusside (SNPID; endothelium-independent) were assessed by laser Doppler flowmetry before and after dietary protocol, with or without 100 mg of indomethacin perorally. Arterial blood pressure, heart rate, serum lipid and liver enzymes, anthropometric measurements, protein expression of COX-1, COX-2, nNOS, iNOS, and eNOS were measured before and after dietary protocol.

Results

PORH and AChID were significantly enhanced, and SNPID remained unchanged in Nutri4 group, while none was changed in Control group following respective diet. PORH was decreased after administration of indomethacin in Nutri4 after dietary protocol. Protein expression of COX-2 was significantly higher in the Nutri4 group compared to Control group after dietary protocol.

Conclusion

Consumption of enriched eggs improves microvascular EDV in healthy young subjects. Results suggest involvement of n-3 PUFAs metabolites via the cyclooxygenases pathway in enhanced reactive hyperemia.

Trial registration

: The study was registered on the Clinical trial under the title: Effect of Enriched QUARTET® Hen Eggs on Cardiovascular Function in Cardiovascular Patients and Healthy Individuals. NCT number: NCT04564690 on September 2020 (https://clinicaltrials.gov/ct2/show/NCT04564690).

Background
Consumption of n-3 polyunsaturated fatty acids (n-3 PUFAs), (i.e. eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA)), has been recognized to reduce risks for cardiovascular (CV) diseases [1]. n-3 PUFAs demonstrated protective CV effects by lowering blood pressure (BP) [2] and exhibiting anti-inflammatory, anti-atherosclerotic and antioxidant properties [3]. For example, the addition of EPA and DHA in daily diet was shown to decrease the concentration of serum lipids, most notably triglycerides, in individuals with hyperlipidemia [4]. Interestingly, additional beneficial effects of n-3 PUFAs consumption is contributed to alteration of vascular tone and blood vessel reactivity [5], both in the healthy persons and in CV patients [1, 2, 5]. Adding fatty acids, such as fish oil, to daily diet induced increased vascular reactivity in healthy patients [4]. Furthermore, the increased proportion of EPA and DHA in the diet increases vasodilator mechanisms in the microcirculation of the forearm, which can contribute to lowering blood pressure [5, 6]. In various clinical trials, it was shown that n-3 PUFAs have a cardio protective effect by improving arterial hemodynamics and reducing arterial stiffness, which can positively interfere with the process of atherosclerosis [7, 8]. Interestingly, cyclooxygenase (COX) metabolites of EPA have been showed to be involved in n-3 PUFAs-mediated vasodilation [9]. Evidence is accumulating that consumption of n-3 PUFAs in a form of functional foods (e.g. n-3 PUFAs enriched hen eggs) may improve microvascular endothelium-dependent vasodilation in healthy young individuals and in patients with metabolic diseases [5, 7, 10]. Importantly, consumption of regular and n-3 PUFAs enriched hen eggs do not have negative effects on any of the measured biological and functional vascular parameters in patients with cardiovascular diseases, as well as in healthy young subjects, suggesting that eggs can be safely consumed in the daily diet [7, 11].

In addition to n-3 PUFAs, other nutritional elements may play an important role in preserving the vascular function, especially ones with antioxidant properties. Vitamin E, which is the most abundant antioxidant soluble in fats, has been described to alleviate hypertension, diabetes mellitus, fatty liver disease, etc. [12–14]. In cells exposed to hypoxia, vitamin E had protective effect by reducing reactive oxygen species (ROS) and cell apoptosis [9, 15], while it may also prevent functional vascular impairment, as observed in diabetic rats [16]. Another very important essential trace element that has a cardio protective role in human health is selenium (Se) [17]. Se is a major component of glutathione peroxidase, an antioxidant enzyme that catalyzes the reduction of hydrogen peroxide to water and oxygen, as well as the reduction of peroxide radicals to alcohols and oxygen, which protects cells from oxidative damage [18]. Se deficiency itself has been reported in patients with stroke, atherosclerosis, and diabetes [19], as well as in hypertension and atherothrombotic diseases [20]. Lutein is also associated with a decrease in blood pressure due to increased nitric oxide (NO) production, a decrease in atherosclerotic lesions, and a decrease in monocyte chemotaxis [21]. Consumption of lutein reduced the risk of metabolic syndrome, and exhibited protection in the progression of early atherosclerosis [22], which was attributed to its antioxidant and anti-inflammatory effects [21]. However, there is a lack of studies in animal models or in humans that evaluate health effects of these nutrients as the components of functional food.

Therefore, the hypothesis of present study was that a diet containing hen eggs enriched with n-3 PUFAs, vitamin E, lutein, and selenium, would improve endothelial function manifested by changes in microcirculatory blood flow. Present study aimed to determine the effect of consumption of these
enriched hen eggs on microvascular reactivity in response to endothelium-dependent and endothelium-independent stimuli in young healthy subjects of both sexes and to identify potential mechanisms involved in altered vascular reactivity.

Materials And Methods

Trial design

The study was registered on the Clinical trial under the title: Effect of Enriched QUARTET® Hen Eggs on Cardiovascular Function in Cardiovascular Patients and Healthy Individuals. NCT number: NCT04564690 [35]. Figure 1. represents CONSORT flow chart of the study

Ethics Approval

The study protocol and procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by the Ethical Committee of the Science Center of Excellence, Josip Juraj Strossmayer University of Osijek (CI: 602-04/14 – 08/06; No: 2158-610714-114) and Ethics Committee of the Medical Faculty Osijek (CI: 602-04/20 – 08/07, Registration number: 2158-61-07-20147).

Inclusion And Exclusion Criteria

Thirty-four young healthy individuals participated in this study. Eligibility criteria included age range between 18 and 30 years, arterial BP values ≤ 120/80 mmHg, normal body mass index (BMI; 18.5–24.9 kg/m²). Exclusion criteria were a history of smoking, hyperlipidemia, renal impairment, hypertension, coronary artery disease, diabetes, cerebrovascular and peripheral artery disease, and taking any drugs or substances that could affect the endothelium. Also, none of the participants have been taking enriched functional food or n-3 PUFAs, lutein, selenium and vitamin E supplementation in any form prior or during the enrollment in the present study. Written informed consent was obtained from each subject.

Sample Size

The statistical program SigmaPlot (version 11.2, Systat Software, Inc., Chicago, USA) was used. The sample size required to show a potentially significant effect was calculated based on preliminary data from the same research group where the primary outcome was an LDF measurement collected from 8 subjects [7]. To detect differences in primary outcomes recorded in this study (PORH measurements) with a significance level of 0.05 and a statistical power of 80% for the paired t-test, the required sample size was 13 subjects per group. For the group with indomethacin primary outcomes recorded in this groups are PORH measurements after intake indomethacin it is 5 subjects per group.
Randomization And Blinding

This was a randomized, double blinded, prospective, placebo-controlled interventional study. After recruitment, a simple randomization procedure was performed by drawing A or B for each subject, i.e. in which group - A (control) or B (Nutri4). The result of such randomization is the difference in the number of subjects that exists between the control and Nutri4, but the number of subjects in both groups met the sample size required for adequate statistical analysis [36].

Participants were recruited and included using a survey questionnaire to meet the inclusion and exclusion criteria of the study, then were given the code ZCI-Q-1-No. or ZCI-Q-2-No., in which ZCI-Q- denotes the name of the project under study ("Scientific Center of Excellence" in which hen eggs enriched with four nutrients are tested: n-3 PUFA, vitamin E, selenium and lutein. Number 1 or 2 denoted group (control or Nutri4 - depending on the eggs to be consumed), and were assigned by the person who divided the eggs. No. is the ordinal number of subjects (1, 2, 3,...).

Intervention

Study protocol lasted 21 days. During those three weeks’ subjects were instructed to eat 3 hen eggs per day (total of 63 eggs). Subjects were divided in two study groups: control group, consuming regular eggs (CTRL, n = 14) and a group consuming enriched eggs (Nutri4, n = 20). Nutri4 consumed approximately 3.29 mg/per day of vitamin E, 1.85 mg per day of lutein, 0.06 mg/per day of selenium and 1026 mg/per day of n-3 PUFAs in 3 eggs per day for 3 weeks), while control group consumed regular hen eggs produced on the same farm (approximately 1.79 mg/per day of vitamin E, 0.33 mg/per day of lutein, 0.05 mg/per day of selenium and 438 mg/per day of n-3 PUFAs) in 3 eggs per day for 3 weeks.

All eggs were the same L size and produced on the poultry farm Marijančanka d.o.o. Marijanci, Croatia. Enriched hen eggs were produced according to the protocol of research group from Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, in which rapeseed oil (1,5%) in feed mixtures fed to laying hens were replaced with mixture of fish (1.5%), linseed (2%) oil and 0,43 selenium mg / kg mixture, 100mg / kg mixture of vitamin E and lutein. The egg content is shown in Table 1 [37, 38].

Subjects were instructed to boil the eggs (10 minutes) before consumption every morning during the three-week protocol. Also, all subjects were instructed to take only the eggs given to them for the purposes of the study (total of 63 eggs) and not to take the other food rich in n-3 PUFAs vitamin E, lutein and selenium, any other enriched functional food or any other supplementation during study protocol. Study was performed in the Laboratory for Clinical and Sport Physiology, Department of Physiology and Immunology at Faculty of Medicine, University of Osijek. Two study visits and all the measurements described below were done on the first day and on the day immediately after the end of the dietary protocol. All measurements and blood sampling occurred in the morning after an overnight fasting.
Subjects were instructed not to undertake any strenuous activity during the 24 h preceding the visit and to avoid caffeine intake in the morning before the study visit.

**Anthropometric And Arterial Blood Pressure Measurements**

Subjects weight (kg) and height (m) were measured to calculate body mass index (BMI). Waist and hip circumference, heart rate (HR) and arterial blood pressure (BP) were measured at the beginning of each visit after 15 min rest in seated position using an automated oscillometric sphygmomanometer (OMRON M3, OMRON Healthcare Inc., Osaka, Japan). The final values of BP and HR were the mean of three repeated measurements.

**Assessment Of Microvascular Endothelium-dependent And Endothelium-independent Vasodilation**

Measurement of microvascular blood flow and reactivity tests were performed in all subjects using laser Doppler flowmetry (LDF) (MoorVMS-LDF, Axminster, UK), before and after corresponding diet protocols, as previously described [8, 11]. Endothelium-dependent vasodilation of skin microcirculation was tested by measurement of microvascular response to vascular occlusion (post-occlusive reactive hyperemia, PORH) and to iontophoresis of acetylcholine (acetylcholine induced dilation, AChID), while endothelium-independent vasodilation was assessed by iontophoresis of sodium-nitroprusside (sodium nitroprusside induced dilation, SNPID). All microvascular tests were performed according to the protocols described in detail in earlier publications of our research [8, 9, 39, 40] group. LDF measurements were performed in the morning after an overnight fasting, in a temperature controlled room (23.5 °C +/- 0.5 °C). Microvascular blood flow was expressed in arbitrary perfusion units (PU) and described as the area under the curve (AUC) using original software provided by the manufacturer (MoorVMS-PC v4.0, Axminster, UK). PORH measurement was expressed as the difference between percentage of flow change during reperfusion and occlusion in relation with baseline (R-O%). AChID and SNPID were expressed as flow increase following ACh or SNP administration compared to baseline flow.

In addition, to test the role of metabolites of cyclooxygenase enzymes (COX-1 and COX-2) in the microvascular responses, in a separate set of experiments, measurements of ACh induced dilation and PORH were conducted in randomly selected subjects prior and 90 minutes after peroral intake of 100 mg of indomethacin [41] at both study visits (n = 6 from CTRL and N = 5 from Nutri4 group).

**Isolation Peripheral Blood Mononuclear Cell (Pbmc)**

Venous blood was sampled in vacutainers with the anticoagulant ethylenediaminetetraacetic acid (EDTA). The venous blood was then mixed with 1 × phosphate-buffered saline (PBS) and layered on a Ficoll-Paque® PLUS solution (density gradient) and centrifuged for 25 min at 800 G (Rotina 380, Hettich
GmbH & Co. KG, Tuttlingen, Germany). After centrifugation, the middle layer of PBMCs was separated and used in further experiments [26].

Protein Expression Of Enzymes Important In The Mechanisms Of Microvascular Reactivity

For the purpose of determining the relative protein expression of iNOS, eNOS, nNOS, COX-1 and COX-2 from isolated PBMC, Western blot was done according to the well-established method at our laboratory [42–44]. Homogenization buffer (50 mM HEPES 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% TRIXON-X), protease inhibitor cocktail 0.4 µl / 100 µl (Sigma Aldrich) was used to homogenize PBMC. 200 µl of homogenization buffer was added to resuspend 1x10^7 PBMC. Everything is well shaken on a mixer (vortex) and kept on ice for 3 minutes. This was followed by centrifugation of the samples for 30 minutes at 15000 x g at 4°C. The supernatant was pipetted to be used for further analysis. Sample preparation for SDS-PAGE electrophoresis followed. Samples were boiled at a ratio of 1:1 at 95°C for 5 minutes with Laemmli buffer, centrifuged briefly (spin-down) and applied to the gel. After electrophoresis, the samples were transferred to a PVDF membrane and then incubated with the primary antibody overnight (COX-1 rabbit PolyAB, Proteintech Europe, UK, #13393-1-AP, 1:500; COX-2 rabbit PolyAB, Proteintech Europe, UK, #12375-1-AP, 1:500; iNOS rabbit PolyAB, Novus Biological, USA, #NB300-605, 1:500; eNOS rabbit PolyAB, Novus Biological, USA, #NB300-500, 1:1000; nNOS rabbit PolyAB, Novus Biological, USA, # NBP1-39681, 1:1000) overnight at 4°C. The next day, the membrane was incubated with appropriate horse radish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit HRP, Abcam, UK, ab205718; goat anti-mouse HRP, Santa Cruz Biotechnology, USA, sc-2005, both in 1:7500 dilution), followed by chemiluminescence imaging using Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the manufacturer's instructions. Protein expression levels were normalized to expression of β-actin and presented as relative protein levels.

Venous Blood Sample Analysis

A venous blood samples were taken after 15 min resting in a seated position at each visit. Blood samples were analyzed for full blood count, fasting blood glucose, creatinine, urea, fasting lipid profile (total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides), and liver enzymes determined by spectrophotometric method, hsCRP and transferrin determined by immunoturbidimetry, plasma electrolytes determined by potentiometry methods. These analyses were performed at the Department of Clinical Laboratory Diagnostics, University Hospital Osijek.

Measurements Of Serum Vitamin E Concentration

Vitamin E concentrations in serum samples were determined according to the existing protocol [46]. First, absolute ethanol was used to denature serum proteins and Xylene was used to separate the supernatant
from proteins. The whole mixture was centrifuged at 3000g for 10 minutes and the supernatant was separated. 2,2-bipyridyl and FeCl3 were then added to the supernatant, resulting in a pink coloration. After 2 minutes of incubation, the absorbance was measured using spectrophotometer (PR 3100 TSC Microplate Reader, Bio-Rad Laboratories, Hercules, California) at 492 nm, and the obtained absorbance was proportional to the serum vitamin E concentration. A five-point calibration curve was also made.

**Serum Selenium Concentration Measurements**

All samples for measurement of serum Se concentration were digested in ultra-pure HNO3 and H2O2 (5:1 ratio) at 180 °C for 60 minutes in a close microwave system CEM Mars 6 (CEM, Matthews, NC, USA). Se concentrations in solutions of digested serum samples were determined by inductively coupled plasma mass spectrometry (ICP-MS) (ICP-MS, Agilent 7500a, Agilent Technologies Inc., California, USA). Each serum sample on the ICP was analyzed by internal pooled plasma control and reference material NIST 1567b (wheat flour, National Institute of Standards and Technology, USA) was used for the control of the analytical method. All samples were analyzed in triplicate. Measurements were performed at the Department for Agroecology and Environment Protection, Faculty of Agrobiotechnical Sciences Josip Juraj Strossmayer University of Osijek.

**Measurements Of Serum Lutein Concentration**

Lutein concentrations in serum samples were determined according to the existing protocols [47, 48]. In 200 ul of serum, we added 1 ml of deionized water and 0.01% ascorbic acid dissolved in absolute ethanol and stirred the mixture. Then we added 2 mL of hexane, stirred and centrifuged at 2500 RPM for 20 minutes. After centrifugation, we separated the supernatant, evaporated the supernatant and determined the concentration of lutein using HPLC. HPLC analysis was performed at the Department of Chemistry, Josip Juraj Strossmayer University of Osijek.

**Statistical Analysis**

The results are presented as arithmetic mean and standard deviation (SD). The Shapiro-Wilkinson test was used to assess normality of data distribution. The differences of the normally distributed numerical variables between the two independent groups were tested by the Student's t-test, in case of deviation from the normal distribution by the Mann-Whitney U-test. A paired t-test was used for intragroup comparisons or Wilcoxon rank sum tests when variables were not normally distributed. The One-Way ANOVA test was used for comparison between multiple groups. The level of statistical significance was determined at p < 0.05.

**Results**
Table 1
Composition of micronutrient ingredients in Regular eggs and Nutri 4 eggs

An L-grade egg with an average weight of 68 g has about 60 g of edible portion.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Regular eggs</th>
<th>Nutri4 eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (mg)</td>
<td>0.595</td>
<td>1.098*</td>
</tr>
<tr>
<td>Lutein (mg)</td>
<td>0.11</td>
<td>0.616*</td>
</tr>
<tr>
<td>Selenium (mg)</td>
<td>0.0183</td>
<td>0.02305*</td>
</tr>
</tbody>
</table>

Fatty acids

<table>
<thead>
<tr>
<th></th>
<th>Regular eggs</th>
<th>Nutri4 eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>∑ SFA</td>
<td>1566 ± 346</td>
<td>1442 ± 185</td>
</tr>
<tr>
<td>∑ MUFA</td>
<td>1976 ± 189</td>
<td>2419 ± 139</td>
</tr>
<tr>
<td>∑ n-6 PUFA</td>
<td>1263 ± 148</td>
<td>747 ± 46*</td>
</tr>
<tr>
<td>LA</td>
<td>1165 ± 140</td>
<td>702 ± 43</td>
</tr>
<tr>
<td>AA</td>
<td>89 ± 9</td>
<td>44 ± 4*</td>
</tr>
<tr>
<td>∑ n-3 PUFA</td>
<td>146 ± 20</td>
<td>342 ± 25*</td>
</tr>
<tr>
<td>ALA</td>
<td>71 ± 11</td>
<td>189 ± 16*</td>
</tr>
<tr>
<td>EPA</td>
<td>n.d.</td>
<td>19 ± 2*</td>
</tr>
<tr>
<td>DHA</td>
<td>75 ± 11</td>
<td>135 ± 11*</td>
</tr>
<tr>
<td>∑ n-6/∑ n-3 PUFA</td>
<td>8.71</td>
<td>2.18*</td>
</tr>
</tbody>
</table>

Results are shown as mean ± standard deviation (SD). *p < 0.05 paired t-test Regular eggs vs. Nutri4 enriched hen eggs

Effects Of Diets On Anthropometric And Biochemical Measurements In Study Population

Characteristics of the study population are presented in Table 2. There was no difference in participants’ age between the CTRL and Nutri4 group. Subjects of both sexes from the Nutri4 and CTRL group were lean and had similar BMI and WHR before the study protocol. No significant change in BMI or WHR in the Nutri4 group and CTRL group occurred after dietary protocol. There was no difference in BP (SBP, DBP and MAP, respectively) between study groups before and after the dietary protocol. The Nutri4 group had significantly higher HR than CTRL group before the dietary protocol and after the three-week dietary protocol (Table 2).

Prior to the dietary protocol, all biochemical parameters were similar between groups. Consumption of eggs did not cause significant changes in serum concentration of HDL cholesterol and triglycerides in
CTRL and Nutri4 group compared to pre-diet measurements. However, the serum LDL cholesterol concentration was significantly increased in CTRL group after a three-week dietary protocol. The iron level in Nutri4 group decreased significantly after dietary protocol (although within the reference interval). There was a significant increase in serum glucose and urea concentration in Nutri4 subjects after egg consumption compared to CTRL group (however, within reference interval). Other measured parameters were similar between groups and not affected by respective dietary protocols. (Table 2).
Table 2
The Effect of Regular and Nutri4 Hen Eggs Consumption on Anthropometric, Hemodynamic and Biochemical Parameters in Healthy Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Nutri4 group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td>N (W/M)</td>
<td>14 (6 / 9)</td>
<td>20 (7 / 13)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>22 ± 3</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>BW (kg)</td>
<td>78 ± 17</td>
<td>77 ± 15</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 3.6</td>
<td>24.0 ± 3.2</td>
</tr>
<tr>
<td>WHR</td>
<td>0.84 ± 0.07</td>
<td>0.84 ± 0.07</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>110 ± 17</td>
<td>108 ± 15</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>72 ± 12</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>85 ± 7</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>HR (beats per minute)</td>
<td>65 ± 8</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>Erythrocytes (x10⁰E12/L)</td>
<td>4.7 ± 0.4</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>141 ± 14</td>
<td>141 ± 16</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39 ± 3</td>
<td>32 ± 16</td>
</tr>
<tr>
<td>Leukocytes (x10⁰E9/L)</td>
<td>6.1 ± 1.9</td>
<td>6.0 ± 1.2</td>
</tr>
<tr>
<td>Thrombocytes (x10⁰E9/L)</td>
<td>201 ± 65</td>
<td>216 ± 64</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>5.0 ± 1.5</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>86 ± 18</td>
<td>87 ± 14</td>
</tr>
<tr>
<td>Urate (µmol/L)</td>
<td>330 ± 67</td>
<td>318 ± 88</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>25 ± 8</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>23 ± 8</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>Gamma - glutamyltransferase (U/L)</td>
<td>14 ± 4</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>139 ± 2</td>
<td>139 ± 2</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.2 ± 0.3</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Iron (µmol/L)</td>
<td>16.4 ± 6.7</td>
<td>13.8 ± 7.1</td>
</tr>
<tr>
<td>Transferrin (g/L)</td>
<td>2.73 ± 0.5</td>
<td>2.78 ± 0.71</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.6 ± 0.3</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Parameter</td>
<td>Control group</td>
<td>Nutri4 group</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>0.6 ± 0.5</td>
<td>1.1 ± 1.5</td>
</tr>
<tr>
<td>cholesterol (mmol/L)</td>
<td>4.3 ± 0.8</td>
<td>4.6 ± 1.5</td>
</tr>
<tr>
<td>triglycerides (mmol/L)</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.5 ± 0.4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.5 ± 0.7</td>
<td>2.9 ± 0.8</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (SD).

BMI- body mass index; WHR- waist-to-hip ratio; SBP- systolic blood pressure; DBP- diastolic blood pressure; MAP- mean arterial pressure; HR- heart rate; HDL- high-density lipoprotein; LDL- low-density lipoprotein. * p < 0.05 before vs. after within the group; † p < 0.05 difference between the groups.

**Free fatty acids profile, vitamin E, lutein and selenium in serum**

Concentration of EPA and vitamin E was significantly increased in the Nutri4 group following enriched eggs consumption compared to the initial value and compared to values of CTRL group. DHA concentration was significantly increased in Nutri4 groups following enriched eggs consumption. The serum concentration of lutein was significantly increased in the Nutri4 group after the dietary protocol, while in the CTRL group there was no statistical change after the dietary protocol. Selenium concentration remained statistically unchanged after the three-week dietary protocol in the CTRL group and in the Nutri4 group (Table 3).
Table 3
The Effect of Regular and Nutri4 Hen Eggs Consumption on Serum Fatty Acids Profile, Vitamin E, Selenium and Lutein in Healthy Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Nutri4 group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td><strong>SFA (µmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4:0 Butyric acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C6:0 Caproic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C8:0 Caprylic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C10:0 Capric acid</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>C11:0 Undecylic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C12:0 Lauric acid</td>
<td>&lt;LOQ</td>
<td>20.80</td>
</tr>
<tr>
<td>C13:0 Tridecylic acid</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>C14:0 Myristic acid</td>
<td>40.4 ± 9.1</td>
<td>36.3 ± 6.6</td>
</tr>
<tr>
<td>C15:0 Pentadecylic acid</td>
<td>12.2 ± 1.6</td>
<td>12.7 ± 1.3</td>
</tr>
<tr>
<td>C16:0 Palmitic Acid</td>
<td>1258 ± 350</td>
<td>1304 ± 332</td>
</tr>
<tr>
<td>C17:0 Margaric acid</td>
<td>13.5 ± 1.7</td>
<td>13.6 ± 2.6</td>
</tr>
<tr>
<td>C18:0 Stearic acid</td>
<td>404 ± 111</td>
<td>416 ± 137</td>
</tr>
<tr>
<td>C20:0 Arachidic acid</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>C21:0 Heneicosanoic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C22:0 Behenic acid</td>
<td>&lt;LOQ</td>
<td>10.2</td>
</tr>
<tr>
<td>C23:0 Tricosanoic acid</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>C24:0 Lignoceric acid</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td><strong>PUFA (µmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:1[cis-9] Myristoleic acid</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>C15:1[cis-10] Cis-10-pentadecenoic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>n-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1[cis-9] Palmitoleic acid</td>
<td>58.3 ± 14.9</td>
<td>54.1 ± 17.7</td>
</tr>
<tr>
<td>Parameter</td>
<td>Control group</td>
<td>Nutri4 group</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td>C17:1[cis-10] cis-10-Heptadecenoic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>n-9 C18:1[trans-9] Elaidic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C18:1[cis-9] Oleic acid</td>
<td>691 ± 218</td>
<td>656 ± 135</td>
</tr>
<tr>
<td>C20:1[cis-11] 11-Eicosenoic acid</td>
<td>7.8 ± 2.0</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>C22:1[cis-13] Erucic acid</td>
<td>9.2</td>
<td>9.1 ± 0.7</td>
</tr>
<tr>
<td>C24:1[cis-15] Nervonic acid</td>
<td>&lt;LOQ</td>
<td>8.7 ± 0.6</td>
</tr>
<tr>
<td>n-6 C18:2[trans-9,12] Linoelaidic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C18:2[cis-9,12] Linoleic acid</td>
<td>1148 ± 300</td>
<td>1170 ± 298</td>
</tr>
<tr>
<td>C18:3[cis-6,9,12] gamma-Linolenic acid</td>
<td>19.9 ± 6.7</td>
<td>17.2 ± 3.7</td>
</tr>
<tr>
<td>C21:2[cis-11,14] Eicosadienoic acid</td>
<td>8.6 ± 1.9</td>
<td>9.5 ± 2.6</td>
</tr>
<tr>
<td>C20:3[cis-8,11,14] Dihomo-gamma-linolenic acid</td>
<td>54.1 ± 16.2</td>
<td>54.1 ± 12.9</td>
</tr>
<tr>
<td>C20:4[cis-5,8,11,14] Arachidonic acid</td>
<td>298 ± 52</td>
<td>354 ± 53</td>
</tr>
<tr>
<td>C22:2[cis-13,16] 13,16-Docosadienoic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>n-3 C18:3[cis-9,12,15] alpha-Linolenic acid</td>
<td>17.2 ± 7.7</td>
<td>16.0 ± 5.1</td>
</tr>
<tr>
<td>C20:3[cis-11,14,17] 11,14,17-Eicosatrienoic acid</td>
<td>N/F</td>
<td>N/F</td>
</tr>
<tr>
<td>C20:4[cis-5,8,11,14] Eicosa-5,8,11,14,17-pentaenoic acid</td>
<td>15.1 ± 6.9</td>
<td>14.1 ± 4.6</td>
</tr>
<tr>
<td>C22:6[cis-4,7,10,13,16,19] cis-4,7,10,13,16,19-Docosahexaenoic acid</td>
<td>54.0 ± 19.9</td>
<td>89.9 ± 37.7 *</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>11.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Vitamin E µg/mL</td>
<td>10.27 ± 3.67</td>
<td>10.30 ± 3.687</td>
</tr>
<tr>
<td>Parameter</td>
<td>Control group</td>
<td>Nutri4 group</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td>Selenium µg/L</td>
<td>62.44 ± 9.37</td>
<td>66.37 ± 10.09</td>
</tr>
<tr>
<td>Lutein µmol/L</td>
<td>0.199 ± 0.104</td>
<td>0.199 ± 0.202</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation (SD). <LOQ - below limit of quantification; N/F - not found * p < 0.05 before vs. after within the group (Control or Nutri4); † p < 0.05 difference between the groups

Skin Microvascular Reactivity To Porh, Acetylcholine And Sodium-nitroprusside

PORH was significantly increased in Nutri4 group compared to CTRL group after the study protocol (Fig. 2A). There was no change in microvascular PORH in the CTRL group after the diet protocol compared to the values before diet (Fig. 2A). There was a significant increase in ACh-induced vasodilation in the Nutri4 group after the diet protocol, while consumption of regular eggs did not affect ACh-induced vasodilation in CTRL group (Fig. 2B). Sodium-nitroprusside – induced dilation (SNPID) was similar between the groups before and after respective dietary protocols (Fig. 2C).

PORH, enhanced after Nutri4 eggs consumption was significantly decreased after administration of 100 mg indomethacin in Nutri4 group, while it remained unchanged in the CTRL group (Fig. 3A). AChID remained unchanged in Nutri4 group and CTRL group after the dietary protocol and after intake 100 indomethacin (Fig. 3B).

Protein Expression Of Enzymes Important In The Mechanisms Of Microvascular Reactivity

There was a significant increase in COX-2 protein expression in the Nutri4 group compared to the measurement before dietary protocol and compared to CTRL group after regular hen eggs consumption. There were no significant differences in the protein expressions of nNOS, iNOS, eNOS and COX-1 in any of studied groups (Fig. 4).

Discussion

This randomized, double-blind, placebo-controlled interventional study examined for the first time the effects of consumption of functionally enriched hen eggs (enriched with four nutrients; n-3 PUFAs, vitamin E, lutein, and Se) on endothelium-dependent and endothelium-independent vascular reactivity in microcirculation of healthy young subjects. The important findings of the present study are: 1)
consumption of Nutri4 hen eggs significantly increased the concentration of n-3 PUFAs, significantly decreased n-6 / n-3 ratio, and increased lutein and vitamin E concentration in the serum of participants, without adversely affecting the lipid profile and other measured biochemical values; and 2) there was a positive effect of consumption of Nutri4 hen eggs on microvascular endothelium-dependent vasodilation, which could be potentially attributed to COX-2 produced metabolites of n-3 PUFAs. This is supported by increased expression of COX-2 protein in the Nutri4 group.

It is well documented that n-6 PUFAs - derived vasoconstrictive prostaglandins mediate impaired endothelium-dependent responses in conditions such as hypertension and high salt dietary intake [23]. On the other hand, n-3 PUFAs compete with n-6 PUFAs for pathways for degradation and production of vasoprotective metabolites [23] e.g. they compete for COX - mediated production of prostaglandins and other mediators [24]. In the case of higher n-6 / n-3 PUFAs ratio, prostaglandine series 2 (PGI$_2$), leukotriene series 4 (LTB$_4$) and thromboxane B series 2 (TXB$_2$) are produced, which have vasoconstrictive effects, act as platelet activators and exhibit pro-inflammatory potential [24]. If the ratio goes to higher concentrations of n-3 PUFAs, then the production of anti-inflammatory and vasodilator mediators such as prostaglandins series 3 (PGI$_3$), leukotrienes series 5 (LTB$_5$) and thromboxane series 3 (TXA$_3$) dominates[25]. In present study there was significant increase in serum concentration of EPA and DHA, as well as a decrease in n-6 / n-3 PUFAs ratio and increased serum concentration of vitamin E and lutein in Nutri4 group. These results confirmed our previous findings that consumption of enriched hen eggs provide means to increase nutrients in blood and potentially alter prostaglandins and leukotrienes balance [26, 27]. That had important impact on microvascular reactivity and microcirculatory blood flow, since PORH and AChID were significantly enhanced in the Nutri4 after enriched eggs intake, while there was no change in the control group. This is in agreement with the study by Stupin et al. where AChID after consumption of n-3 PUFAs enriched hen eggs was significantly increased [8]. Hereby, results suggest that mechanisms of enhanced PORH may be related to increased production of COX metabolites, presumably via COX-2 pathway, since peroral administration of indomethacin, non-selective blocker of COX-1,2 attenuated PORH, and expression of COX-2 protein was increased in Nutri4 group. Interestingly, endothelial selenoproteins control the balance of vascular tone by maintaining the balance of superoxide anion/NO, and the synthesis of eicosanoids by regulating the activity of COX1, COX 2 and lipoxygenase [25, 27]. Previously, we have demonstrated that low concentrations of selenium intake (0.030 mg/kg) can cause increased oxidative stress and decreased AChIR response in rats [28]. Since eggs were enriched with nutrients with antioxidant properties (vitamin E, lutein and selenium), one may speculate that part of beneficiary effects on microvascular blood flow may be attributed to alteration of oxidative stress and anti-oxidative defence mechanisms. For example, Barić et al. [9] showed that the use of vitamins E (800 IU/day) and C (1000 mg/day) prevented increased oxidative stress and impairment of microcirculatory function in subjects on a HS diet. However, in present study, serum markers of oxidative stress were not altered by dietary protocols (data not shown).

During the three-week dietary protocol, the biochemical, anthropological and hemodynamic parameters were assessed. Although some of them (e.g. iron, creatinine and glucose) were significantly changed
after dietary protocol, all observed changes were within the reference interval and as such are not physiologically relevant. Similarly, in CTRL group there was a significant increase in LDL after a three-week dietary protocol, however, slightly above referent values. Furthermore, consumption of eggs has not altered liver enzymes or hsCRP, suggesting lack of their potential noxious effects.

Coronary heart disease, acute myocardial infarction, ischemic heart disease is the most often underlined by dyslipidemia. Dietary habits may significantly affect serum lipoproteins [29, 30]. It has been shown that in patients with hypertriglyceridemia n-3 PUFAs in doses of 2 to 4 grams per day lower triglycerides by 25–30%, while total cholesterol does not change [31, 32]. In double-blind controlled study by Tousoulis et al. n-3 PUFAs supplementation (46% EPA and 34% DHA) of 2 grams for 12 weeks improved endothelial function and arterial stiffness in patients with metabolic syndrome [33]. Likewise, long-term consumption of EPA (1600 mg/day) increases NO-dependent and endothelium-independent vasodilation in patients with coronary artery disease [34]. These results are in agreement with our previous study in healthy subjects in which the consumption of n-3 PUFAs enriched eggs showed a beneficial effect on microvascular reactivity, endothelium-dependent vasodilatation and blood pressure, as well as favorable anti-inflammatory properties [7, 8]. Thus, consumption of functional food with beneficial ratio of n-6 /n-3 PUFAs and with increased content of n-3 PUFAs, together with other nutrients may serve as a novel mode in prevention of cardiovascular diseases. This is supported by results of present study that showed significantly increased serum concentration of n-3 PUFAs, lutein and Se and decreased n-6 /n-3 ratio, even in healthy young participants.

There are several limitations to this study. We have not measured the n-3 PUFAs metabolic products of COX-1 or COX-2 pathway. However, there is a clear increase in protein expression of COX-2 in Nutri4 group, while expression of other assessed enzymes was not altered with dietary protocol. Thus observed enhanced endothelium-dependent vasodilation could be attributed to increased concentration of n-3 PUFAs and presumably their metabolites in serum and /or vasculature. Second, although Se levels were not significantly increased, they exhibited tendency to increase in Nutri4 group; therefore, the time period of consumption or amount of eggs of might be extended.

**Conclusion**

Present study is the first randomized controlled trial to evaluate the effects of consumption of functional eggs on microvascular function in healthy humans. Consumption of eggs enriched with n-3 PUFAs, vitamin E, selenium and lutein has a beneficial effect on the microcirculation of healthy young subjects. These beneficial effects can be attributed to mostly n-3 PUFAs components of enriched eggs. Consumption of enriched eggs enhanced microcirculatory blood flow, which could be, at least partly, mediated by vasodilator metabolites of the COX-2 pathway of n-3 PUFAs degradation. Likewise, consumption of hen eggs does not have a negative effect on biochemical parameters, such as serum lipid profile and liver enzymes’ concentration, thus rendering the eggs healthy dietary choice.

**Abbreviations**
EDV
Endothelium-dependent vasodilation

n-3 PUFA
n-3 polyunsaturated fatty acids

PORH
Post-occlusive reactive hyperemia

ACHID
Acetylcholine

SNPID
Sodium nitroprusside

EPA
Eicosapentaenoic acid

DHA
Docosahexaenoic acid

ALA
Alpha-linolenic acid

CV
Cardiovascular diseases

BP
Blood pressure

COX
Cyclooxygenase

ROS
Reactive oxygen species

Se
Selenium

NO
Nitric oxide

kg
Weight

m
Height

BMI
Body mass index

HR
Heart rate

LDF
Laser Doppler flowmetry

AUC
Area under the curve
PBMC
Peripheral Blood Mononuclear Cell
EDTA
Ethylenediaminetetraacetic acid
PBS
Phosphate-buffered saline
iNOS
Inducible nitric oxide synthase
eNOS
Endothelial nitric oxide synthase
nNOS
Neuronal nitric oxide synthase
HRP
Horse radish peroxidase
HDL
high-density lipoprotein
LDL
low-density lipoprotein
hsCRP
High-sensitivity C-reactive protein
PGI₂
Prostaglandine series 2
LTB₄
leukotriene series 4
TXB₂
thromboxane B series 2
PGI₃
prostaglandins series 3
LTB₅
leukotrienes series 5
TXA₃
thromboxane series 3

Declarations

Author Contributions

I.D; Resources: I.D; Supervision, Z.M. and I.D; Validation, G.K and I.D; Visualization, P.Š, Z.M and A.S; Writing – original draft: P.Š and I.D; Writing – review & editing: P.Š, Z.M and I.D.

Availability of data and materials

The data presented in this study are available on request from the corresponding author.

Consent for publication

Not applicable.

Funding

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Ethics approval and consent to participate

The study protocol and procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by the Ethical Committee of the Science Center of Excellence, Josip Juraj Strossmayer University of Osijek (Cl: 602-04/14-08/06; No: 2158-610714-114) and Ethics Committee of the Medical Faculty Osijek (Cl: 602-04/20-08/07, Registration number: 2158-61-07-20147). All the participants provided written informed consent for their clinical data to be used for research purposes.

Competing interests

The authors declare that they have no competing interests.

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References


34. Tagawa H, Shimokawa H, Tagawa T, et al. Long-Term Treatment with Eicosapentaenoic Acid Augments Both Nitric Oxide-Mediated and Non-Nitric Oxide-Mediated Endothelium-Dependent


**Figures**

**Figure 1**

Consort study protocol
Figure 2

Panel A. Post-occlusive reactive hyperemia (PORH) before and after the dietary protocol. Results are shown as mean ± standard deviation (SD); * p < 0.05 before or after within the group (Control group or Nutri4 group).
Panel B. Forearm skin microcirculation by iontophoresis - AChID before and after the dietary protocol. Results are shown as mean ± standard deviation (SD); * p < 0.05 before or after within the group (Control group or Nutri4 group).

Panel C. Forearm skin microcirculation by iontophoresis sodium nitroprusside induced dilation - SNPID before and after the dietary protocol.
Figure 3

Panel A. Post-occlusive reactive hyperemia (PORH) before and after the dietary protocol with/ without administration of indomethacin. This part of measurements were performed on a separate set of participants, due to measurements design. Results are shown as mean ± standard deviation (SD); *p < 0.05 paired t-test before and after dietary protocol with administration indomethacin. † p < 0.05 paired t-test before and after dietary protocol without administration indomethacin.

Panel B. Forearm skin microcirculation by iontophoresis-AChID before and after administration of 100 mg indomethacin. This part of measurements were performed on a separate set of participants, due to measurements design.
Figure 4

Relative protein expression and representative blots of (a) COX-1 – cyclooxygenase 1; (b) COX-2 – cyclooxygenase 2; (c) iNOS – inducible nitric oxide synthase; (d) eNOS – endothelial nitric oxide synthase; and (e) nNOS - neuronal nitric oxide synthase in isolated peripheral blood mononuclear cells (PBMCs), determined by Western blot method. Results are shown as mean + standard deviation (SD); *p < 0.05 paired t-test before and after dietary protocol (Control or Nutri4); #p < 0.05 One-way ANOVA test difference between the groups.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ConsortChecklistunjaraetal.2022.doc