Original Contribution

Effect of fish and krill oil supplementation on glucose tolerance in rabbits with experimentally induced obesity

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Abstract

Purpose  This study was conducted to investigate the effect of fish oil (FO) and krill oil (KO) supplementation on glucose tolerance in obese New Zealand white rabbits.

Methods  The experiments were carried out with 24 male rabbits randomly divided into four groups: KO—castrated, treated with KO; FO—castrated, treated with FO; C—castrated, non-treated; NC—non-castrated, non-treated. At the end of treatment period (2 months), an intravenous glucose tolerance test (IVGTT) was performed in all rabbits.

Results  Fasting blood glucose concentrations in FO and KO animals were significantly lower than in group C. The blood glucose concentrations in FO- and KO-treated animals returned to initial values after 30 and 60 min of IV-GTT, respectively. In liver, carnitine palmitoyltransferase 2 (Cpt2) and 3-hydroxy-3-methyl-glutaryl-CoA synthase 2 (Hmgcs2) genes were significantly increased in FO-fed rabbits compared with the C group. Acetyl-CoA carboxylase alpha (Acaca) expression was significantly reduced in both KO- and FO-fed rabbits. In skeletal muscle, Hmgcs2 and Cd36 were significantly higher in KO-fed rabbits compared with the C group. Acaca expression was significantly lower in KO- and FO-fed rabbits compared with the C group.

Conclusion  The present results indicate that FO and KO supplementation decreases fasting blood glucose and improves glucose tolerance in obese New Zealand white rabbits. This could be ascribed to the ameliorated insulin sensitivity and insulin secretion and modified gene expressions of some key enzymes involved in \( \beta \)-oxidation and lipogenesis in liver and skeletal muscle.

Keywords  Fish oil · Krill oil · Glucose tolerance · Obesity · Gene expression · Lipogenesis
Introduction

Obesity is often associated with insulin resistance (IR) and deterioration of lipid and glucose metabolism, which are the hallmarks of metabolic syndrome [1–6]. Briefly, IR can be defined as an inadequate response by insulin target tissues, such as skeletal muscles, adipose tissue and liver, to insulin exposure [7–10]. The mechanisms of decreased insulin sensitivity include reduced insulin-stimulated glucose uptake and metabolism in skeletal muscle and adipose tissue, impaired inhibition of hepatic glucose production, i.e., gluconeogenesis and glycogenolysis, and a reduced ability of insulin to inhibit lipolysis in adipose tissue [2, 10, 11]. In humans, IR can be manifested as one of the following three symptoms: diabetes mellitus type 2 (plasma fasting glucose concentration >7 mmol/l); impaired glucose tolerance (plasma glucose concentration 2 h after glucose oral tolerance test between 7.8 and 11 mmol/l); and impaired fasting glucose (plasma glucose concentration between 6.1 and 6.9 mmol/l).

There is a growing body of evidence showing that the consumption of products containing marine n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) can positively affect IR [4, 11–16].

Two possible sources of marine n-3 LC-PUFAs are fish oil (FO) and krill oil (KO). KO is proposed as a nutritional supplement during the last years, and an increasing number of studies demonstrate health benefits in rodents (mice and rats) as well as humans [14, 16–18]. KO is extracted from Antarctic krill (Euphausia superba) and has a unique chemical composition. Both FO and KO are rich in n-3 LC-PUFAs, but KO contains the fatty acids predominantly in the form of phospholipids (PL) rather than triglycerides (TG) as in FO [16, 17]. In addition to its high content in PL, KO contains the antioxidant astaxanthin that gives KO its dark red color and might help to protect the unsaturated bonds from oxidative damage [17]. The preclinical studies have shown that exogenous KO supplementation improves lipid profile, decreases body weight, reduces endocannabinoid biosynthesis, decreases liver fat infiltration and oxidative stress and increases hepatic β-oxidation [14, 18–22]. The clinical studies demonstrate that KO improves blood lipid profile, changes endocannabinoid concentration, reduces oxidative damage and increases blood level of n-3 PUFAs [15, 23–27]. However, despite these studies indicating that dietary KO supplementation has beneficial metabolic and anti-inflammatory effects in rodents and humans, there is little basic research investigating its effect on glucose tolerance and insulin resistance.

Rabbits have several advantages over mice as an animal model to study various obesity-associated metabolic abnormalities such as dyslipidemia, atherosclerosis, MS and IR, since their lipid profile and metabolism is similar to that of humans (so-called LDL mammals) and differ from mice and rats (so-called HDL mammals). In addition, they have high levels of ApoB-containing lipoproteins and cholesterol ester transfer proteins and are very susceptible to the development of atherosclerosis, with lesions resembling those in humans [28–31].

Studies in rodents, and to a lesser extend in humans, have demonstrated that FO has positive effects on some parameters of glucose homeostasis [4, 11, 13, 32–35]. However, no data on the effects of FO and KO on insulin resistance and β-cell function are available in rabbits.

Therefore, this study was conducted to investigate the effect of FO and KO supplementation on glucose tolerance in rabbits with experimentally induced obesity by castration.

Materials and methods

Animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, and the Guidelines of the Animal Welfare Act, and were approved by the Commission of Ethics at the Faculty of Veterinary Medicine of Trakia University, Stara Zagora.

The experiments were carried out with 24 male New Zealand white rabbits. At the beginning of the experiment, they were between 3 and 3.5 months old. The animals were housed in individual cages (80 × 60 × 40 cm). The light/dark regime corresponded to the circadian cycle. The rabbits were given free access to water. They were fed a commercially available standard chow diet for adult rabbits given as dry pellets. During the experimental period, all rabbits received the same amount of energy by the diet ≈450 kcal/daily. Before the randomization to groups, initial body weight was determined and blood samples were taken from each rabbit for the determination of the pre-castrations values of blood glucose and plasma insulin.

The rabbits were randomly divided into four groups: KO—castrated, treated with KO; FO—castrated, treated with FO; C—castrated, full diet fed; and NC—non-castrated.

The castration was used to induce obesity in rabbits [31]. The castration of the rabbits was performed under general anesthesia using Anaket 10 %. For surgery, the rabbits were laid on their backs, the fur in the scrotal area was depilated, and the skin was disinfected. The scrotal wounds after castration remained open [31].

The KO and FO were kindly provided by Aker Bio-Marine Antarctic AS Oslo, Norway. They were given as gelatinous capsules at a dose of 600 mg omega-3 PUFA daily for a period of 60 days.

On the day before intravenous glucose tolerance test (IVGTT), body weight, body weight gain and body mass
index (BMI) were determined as markers of obesity. BMI was calculated using the equation = body weight (kg)/ height² (m). The height was measured as distance between the shoulder joint and the end of the paw at the lateral position of the rabbit.

At the end of the treatment period of 2 months after castration, an intravenous glucose tolerance test was performed in all rabbits as previously described [31, 36, 37]. Briefly, food was removed for 12 h overnight, and a bolus of 40 % glucose (0.6 g/kg) was injected through the ear vein. Blood samples for the determination of glucose and insulin concentration were obtained at 0, 10, 30, 60 and 120 min after glucose administration. The blood samples were centrifuged immediately after the collection at 800×g for 15 min. Plasma for the determination of insulin was stored in plastic tubes at −20°C until assayed. Glucose concentration was measured in whole blood.

Blood glucose and insulin analysis

The blood glucose concentration was measured immediately after collection of the samples with a blood glucose monitoring system (BIONIME Gmbh, Heerbrugg, Switzerland) based on the glucose oxidase method using one drop of whole blood. Plasma insulin concentration was measured by radioimmunoassay with a commercially available kit adapted for rabbits (Immunotech, Prague, Czech Republic).

Indexes of insulin sensitivity

The indexes of insulin sensitivity were calculated as described in cats [38]. Fasting insulin (I₀) and fasting insulin to glucose (G₀) ratio (I₀/G₀) were determined before glucose injection. Insulin concentration (I₆₀ min and I₁₂₀ min) and insulin to glucose ratio (I₆₀ min/G₆₀ min and I₁₂₀ min/G₁₂₀ min) were calculated also after 60 and 120 min after glucose infusion.

The following kinetic parameters, indicative for the “fate” of glucose, were estimated: half-life of plasma glucose (t½ glucose) and area under the curve (AUC) of glucose and insulin concentrations (AUC glucose 0–120 min and AUC insulin 0–120 min). Kinetic parameters were calculated with Phoenix 6.01 (Pharsight Corporation, Mountain View, USA). AUC glucose 0–120 min and AUC insulin 0–120 min were calculated by the trapezoidal rule. t½glucose was calculated by linear regression analysis of the semilogarithmic plot of glucose concentration versus time.

Some simplified estimates of insulin sensitivity were calculated from insulin and glucose values at baseline: HOMA insulin resistance, QUICKI and the Bennett index [38–41].

QuickI = 1/logI₀ + logG₀ [38, 40, 42]; and (3) Bennett index = 1/logI₀ × logG₀ [38, 41], where I₀ is the amount of fasting insulin (µU/ml) and G₀ is the fasting glucose value (mmol/l). Higher HOMA insulin resistance and lower QUICKI and Bennett indexes are indicators of increased insulin resistance.

Indexes of β-cell function

HOMA β-cell was calculated using the equation:

HOMA β-cell = (20 × I₀)/(G₀ − 3.5) [39, 42].

The indexes characterizing the first or early phase of insulin secretion and insulin secretion during the first and second hours after glucose loading were calculated as shown in dogs [43, 44]. The highest values of insulin and glucose were considered peak values, and the increments of insulin and glucose concentration above their respective fasting levels were considered as ΔI and ΔG [43]. Early-phase insulin secretion in response to glucose infusion was calculated as the insulinoergic index (ΔI/ΔG), AUC for insulin determined from 0 to 10 min (AUC insulin 0–10 min) and insulin to glucose ratio after 10 min (I₁₀ min/G₁₀ min). Insulin secretion during the first and second hour after IVGGT was calculated as AUC insulin 0–60 min and AUC insulin 0–120 min, respectively [43, 44].

Histological examination

Materials for histological examination were taken from the liver after the animals were killed. Briefly, after fixation in Bouin’s fixative and in 10 % neutral-buffered formalin, the tissue specimens were embedded in paraffin, cut into 5–6-µm-thick section and stained with hematoxylin and eosin.

Enzyme activity

Frozen liver samples were homogenized and the post-nuclear fraction isolated as described earlier [45]. The assay for carnitine palmitoyltransferase (CPT)-2 was performed according to Bremer [46], but with some modifications: The reaction mix contained 17.5 mM HEPES pH 7.5, 52.5 mM KCl, 100 mM palmitoyl-CoA and 0.01 % Triton X-100. The reaction was initiated with 100 µM [methyl-14C]-L-carnitine (1,100 cpm/nmol), and 35 µg total protein was used [47]. The activity of fatty acyl-CoA oxidase (ACOX)-1 and fatty acid synthase (FAS) were measured in post-nuclear fractions using 20 µg protein and 60 µg, respectively, as described by Madsen et al. [48] and Skorve et al. [47].

Gene expression analysis

Total RNA was purified and isolated from homogenized pieces of liver and muscle of approximately 20 and 30 mg,
respectively, using the RNAeasy Mini Kit from Qiagen (Hilden, Germany). For muscle samples, an extra step of proteinase K treatment was added (Qiagen). The quantity of the RNA was measured spectrophotometrically using NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA). The purity of the RNA was assayed using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA). The quality limit for further use of RNA was set to a RNA integrity number (RIN) ≥ 7 (out of 10). cDNA was obtained using the High Capacity Reverse Transcriptase Kit with RNase inhibitor (Applied Biosysstem, Foster City, USA). Real-time PCR was performed on an ABI prism 7900 H sequence detection system (Applied Biosystems) using 384-well multiply PCR plates (Sarstedt Inc., Newton, USA), SYBR Select Master Mix from Applied Biosystems and gene-specific primers from Sigma–Aldrich (Table 1). Hypoxanthine phosphoribosyltransferase (Hprt1) and 18s were used as reference genes. For 18s, primers from the 18s Genomic Control Kit (#RT-CKFT-18s) were used (Eurogentec, Seraing, Belgium). Dilutions of pooled cDNA were used for the standard curves. NormFinder (http://moma.dk/normfinder-software) was used to assess the reference genes, and hepatic mRNA levels were normalized to Hprt1, while muscle tissue was normalized to 18s. Gene names and sequences of specific primers, including reference genes, are presented in Table 1.

Statistical analysis

The statistical analyses were performed using Statistica version 7.1 for Windows (StatSoft Inc., USA, 1984–2002). The descriptive statistical tests, including the mean and standard error of the mean, were calculated according to the standard methods. The ANOVA test was used to evaluate the difference between means of glucose, insulin and pharmacokinetic parameters between groups. When the effect of groups was significant, the differences between groups were determined by means of the LSD test of the post hoc procedure. For the difference between means of mRNA levels and enzyme activity, Dunnet’s multiple comparison test, comparing all groups to control, was used post hoc. The paired t test was applied to assess the difference of quantitative variables between dependent groups (concentrations of glucose and insulin at different times of sampling). All data are presented as mean ± standard error of the mean (mean ± SEM). The significance of differences was preset at P < 0.05.

Results

Body weight

There were no group differences in pre-castration body weights (Table 2). After 60 days of treatment, the mean body weights, body weight gain and BMI of the FO and KO groups were lower compared with group C (Table 2).

Blood glucose and plasma insulin concentration

Pre-castration values of glucose and insulin concentration were not different among groups (Figs. 1, 2). Fasting blood glucose concentrations at 0 min of IVGTT in FO and KO were significantly lower than in C animals (Fig. 1). Exogenous glucose injection caused a sharp increase in blood glucose concentration after 10 min, the highest values being found in group C (Fig. 1). In all groups, a gradual decrease in glucose level was found over time. After 30 min, the glucose concentration had already returned to the initial values in the FO group and was not statistically different from that in NC animals. After 60 min, glucose concentration in the KO group returned to baseline (P > 0.05) and was lower compared with C animals.

Fasting plasma insulin in the C group was significantly higher when compared to the other three groups—KO, FO and NC (Fig. 2). Glucose injection caused a marked increase in insulin concentration after 10 min, the peak values being found in the C and FO groups. Thereafter, an apparent decrease in plasma insulin concentration was noted at the 30th min. The reduction was most prominent

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hprt</td>
<td>Hypoxanthine phosphoribosyltransferase</td>
<td>cccacgcgttgattagtg</td>
<td>gctccactctctctcata</td>
</tr>
<tr>
<td>Irs1</td>
<td>Insulin receptor substrate 1</td>
<td>actactcactgccaaggtcc</td>
<td>atagagaagcgacagacgc</td>
</tr>
<tr>
<td>Pklr</td>
<td>Pyruvate kinase liver and RBC</td>
<td>tacattgacgacgggctcat</td>
<td>tccgcacaaagagccaaaa</td>
</tr>
<tr>
<td>Hmgcs2</td>
<td>3-Hydroxy-3-methyl-glutaryl-CoA synthase 2 (mitochondrial)</td>
<td>cacacacacaggaacagt</td>
<td>ggaacacggaagagaaaaa</td>
</tr>
<tr>
<td>Cpt2</td>
<td>Carnitine palmitoyltransferase 2</td>
<td>agegacacacacacttcaaa</td>
<td>aacgcggatgtaagagc</td>
</tr>
<tr>
<td>Cd36</td>
<td>CD36 molecule/fatty acid translocase</td>
<td>tgctagacatgcaaggt</td>
<td>agegcgttgctctgtaaag</td>
</tr>
<tr>
<td>Acaca</td>
<td>Acetyl-coenzyme A carboxylase alpha</td>
<td>gggtcagtctctctaactct</td>
<td>actccacagatccattc</td>
</tr>
</tbody>
</table>
(more than ten times) in FO-treated animals. At the same time point, plasma insulin levels in C animals were significantly higher than in FO, KO and NC groups. After 60 and 120 min, there were no differences between groups.

Indexes of insulin resistance

Similar to fasting insulin, the insulin to glucose ratio at baseline in C animals tended to be higher than in KO, FO
and NC groups (Table 3). AUCglucose 0–120 min in group C was significantly higher than in KO, FO and NC animals. The half-life of plasma glucose ($t_{1/2}$ glucose) in KO and FO groups was significantly lower than in group C and similar to that in NC group. AUCinsulin 0–120 min in C was significantly higher than in KO and NC groups.

The HOMAins. res. index in the C group tended to be higher than in KO, FO and NC groups, while no differences between KO, FO and NC rabbits were found.

Indexes of β-cell function

The insulinogenic index ($AI/AG$) in FO animals was significantly higher than in C, KO and NC groups (Table 4). HOMA-β-cell index in group C was higher when compared to KO, FO and NC groups. The first phase of insulin secretion measured as AUCinsulin 0–10 min in the C group was higher than in KO and NC groups. Insulin secretion during the first hour after glucose injection (AUCinsulin 0–60 min) in C and FO was significantly higher as compared to NC. Insulin secretion during the second hour in the C group, measured as AUCinsulin 60–120 min, was significantly higher than after KO and FO treatment.

Gene expression in liver and skeletal muscle

The hepatic expression of genes involved in lipid and glucose metabolism was determined. The PPARα-regulated gene, carnitine palmitoyltransferase 2 (Cpt2), was significantly increased in FO-fed rabbits as well as NC rabbits, compared with the C group (Fig. 3a). Another PPARα-regulated gene, 3-hydroxy-3-methyl-glutaryl-HMG CoA synthase 2 (Hmgcs2), was also significantly increased in FO-fed rabbits and NC rabbits compared with C (Fig. 3b). The fatty acid transporter Cd36 was, however, not affected by any of the treatments (Fig. 3c). The rate-limiting gene in lipogenesis, acetyl-CoA carboxylase alpha (Acaca), was significantly reduced at the mRNA level in both KO- and FO-fed rabbits (Fig. 3d). The protein encoded by the gene pyruvate kinase (Pklr1) is the rate-limiting step in glycolysis, and its mRNA level was significantly reduced compared with C in FO-fed rabbits (Fig. 3e). The gene expression of insulin receptor substrate 1 (Irs1) was not significantly affected by KO or FO treatment (Fig. 3f).

In skeletal muscle, the Cpt2 mRNA level was significantly higher in NC rabbits, while Hmgcs2 and Cd36 were significantly higher in KO-fed and NC rabbits compared with the C group (Fig. 4a–c). Acaca expression was significantly lower in KO- and FO-fed rabbits and significantly higher in NC rabbits compared with the C group (Fig. 4d). The gene expression of Irs1 was not significantly affected (Fig. 4e).

Table 3 Insulin resistance indexes in rabbits

<table>
<thead>
<tr>
<th>Indexes of insulin sensitivity</th>
<th>Abbreviations</th>
<th>KO vs C</th>
<th>FO vs C</th>
<th>C vs NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin/glucose ratio at baseline</td>
<td>I0/G0</td>
<td>0.43 ± 0.10</td>
<td>0.50 ± 0.09</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>Insulin/glucose ratio at 60 min</td>
<td>I60/G60</td>
<td>0.27 ± 0.07</td>
<td>0.42 ± 0.07</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Insulin/glucose ratio at 120 min</td>
<td>I120/G120</td>
<td>0.27 ± 0.07</td>
<td>0.54 ± 0.09</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>HOMA insulin resistance index</td>
<td>0.97 ± 0.06</td>
<td>1.74 ± 0.17</td>
<td>0.74 ± 0.07</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>QUICKI index</td>
<td>0.56 ± 0.07</td>
<td>0.69 ± 0.09</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Benet index</td>
<td>0.25 ± 0.07</td>
<td>0.21 ± 0.09</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

All values are mean ± SEM: KO—castrated and fed krill oil; FO—castrated and fed fish oil; C—castrated non-treated; NC—non-castrated non-treated; NS—not significant.
Hepatic enzyme activity

Liver CPT2 activity and ACOX1 activity were not affected by KO or FO diets compared with the C diet (Fig. 5a, b). FO demonstrated a significantly lower level of FAS activity compared with C (Fig. 5c).

Histological examination

Light microscopy of liver samples in rabbits from group C showed bright vacuoles in the hepatocytes, indicating marked fat infiltration. No fat infiltration was found in liver samples from FO- and KO-treated animals, the histological pictures being similar to that in non-castrated non-treated rabbits (Fig. 6).

Discussion

In the current study, we used a model of obesity based on the castrated male New Zealand white rabbits developed in our laboratory. The generation of this model has been previously described [31]. This is the first study demonstrating the effect of KO and FO on glucose homeostasis parameters in this model of obesity in rabbits. The major finding in this study was that FO and KO supplementation decreased fasting blood glucose and improved glucose tolerance in obese New Zealand rabbits. In addition, FO and KO affected the expression levels and/or activity of some key enzymes involved in β-oxidation and lipogenesis in liver and skeletal muscle.

The results from IVGTT indicated marked hyperinsulinemia in castrated non-treated rabbits, which is in accordance with our previous results [31] and is an important feature of early stage of decreased insulin sensitivity in obesity [7, 9, 49, 50]. This was further confirmed by the calculated indexes of β-cell function (HOMAβ-cell, AUCinsulin 0–10 min, AUCinsulin 0–60 min, AUCinsulin 60–120 min), showing a compensatory increase in insulin secretion both at fasting and during the first 2 h after glucose loading after IVGTT. The significant reduction in plasma insulin concentration at 0 min and after 30 min during the IVGTT and the observed changes in some of the simplified indexes of insulin resistance after treatment (I0/G0 ratio; HOMAins. res.; AUCglucose 0–120 min, AUCinsulin 0–120 min and t1/2 glucose) suggest improvement of glucose tolerance in FO- and KO-treated animals.

Previously obtained results in our laboratory showed that castration in male New Zealand white rabbits caused a marked increase in body weight [31]. In addition, they developed obesity resembling human visceral type of obesity which is one of the main predisposing factor for insulin resistance in humans [3–7, 51, 52], as well as in

### Table 4 - β-cell function indexes in rabbits

<table>
<thead>
<tr>
<th>Indexes of β-cell function</th>
<th>Abbreviations</th>
<th>KO (fish oil)</th>
<th>FO (krill oil)</th>
<th>C (control)</th>
<th>NC (non-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulinogenic index</td>
<td>D</td>
<td>1.53 ± 0.5</td>
<td>6.25 ± 0.3</td>
<td>5.00 ± 1.0</td>
<td>5.00 ± 1.0</td>
</tr>
<tr>
<td>Insulin/glucose ratio at 10 min</td>
<td>I10min/G10min</td>
<td>2.15 ± 0.3</td>
<td>5.00 ± 1.0</td>
<td>5.00 ± 1.0</td>
<td>5.00 ± 1.0</td>
</tr>
<tr>
<td>HOMAβ-cell index</td>
<td></td>
<td>19.4 ± 3.5</td>
<td>47.64 ± 10.2</td>
<td>164.7 ± 10.2</td>
<td>427.8 ± 10.2</td>
</tr>
<tr>
<td>AUCinsulin 0–10 min µU/ml</td>
<td></td>
<td>469.4 ± 10.2</td>
<td>1,641.7 ± 10.2</td>
<td>2,224.5 ± 10.2</td>
<td>427.8 ± 10.2</td>
</tr>
<tr>
<td>AUCinsulin 0–60 min µU/ml</td>
<td></td>
<td>189.3 ± 3.5</td>
<td>574.6 ± 10.2</td>
<td>783.4 ± 10.2</td>
<td>427.8 ± 10.2</td>
</tr>
<tr>
<td>AUCinsulin 60–120 min µU/ml</td>
<td></td>
<td>374.1 ± 3.5</td>
<td>574.6 ± 10.2</td>
<td>783.4 ± 10.2</td>
<td>427.8 ± 10.2</td>
</tr>
</tbody>
</table>

All values are mean ± SEM: KO—castrated and fed krill oil; FO—castrated and fed fish oil; C—castrated non-treated; NC—non-castrated non-treated; NS—not significant.
rabbits [30, 31, 37]. In our current study, the castrated rabbits (C) received the same amount of energy by the diet as non-castrated rabbits (NC) but gained significantly more weight proving that castration is an obesogenic factor.

The improved glucose homeostasis observed in our study could partly be attributed to PPARs activation by the omega-3 PUFAs in FO and KO. Two main mechanisms of an insulin sensitizing effect of PPARγ activation in adipose tissue have been revealed: Stimulation of adipocyte differentiation leading to protection of non-adipocyte tissue (skeletal muscle, liver) against excessive lipid accumulation and stimulation of an adequate secretion of some adipokines such as adiponectin and leptin which are important mediators of insulin action in insulin-sensitive
tissue [32, 53, 54]. In contrast to group C, where the histological analysis showed an abundant liver fat infiltration, the liver samples of FO- and KO-supplemented rabbits were similar to that of the NC group, which may in part explain the improved insulin sensitivity in these rabbits since fatty liver and simple steatosis in humans is often accompanied by insulin resistance and deterioration in glucose metabolism [55, 56]. Our data are in accordance with the results in rats and mice showing that FO and KO suppress hepatic steatosis by decreasing TG accumulation in liver [14, 18, 58]. This effect of omega-3 PUFAs might be due to the combined effects of inhibition of lipogenesis and stimulation of fatty acid oxidation [57–59]. Omega-3 PUFAs down-regulate the mature form of sterol regulatory element-binding protein 1, which is the main activator of genes encoding for lipogenic enzymes such as fatty acid synthase, acetyl-CoA carboxylase and stearoyl-CoA desaturase [57, 58]. At the same time, omega-3 PUFAs up-regulate the activity of PPARα in liver, which is the main activator of β-oxidation [58, 60]. This was confirmed by our data on the expression levels of some hepatic genes involved in lipid metabolism. For example, the hepatic gene expression of one of the rate-limiting enzymes in de novo lipogenesis, acetyl-CoA carboxylase, was significantly lower in rabbits fed FO and KO than in C. However, the hepatic activity of the other key enzyme in fatty acid synthesis, the fatty acid synthase complex (FAS), was affected by FO only. Therefore, the explanation of these results needs additional studies as the histological analysis of liver samples showed that in contrast to group C, no fat infiltration in both FO- and KO-supplemented groups was found. We found that FO increased expression of the PPARα-dependent gene Cpt2 in liver and to a lesser extend in skeletal muscle and Hmgcs2 in liver. In addition to its role as a rate-limiting enzyme in ketogenesis and cholesterol synthesis, recently it has been shown that mitochondrial Hmgcs2 is involved in stimulation of β-oxidation by its ability to interact with PPARα and act as co-activator to up-regulate transcription from PPRE of its own gene [61, 62]. FO and KO did not significantly affect the concentration of mRNA encoding for the transmembrane fatty acid transporting protein—CD36 in liver. At the same time, KO increased the expression of Cd36 and Hmgcs2 genes in skeletal muscles. In FO- and KO-supplemented animals, there were no significant changes in Irs1 expression in liver and skeletal muscle, suggesting no marked effect of exogenous FO and KO on the early steps of the insulin transduction pathway. Therefore, the observed amelioration of glucose tolerance and fasting blood glucose concentration by FO and KO might be at least in part due to the enhanced intracellular transport and oxidation of non-esterified fatty acid in skeletal muscles and/or liver, as their acyl-CoA derivatives are one of the main factors decreasing insulin sensitivity [7, 9, 63]. The results of Neschen et al. [32] showed that omega-3 fatty acids protect from high-fat-induced hepatic insulin resistance in a PPARα activation manner in rats. Interestingly, in liver, CPT-2 was increased by FO at the mRNA level only while the activity of this enzyme did not change after KO and FO administration. In addition, no effect of treatment on ACOX-1
activity was found. We hypothesize that this is due to the fact that omega-3 PUFAs are ligands of PPARs and as such they predominantly regulate gene expressions at the transcriptional level, while the activity of CPT-2 and other enzymes involved in lipid and glucose metabolism is probably also regulated at posttranscriptional, translational or posttranslational levels. Therefore, in order to prove this hypothesis, additional studies are needed to measure the same parameters at mRNA and at protein levels. The reduction in gene expression of the key enzyme of glycolysis, PKLR1, indicated decreased glucose degradation in liver and, therefore, some glucose-sparing effect of omega-3 PUFA treatment, especially in the form of FO. As such glucose remains available as an energy source (for example in neurons and red blood cells) or for glycogen synthesis in skeletal muscle and liver.

On the other hand, it has been found that in high-fat-fed rats FO improved glucose tolerance by enhancing insulin secretion from pancreatic β-cells, which was also confirmed in an in vitro study on pancreatic islets [12]. Our results are in line with this data as FO-treated rabbits exhibited enhanced first phase of insulin secretion (AUC insulin 0–10 min), greater insulinogenic index (ΔI/ΔG), describing the increment of insulin after glucose infusion, and enhanced insulin secretion during the first and second hour after glucose injection during the IVGTT (AUC insulin 0–60 min and AUC insulin 60–120 min).

In a recent study, Tandy et al. [14] found that dietary KO supplementation reduced hepatic steatosis and blood glucose concentration in high-fat-fed mice in a dose-dependent manner. It has been suggested that omega-3 PUFA ingested in the form of phospholipids, as in KO, is characterized by a higher bioavailability and tissue uptake probably resulting in different molecular effects and tissue specificities [16–18, 20]. It has been reported that the effects of KO on lipid and glucose metabolism were stronger than those observed with FO, which may be attributed to an increased efficacy of omega-3 PUFA in PL form (KO) compared with the TG form (FO) and to their different tissue distribution [16]. However, in our study, the

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**Fig. 6** Morphological features of liver. **a** Castrated non-treated rabbits (group C); **b** non-castrated (group NC); **c** castrated and fed krill oil (group KO); **d** castrated fed fish oil (group FO); **vc** vena centralis; **arrow** normal hepatocytes; **arrowhead** hepatocytes with fat infiltration; H/E (bar a, b, c, d = 25 μm, ×40)
effects of KO and FO on fasting glucose and glucose tolerance in obese rabbits were similar. In humans, it has been found that the metabolic effects of KO were essentially similar to those of FO, but at a lower dose of EPA and DHA [15]. In rabbits, however, FO and KO have similar effects on glucose homeostasis parameters with the same dose of EPA and DHA.

One limitation of this investigation is that the differential effects of KO and FO compared with castrated nontreated rabbits (group C) could be mediated by the differences in body weight. In addition, we determined the concentration of key enzymes and factors involved in lipid and glucose metabolism predominantly at mRNA level. Therefore, more studies are needed to measure the same parameters at protein level in order to better understand the molecular mechanism of improved glucose homeostasis after FO and KO treatment.

In conclusion, the present results indicated that both FO and KO supplementation decreased fasting blood glucose, improved glucose tolerance and reduced hepatic steatosis in obese New Zealand white rabbits. These effects could be ascribed to the ameliorated insulin sensitivity and insulin secretion by omega-3 PUFAs as shown by the changes in calculated indexes of insulin resistance and \(\beta\)-cell function and the modified gene expression levels of key enzymes and factors involved in \(\beta\)-oxidation and lipogenesis in liver and skeletal muscles. We found that the effects of FO and KO on investigated glucose homeostasis parameters were similar, suggesting the eventual use of KO as an alternative source of omega-3 PUFA in individuals with metabolic syndrome and obesity to improve insulin resistance and \(\beta\)-cell function.

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Conflict of interest The authors declare no conflict of interest.

References


