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Distinct effects of virgin coconut oil supplementation on the glucose and lipid homeostasis in non-diabetic and alloxan-induced diabetic rats

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ABSTRACT

Non-diabetic and alloxan-induced diabetic rats were fed with standard laboratory food enriched with 20% virgin coconut oil for 16 weeks. In non-diabetic animals coconut oil improved insulin sensitivity and ability to control glycaemia and decreased the serum triglycerides for almost 50% in comparison with controls. Supplementation with coconut oil caused liver steatosis in both non-diabetic and diabetic animals. However, the severity of steatosis was lower in diabetic animals compared to non-diabetic animals. Coconut oil had no effects on heart histology, ascending and abdominal aorta wall thickening and atherosclerotic plaques development neither in non-diabetic nor in diabetic animals. While alloxan treatment caused Type I diabetes in rats, supplementation with coconut oil in combination with the alloxan unexpectedly resulted in Type II diabetes. The development of severe insulin resistance and deterioration in serum lipid profile implied that the use of coconut oil is contra-indicated in diabetic condition.

1. Introduction

The idea that the type of dietary regime could be linked with the cardiovascular diseases (CVD) aroused in the mid-20th century (Blackburn, 2012). Cornelis D. de Langen first noticed that native Javanese rarely exhibited thrombosis and emboli, probably because of the local vegetarian diet with low cholesterol and other lipid content (De Langen, 1935). Similarly, Isidore Snapper observed that CVD were practically non-existent among low-income individuals in North China owing to the cereal-vegetable based diet (Snapper, 1941). Based on the results of the study conducted on Minnesota businessmen, Ancel Keys argued that hypercholesterolemia was significantly correlated with CVD development (Keys et al., 1963). This study was the first systematic cohort study which measured a number of potential risk factors for their value in predicting heart attacks in healthy participants. It had no a priori hypotheses, and it focused on a group consisting of a single socioeconomic class, with an idea to compare men who developed CVD to those who did not develop it. The investigators found the increasing risk of developing CVD with higher cholesterol level. Strength of this study was a long follow-up period; however, the weakness was the inadequate sample size for CVD endpoints, and the fact that obtained data could not be generalized to any larger groups than those of high socioeconomic status who were successful in business and professional life. In 1957, the American Heart Association proposed that modifying dietary fat could reduce the incidence of CVD (Page, Stare, Corcoran, Pollack, & Wilkinson, 1957). The first Dietary Guidelines for Americans proposing to avoid excess saturated fatty acids (SFA) came in 1980 (Service, 1980). In the following decades, the recommendation to decrease dietary SFA became central dogma how to reduce CVD risk (Jacobson et al., 2015).

However, there is an increasing body of evidence that this issue is not that straightforward (Bier, 2016). First of all, SFA reduction itself has a minor effect on CVD risk (Nettleton, Brouwer, Geleijnse, & Hornstra, 2017). Secondly, not all SFA are atherogenic – the effect of total SFA intake on CVD risk depends on the type of SFA consumed (Khosla & Khosla, 2017). Inflammation is also an important factor in CVD: the initiating event in atherosclerosis development is endothelial dysfunction that causes vascular inflammation and subsequent plaque

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formation (Jaarin, Norliana, Yusof, Nursyafiza, & Mohd Saad, 2014). The current nutritional guidelines advising a decrease in animal fat consumption caused an increase in the use of vegetable fats relatively high in omega-6 fatty acids. One of the main concerns regarding dietary omega-6 fats is related to their conversion to pro-inflammatory eicosanoids (Alshatwi & Subash-Babu, 2018).

Virgin coconut oil (VCO) contains more than 90% of SFA and less than 10% of mono-unsaturated fatty acids, with medium chain fatty acids being the largest fraction (60%) of SFA (Ghani et al., 2018). Owing to its chemical composition, VCO can be useful for studying the effects of SFA on the lipid profile disruption. It is well known that diabetes often presents with a typical dyslipidaemia, and that these lipid changes may not only be the consequence of diabetes, but they may also cause disturbances of glucose metabolism (Parhofer, 2015). Increase in triglycerides level leads to free fatty acids level elevation, which induces insulin resistance and β-cell dysfunction through disruption or modulation of the signalling cascade that links insulin receptors with glucose transporters. In addition, hypertriglyceridemia may induce subclinical inflammation leading to the insulin resistance and β-cell dysfunction (Briaud, Harmon, Kelpe, Segu, & Poitout, 2001; Lee et al., 1994; Rachek, 2014). Considering this, the aim of the study was to examine the effect of virgin coconut oil as a food supplement on glucose and lipid homeostasis in non-diabetic and alloxan-induced diabetic rats.

2. Materials and methods

2.1. Animals and treatments

All animal procedures were performed in compliance with the ARRIVE guidelines and Directive 2010/63/EU. In accordance to National legislation, all animal procedures were approved by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management, License number 323-07-10153/2016-05/1.

Wistar strain (*Rattus norvegicus*) 3–3.5 months old male rats were used for the experiment. The animals were acclimated to 22 ± 1 °C and maintained under 12 h light/dark period, with *ad libitum* access to tap water and food. Rats were randomly divided into four groups and housed in pairs in cages with six rats per group. The experiment took 16 weeks.

The rats of the control (Con) group were fed on standard commercial rat food (Veterinary Institute, Subotica, Serbia). A single intraperitoneal (i.p.) injection of saline (5 ml/kg body mass) was administered on the first day of the experiment to allow comparability with other experimental groups (see below).

The rats of non-diabetic virgin coconut oil (VCO) group were fed on standard commercial rat food enriched with virgin coconut oil (Granum®, Hajdukovo, Serbia). Based on the manufacturer data, the most abundant compounds of VCO are caprylic (C8:0) – 6.57%, capric (C10:0) – 5.78%, lauric (C12:0) – 48.51%, myristic (C14:0) – 19.50%, palmitic (C16:0) – 9.02%, stearic (C18:0) – 3.57%, and oleic acid (C18:1-trans-9) – 5.39%. VCO was added in the quantity to achieve the final concentration of approximately 20% of food and rigorously stirred for 10 min to allow equal oil distribution. The food was prepared weekly: based on the measurements for 16 weeks, an average VCO concentration in food was 21.402 ± 0.002%. Additionally, the single i.p. injection of saline (5 ml/kg body mass) was administered on the first day of the experiment to allow comparability with other experimental groups (see below).

The rats of alloxan-induced diabetic (ALX) group were fed on standard commercial rat food. The single i.p. injection of alloxan (150 mg/kg body mass, dissolved in 5 ml of saline) was administered on the first day of the experiment.

The rats of diabetic virgin coconut oil (ALX + VCO) group were fed in the same way as the VCO group rats and administered with the same alloxan treatment as the ALX group rats.

Body mass, body mass gain, and food and water intake were measured weekly throughout the experiment. The results were expressed as a time course of measured values and recalculated into the area under curve (AUC) values.

2.2. Sample preparation

At the end of experiment the animals were killed by decapitation using Harvard guillotine, and blood and tissue samples were collected.

Blood was incubated at room temperature for 45 min to allow clot formation. Clot was removed by centrifugation at 2000*g* for 10 min in a refrigerated centrifuge. The resulting supernatant was immediately transferred into a clean polypropylene tube using a Pasteur pipette (Tuck et al., 2009).

The serum and the tissue samples were stored at −80 °C until the analysis.

2.3. Glycaemia measurement

Weekly glycaemia, oral glucose tolerance test (OGTT), and insulin tolerance test (ITT) were measured using fresh tail capillary blood samples and handy Wellion CALLA Light blood glucose test strips system. The results were expressed as a time course of blood glucose measurements and recalculated into the area under a curve (AUC) values.

Weekly glycaemia was measured after 16 h of overnight fast (Bowe et al., 2014), with the last point measured prior to decapitation. Fasting started at 4 p.m., with glycaemia measurement at 8 a.m. the following day. Both time points were carefully chosen in respect with rats' circadian rhythm, corresponding to the Zeitgeber time (ZT) point 10 and 2. As nocturnal animals, rats consume 80% of their daily food intake during the period between 5 p.m. and 6 a.m. (Sidlo, Zaviacic, & Kvasnicka, 1995), while at ZT 2 they exert very high efficacy in glycaemic regulation (la Fleur, Kalsbeek, Wortel, Fekkes, & Buijs, 2001).

OGTT was performed in the last week of experiment under the same fasting conditions as weekly glycaemia measurement. Glucose was administered by oral gavage (2 g/kg b.m./10 ml water). Blood samples were taken previously (time point 0), and +30, +60, +90, and +120 min after glucose administration.

ITT was performed after 6 h of fasting, two days after OGTT measurement. Animal fasting started at 8 a.m., with glycaemia measurement at 2 p.m. These time points were chosen in order to allow animals to feed enough overnight to avoid the risk of severe hypoglycaemia induced by insulin. Insulin (Actrapid; Novo Nordisk) was administered by an intraperitoneal injection (0.75 units/kg b.m./ml saline). Blood samples were taken previously (time point 0), and +15, +30, +45, +60, and +90 min after insulin administration.

2.4. Serum insulin concentration measurement

Fasting serum insulin was measured commercially at the Institute for Application of Nuclear Energy (INEP), Belgrade, Serbia, using radioimmunoassay (RIA) assay kit made by the INEP. The INEP rat insulin RIA assay utilizes ^{125}I-labelled insulin and an insulin antiserum made in guinea pigs against rat insulin. Using double antibody/PEG technique, the INEP rat insulin RIA assay is able to determine the serum insulin level with detection limit of 0.06 mIU/L. The method is based on the competition between sample insulin and labelled insulin for binding insulin antibodies. As a result, the amount of labelled insulin bound to antibodies decreases as the concentration of sample insulin increases, which is measured after separating antibody-bound labelled insulin from free labelled insulin and counting both fractions. The insulin concentration is calculated from the standard curve made by series of increasing concentrations of standard unlabelled insulin. All measurements were performed on the Wallac Wizard 1470 automatic gamma counter.

Homeostasis Model Assessment Insulin Resistance (HOMA1-IR) index and Quantitative Insulin Sensitivity Check (QUICKI) are surrogate measures of insulin resistance that are routinely used in human clinical studies (Muniyappa, Lee, Chen, & Quon, 2008). However, experimental results confirmed that both HOMA-IR and QUICKI indexes could be used as reasonably reliable approximation of direct measures of insulin resistance in rats and mice (Cacho, Sevillano, de Castro, Herrera, & Ramos, 2008; Lee et al., 2008). Using fasting serum insulin concentration and the last day glycaemia measurement (i.e. the glycaemia measurement prior to animal killing), a HOMA1-IR index [insulin (μU/l) × glucose (mmol/l)/22.5] and QUICKI index [1/(log(insulin) + log (glucose))] were calculated in order to estimate glucose homeostasis.

2.5. Serum lipids measurement

Fasting serum concentration of total triglycerides, total cholesterol and the HDLC were measured using Roche Cobas C501 Chemistry analyser, using the TRIGL, CHOL2, and HDLC4 reagent cassette. Serum non-HDLC fraction was calculated as the difference between total and HDL cholesterol.

2.6. Serum AST, ALT and AP activities measurement

Activities of ALT, AST and AP were measured using Roche Cobas C501 Chemistry analyser, using ALTL, ASTL, and ALP2L reagent cassette.

2.7. Serum haptoglobin concentration measurement

Serum haptoglobin concentration was measured using the immunoturbidimetric test on AU400 Chemistry analyser (Beckman Coulter Inc., USA).

2.8. Whole liver homogenate preparation

Livers (200–400 mg) were homogenized in sucrose buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA) supplemented with 1 × phosphatase inhibitor Mix I (Serva). The homogenate was centrifuged at 100,000*g* for 90 min at 4 °C and stored at −80 °C in aliquots until use.

2.9. Liver nuclear extracts preparation

Livers were homogenized in the buffer containing 2 M sucrose, 10 mM HEPES pH 7.6, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM spermidine, 1 mM PMSF, 1 mM DTT and 10% glycerol. After filtering the homogenate through two layers of cheesecloth, the nuclei were pelleted by centrifugation at 72,000*g* in a SW 28 rotor (Beckman L7-55) for 30 min at 4 °C. The pelleted nuclei were resuspended in lysis buffer (10 mM HEPES pH 7.6, 100 mM KCl, 3 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). To precipitate chromatin, $(NH_4)_2SO_4$ pH 7.9 was added slowly with constant stirring to a final concentration of 0.36 M. Chromatin was sedimented by centrifugation (82,000*g*, Beckman Ti 50 rotor, 60 min, 4 °C). Nucleoproteins were precipitated from the supernatant after the addition of crystallized $(NH_4)_2SO_4$ to the final concentration of 2.6 M and sedimented by centrifugation at 82,000*g* in a Ti 50 rotor for 30 min at 4 °C. Nuclear extracts were dialyzed overnight against 25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol, resuspended in dialysis buffer and frozen in small aliquots at −80 °C.

2.10. Western immunoblot analysis

Protein samples of serum (1 μl), whole liver homogenates (20 μg), and nuclear extracts (25 μg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Pharmacia Biotech), which were blocked in solution (0.2% Tween 20, 50 mM Tris–HCl pH 7.6, 150 mM NaCl) containing 5% non-fat condensed milk. After protein transfer, PVDF membranes were incubated with a primary antibody for 1.5 h at room temperature.

The following primary antibodies were used for the Western blotting: goat polyclonal anti-HMGB1 for serum analysis (K-12; Santa Cruz Biotechnology); rabbit polyclonal antibodies specific to HMGB1 (ab18256; Abcam), NF-kB p65 (C-20, Santa Cruz Biotechnology), Nrf2 (ab31163, Abcam), β-actin (ab8227; Abcam) and rabbit monoclonal anti-Hp antibody (ab131236, Abcam) for the analysis of liver homogenates and nuclear fractions. The blots were probed with horseradish peroxidase-conjugated secondary antibody purchased from Santa Cruz Biotechnology – bovine anti-rabbit IgG pr (sc 2379), bovine anti-goat IgG (sc 2378); Abcam – goat anti-rabbit IgG (ab 97051). The immunoreactive bands were identified by the ECL detection system (Santa Cruz Biotechnology). The bands were visualized and quantified with TotalLab (Phoretix) electrophoresis software (ver. 1.1). For reprobing, the membranes were incubated in 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris–HCl pH 6.8 for 35 min at 50 °C, and then rinsed three times, blocked, and probed again with another antibody. All immunoblot analyses were obtained from at least three independent experiments.

2.11. COMET assay

Preparation of single-cell suspensions – For the comet test, a single-cell liver suspension was prepared (Wilson, Pascoe, Parry, & Dixon, 1998). Liver parts were excised and chopped separately 10 times in 0.2 ml of HBSS using two fresh scalpel blades in a scissor-like movement on a Petri dish. The livers were then washed off gently into a 15 ml centrifuge tube with a further 2.8 ml HBSS and 0.03 ml of 0.1% trypsin. The suspension was gently rocked for 10 min at room temperature, after which 10 ml of HBSS was added and the suspension was passed through a 40 μm sieve to remove any large fragments that remained. After centrifugation (800*g* for 5 min), the supernatant was discarded and the pellet was carefully resuspended in 1 ml of HBSS. Cell viability was measured by trypan blue dye exclusion method (Altman, Randers, & Rao, 1993), and cell density was adjusted to 3×10^5 cell/ml.

Comet test – The assay was performed as described by Tice et al. (2000). Microscopic slides were pre-coated with 1% normal melting point (NMP) agarose and air-dried for 24 h at room temperature. Cell suspension (30 μl) was mixed with 70 μl of 1% LMP (low melting point) and added to the slides previously coated with of 1% NMP agarose. The slides were covered with glass coverslips and placed at 4 °C for 5 min. After the coverslips were gently removed, the slides were submerged into ice-cold lysing solution (2.5 M NaOH, 0.1 M EDTA, 0.01 M Tris, 1%TritonX100, pH10) and placed at 4 °C for at least 1 h. After lysis, the slides were placed in a horizontal gel-electrophoresis chamber and loaded with freshly made ice cold electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min at 4 °C to allow DNA unwinding and expression of alkali-labile sites. The samples were then electrophoresed for 20 min at 25 V and 300 mA at 4 °C. Finally, the slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with 20 μl ethidium bromide (5 μg/ml) and analysed using fluorescence microscope (Leica) and image analysis software (Comet IV, Perceptive Instruments). Fifty nuclei were analysed per experimental point (in triplicate), and the percentage of the fluorescence in the comet tail was used as a measure of DNA damage.

2.12. Histology analysis

Liver, heart, ascending aorta and abdominal aorta samples were collected and fixated in 4% formaldehyde solution. After fixation, the samples were dehydrated in a series of increasing ethanol solutions (70%, 96%, and 100%) followed by an immersion in a clearing agent

(xylene). The tissue samples were then embedded with paraffin wax and cut into serial 4 μm thick sections. Every tenth section was mounted on glass slides and stained with haematoxylin and eosin (H&E) by a standard procedure. From each animal, 2–3 H&E stained sections for each tissue were analysed by two independent histologists using the Leica DM4000 B LED microscope with digital camera Leica DFC295 and Leica Application Suite (LAS, v4.4.0) software system and Olympus BX41 light microscope and Olympus C5060A-ADU digital camera C5060-ADU) with analySIS 5.0 software (Soft Imaging System, Olympus). The microscopes, cameras and software were used to obtain digital photographs of histological sections.

Changes in the thickness of the tunica media of ascending and abdominal aorta were investigated by measuring the distance between the first and the last elastic lamina, i.e. internal and external elastic lamina, using ImageJ software (version 1.48; NIH, Bethesda, USA; free download from http://rsbweb.nih.gov/ij). Two sections per animal for each aorta were used to measure their thickness. The measurement was done so that each section was measured in three different places within the aortic circle, making a total of six measurements per each animal.

2.13. Statistical analysis

Where appropriate, the results were expressed graphically as the time-course curves, which were subsequently recalculated into area under a curve (AUC) values. The single time-point measurements were presented as the mean ± standard error of mean, and the AUC values were presented as the percentage of controls. The level of statistical significance was defined as $p < 0.05$. The data were checked for normality using Lilliefors and Kolmogorov-Smirnov tests. Differences in investigated parameters between the groups were calculated using One-way ANOVA. When significant differences were found, pairwise comparisons were performed using Holm-Sidak *post hoc* tests. Statistical package SIGMAPLOT was used for all the analyses and graphical presentations.

3. Results and discussion

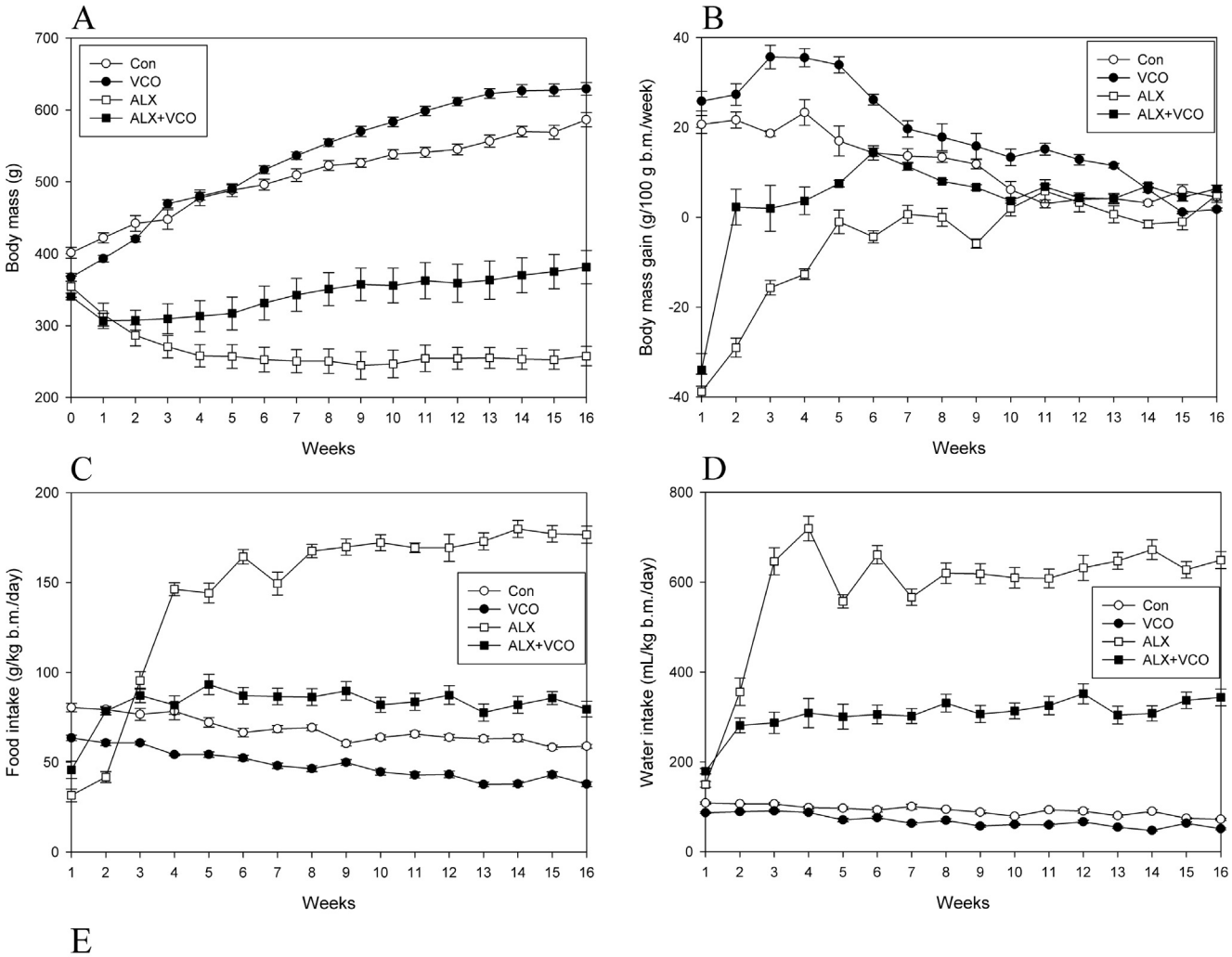
The use of alloxan is one of the most frequent approaches to chemical diabetes induction in a wide variety of animal species (Radenkovic, Stojanovic, & Prostran, 2016). It causes destruction of rat β-pancreatic cells in dosage between 150 mg/kg and 200 mg/kg, with the intraperitoneal route of administration as the safest way to avoid toxic effects and to reduce overall mortality (Federiuk, Casey, Quinn, Wood, & Ward, 2004). As can be seen from our results, alloxan treatment led to a body mass decrease (Fig. 1A), accompanied with an increase in food (Fig. 1C,E) and water intake (Fig. 1D,E). As a result, body mass gain of normally fed diabetic rats was decreased in comparison with control animals (Fig. 1B,E). These changes were especially prominent during the first 4–5 weeks after alloxan application, indicating an intense diabetes progression. At the end of this period, body mass gain and food and water intake were stabilized and remained inside tight boundaries until the end of the experiment.

However, adding coconut oil to the diet of diabetic animals reduced these changes (Fig. 1), shortening the period during which body mass gain and food and water intake became stabilized for 50% (4–5 weeks in ALX animals versus two weeks in ALX + VCO animals). As a result, ALX + VCO group of animals had higher body mass gain and lower food and water intake compared to ALX animals. The same effect was also present in non-diabetic animals – VCO rat group had higher body mass gain and lower food and water intake compared to controls (Fig. 1B-E). We assume that coconut oil decreases food and water intake and increases body mass gain in both non-diabetic and diabetic animals because it is energetically highly efficient food, with a high content of fatty acids which can be metabolized into metabolic water (Mellanby, 1942; Rutkowska, Sadowska, Cichon, & Bauchinger, 2016).

In non-diabetic animals, VCO lowered weekly glycaemia in comparison with normally fed controls (Fig. 2A,B,G). This effect was present only during the first nine weeks of the experiment. The lowest circulating glucose values were recorded after the second week of the experiment. However, blood glucose was slowly rising over the following seven weeks, ultimately reaching the control values (Fig. 2B). We suppose that the key factor was the quantity of coconut oil ingested by animals – with the progress of experiment animals consumed less food, including coconut oil in it, so its hypoglycaemic effects became weaker. In non-diabetic animals coconut oil also lowered OGTT (Fig. 2C,D,G) and ITT (Fig. 2E,F,G) glycaemia, but did not change fasting serum insulin concentration (Fig. 2G). The changes in OGTT and ITT glycaemic response confirmed better insulin sensitivity and the ability to control glycaemia in animals supplemented with coconut oil compared to normally fed controls (Fig. 2G). The coconut oil hypoglycaemic effect could be accounted for by its high content of the lauric acid, which has insulinotropic properties (Garfinkel, Lee, Opara, & Akwari, 1992; Iranloye, Oludare, & Olubiyi, 2013), and polyphenols, which enhance sensitivity to insulin and reduce insulin resistance (Narayanankutty et al., 2016; Siddalingaswamy, Rayaorth, & Khanum, 2011). We have also previously shown that supplementation with VCO increases faecal abundance of probiotic bacteria, such as *Lactobacillus*, *Allobaculum* and *Bifidobacterium* species (Djurasevic et al., 2018) in a manner similar to that of metformin (Wu et al., 2017). Although it is not clear how alterations in gut microbiota may promote beneficial effects in glucose homeostasis, a potential mechanism includes an increased production of short-chain fatty acids (De Vadder et al., 2016; Koh, De Vadder, Kovatcheva-Datchary, & Backhed, 2016). Although it is not clear how alterations in gut microbiota may promote beneficial effects in glucose homeostasis, a potential mechanism includes an increased production of short-chain fatty acids (De Vadder et al., 2016; Koh et al., 2016). Short-chain fatty acids (SCFAs) are produced in the colon and the caecum of the host under the anaerobic fermentation of non-digestible dietary fibres, with the acetate, propionate, and butyrate representing 95% of the whole SCFAs content. SCFAs significantly affect glucose homeostasis through improved gut barrier function, reduced inflammation, and improved insulin sensitivity. Deficiency in SCFAs synthesis has been associated with diabetes pathophysiology. It was shown that supplementation with the dietary fibres increased amount of plasma propionate, which causes a reduction in post-prandial insulin release and improved glucose homeostasis through improved pancreatic β-cell function. Acetate and butyrate, on the other hand, may also play a role in maintaining β-cell function, by modulation of cytotoxic T cells action (Chambers, Preston, Frost, & Morrison, 2018). It should be noted the OGTT curve in VCO animals had an unusual shape – a slow glycaemia increase after glucose ingestion, followed with no consequent glycaemia fall (Fig. 2D). This type of curve could be a sign of early insulin resistance (Tschritter et al., 2003), or a sign of a decreased glucose absorption insufficient to trigger insulin release (Sulaiman & Ooi, 2014). Since ITT confirmed that VCO does not cause insulin resistance, we presume that OGTT curve implicates some kind of coconut oil interference with intestinal absorption of glucose (Kwon et al., 2007; Proenca et al., 2017).

In diabetic animals coconut oil did not change weekly glycaemia (Fig. 2A,G), OGTT (Fig. 2C,G) and ITT (Fig. 2E,G) in comparison with normally fed diabetic rats. However, it increased fasting serum insulin concentration up to the control level (Fig. 2G). As a result, there was a switch between diabetes Type 1 in ALX animals, caused by a 60% decrease in insulin secretion, into diabetes Type 2 in ALX + VCO animals, caused by insulin resistance. Accordingly, HOMA1-IR and QUICKI indexes confirmed a decrease in insulin sensitivity and the ability to control glycaemia in rats of both ALX and ALX + VCO groups, with the latter having almost three times higher HOMA1-IR index, and around 15% lower QUCKI index in comparison with normally fed diabetic rats (Fig. 2G).

It is unclear why coconut oil developed insulin resistance only in diabetic animals. There are two pathways by which alloxan causes



E

	Con	VCO	ALX	ALX+VCO
Body mass gain	100.00±3.11	144.24±5.15[a]	-80.44±11.21[ab]	31.97±4.64[abc]
Food intake	100.00±2.39	71.34±1.95[a]	218.14±4.40[ab]	122.77±6.09[abc]
Water intake	100.00±1.57	74.23±2.30[a]	646.64±19.48[ab]	334.47±20.25[abc]

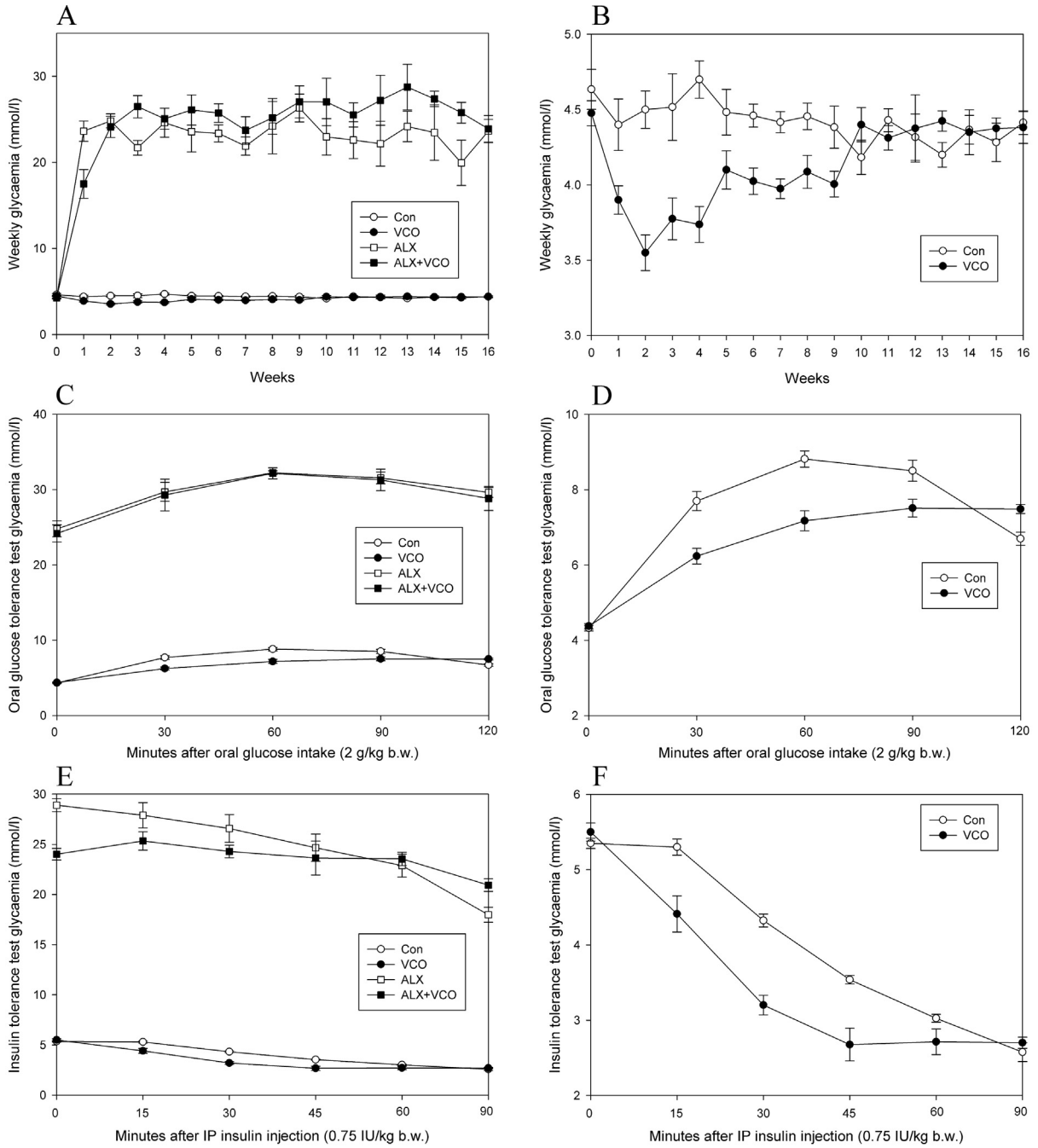
Fig. 1. Time-course curves of (A) weekly body mass (g), (B) body mass gain (g/100 g b.m./week), (C) food intake (g/kg b.m./day), and (D) water intake (ml/kg b.m./day) in rats of control (Con), virgin coconut oil (VCO), alloxan (ALX) and alloxan + virgin coconut oil (ALX + VCO) groups. Data are given as mean ± standard error. (Part E) The area under a curve values (presented as % of control) of body mass gain, food intake and water intake. Minimal significant level: $p < 0.05$. Significantly different: [a]in respect to Con; [b]in respect to VCO; [c]in respect to ALX.

diabetes (Lenzen, 2008). In the presence of intracellular thiols alloxan generates different reactive oxygen species, including hydroxyl radicals, ultimately resulting in beta cell destruction and insulin-dependent diabetes. But alloxan also selectively inhibits glucose-induced insulin secretion through the oxidation of glucokinase thiol groups, thus impairing glucose-sensing ability of beta cells. Given the importance of ROS role in both pathways, it is clear that preserved insulin secretion in rats of ALX + VCO group is a proof of protective antioxidant potential of coconut oil, just as we have previously shown (Đurašević et al., 2019). This VCO protective role could also explain a shortening of the period during which body mass gain and food and water intake become stabilized in ALX + VCO group compared with normally fed diabetic animals.

However, if coconut oil protects beta cells from oxidative stress caused by alloxan and restores normal insulin secretion, how could insulin resistance develop under the same circumstances? There have been many hypotheses concerning the link between high-fat diet and insulin resistance. They include inhibition of GLUT4 (Wolf, 2008) and attenuation of insulin signalling pathways (Blachnio-Zabielska, Grycel, Chacinska, & Zabielski, 2016) by intracellular accumulation of lipids in skeletal muscle and liver; low adiponectin serum level involved in promotion of the insulin resistance (Medina-Urrutia et al., 2015), or inflammation caused by high-fat diet (Sears & Perry, 2015).

In order to assess inflammation, we examined the changes in expression of haptoglobin and HMGB1 protein in the liver and serum, as well as the relationship between nuclear expression of NF-kB p65 and Nrf2 in liver cells (Fig. 3). Our results showed that coconut oil, while having no effects on Hp in non-diabetic animals, decreased serum and liver levels of this protein in diabetic animals (Fig. 3A). As haptoglobin is an acute-phase protein, any inflammatory process may increase its serum or liver level. Based on our results, it is clear that diabetes induces both local (hepatic) and systemic (serum) inflammatory response, and that VCO acts anti-inflammatory by lowering serum and liver haptoglobin levels previously increased by diabetes.

Diabetes also increased serum and liver levels of HMGB1 protein (Fig. 3B). Unlike haptoglobin, coconut oil further increases HMGB1



G

	Con	VCO	ALX	ALX+VCO
Weekly glycaemia	100.00±1.62	93.21±1.67[a]	514.71±19.22[ab]	561.86±9.26[ab]
OGTT	100.00±2.33	87.95±1.79[a]	395.29±4.77[ab]	390.50±10.09[ab]
ITT	100.00±1.26	84.86±3.03[a]	602.63±6.65[ab]	591.93±10.61[ab]
Insulin	21.421±0.88	22.21±1.90	7.93±0.61[ab]	23.02±0.86[c]
Last day glycaemia	4.33±0.08	4.38±0.11	23.69±1.36[ab]	22.23±0.59[abc]
HOMA1-IR	4.12±0.18	4.37±0.43	8.26±0.69[ab]	22.76±1.13[abc]
QUICKI	0.510±0.005	0.508±0.005	0.443±0.005[ab]	0.370±0.003[abc]

Fig. 2. Time-course curves (mmol/l) of weekly glycaemia, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) in rats of control (Con), virgin coconut oil (VCO), alloxan (ALX) and alloxan + virgin coconut oil (ALX + VCO) groups. Panels A,C,E – data for all animal groups; B,D,F – inset for Con and VCO rat groups only. Data are given as mean ± standard error. Part G) The area under a curve values of weekly glycaemia, OGTT and ITT (presented as % of control); the serum insulin concentration (μU/l) and glycaemia on the last day of the experiment (mmol/l), and HOMA1-IR and QUICKI indexes (data are given as mean ± standard error). Minimal significant level: $p < 0.05$. Significantly different: [a]in respect to Con; [b]in respect to VCO; [c]in respect to ALX.

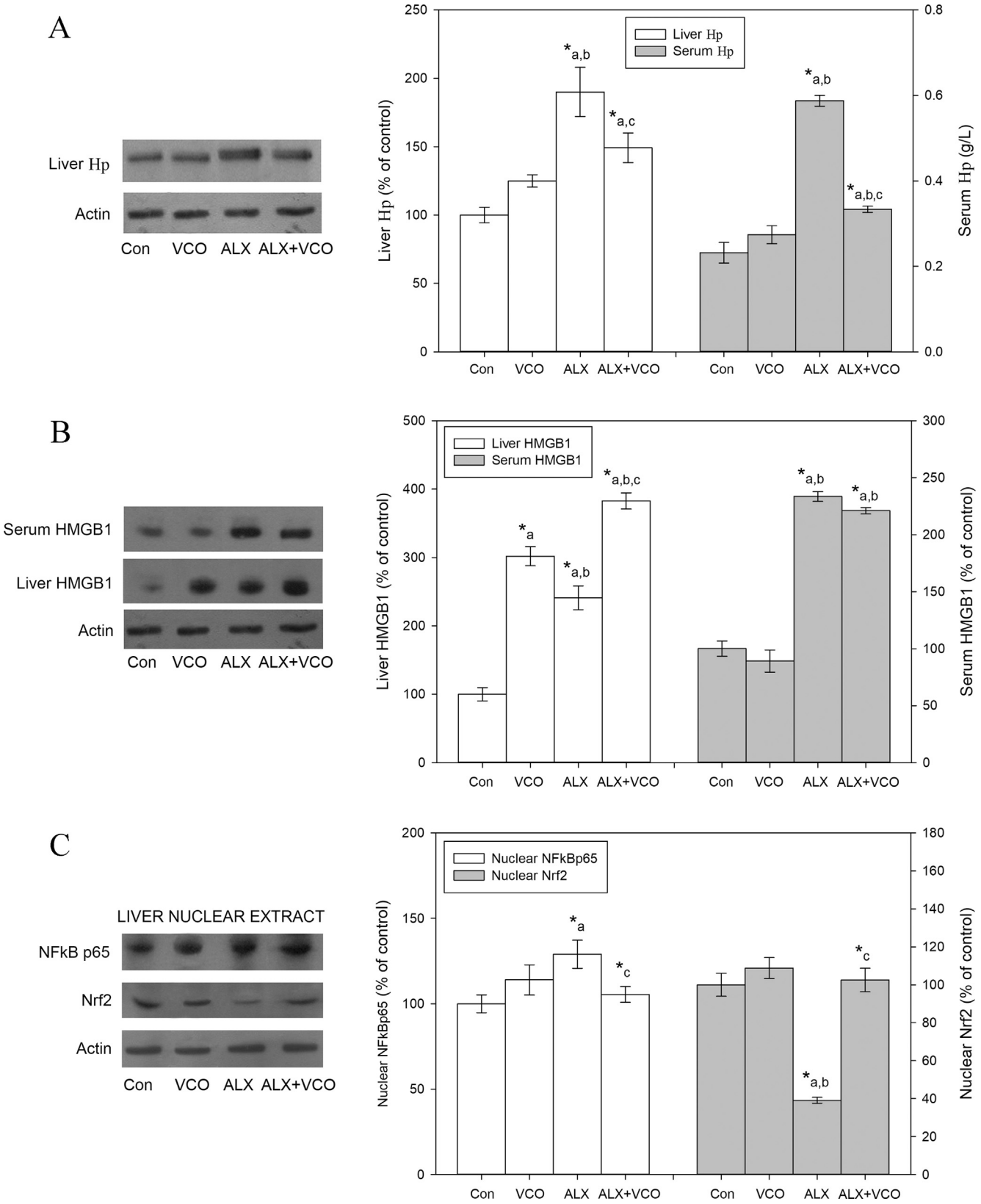


Fig. 3. Representative immunoblots of protein expression levels: (A) haptoglobin (Hp) in serum and whole liver homogenates, (B) HMGB1 in serum and whole liver homogenates, (C) NFkB p65 and Nrf2 level in liver nuclear extracts in rats of control (Con), virgin coconut oil (VCO), alloxan (ALX) and alloxan + virgin coconut oil (ALX + VCO) groups. β actin was used as a loading control. Data are given as mean ± standard error. Minimal significant level: $p < 0.05$. Significantly different: [a]in respect to Con; [b]in respect to VCO; [c]in respect to ALX.

content in the liver of diabetic animals, without affecting its serum concentration (Fig. 3B). The same effect was present in non-diabetic animals – in VCO animals liver level of HMGB1 protein was increased in comparison with controls, while serum level was unchanged (Fig. 3B). HMGB1 protein is an inflammatory mediator and a necrotic marker. It contributes to inflammation through the activation of signalling pathways leading to nuclear translocation of NF-kB p65 and nuclear export of Nrf2 (Jovanovic Stojanov et al., 2018). In our experiment, the increased level of serum and liver HMGB1 affected only normally fed diabetic animals, rising the nuclear content of NF-kB p65, and decreasing the nuclear content of Nrf2 (Fig. 3C). It seems that coconut oil supplementation counteracted these effects: increased liver HMGB1 level had no effects on the NF-kB p65 and Nrf2 nuclear content in the liver of VCO group of animals, while in the ALX + VCO rat group the nuclear content of NF-kB p65 was even decreased, and the nuclear content of Nrf2 restored (Fig. 3C). These results proved that diabetes causes inflammation on both local and systemic levels, while VCO acts as an anti-inflammatory agent. In relation to the above, inflammation cannot explain VCO-induced insulin resistance in diabetic animals, or at least inflammation evaluated by the level of Hp, HMGB1, NF-kB p65, and Nrf2.

Coconut oil and alloxan independently increased liver enzyme activities in the serum, and increased DNA damage in liver cells (Table 1). In case of animals fed by coconut oil, this can be explained by liver steatosis development (Saadati et al., 2019), since all liver samples from VCO group showed microvesicular steatosis characterized by small and diffusely dispersed lipid vacuoles in the hepatocyte cytoplasm (Fig. 4B). Liver fat accumulation is known to be induced by VCO (de Moura e Dias et al., 2018), probably due to its high content of medium chain fatty acids which after absorption in the small intestine are transferred directly by portal vein into the liver, and not into lymphatic fluid (You, Ling, Qu, & Bistrian, 2008). This is especially important in case of our experiment, since the average oil consumption in the VCO and ALX + VCO rat groups was 11.015 ± 0.259 and 17.487 ± 0.285 g/kg b.m./day, which, calculated for a man weighing 60 kg (Blanchard & Smoliga, 2015), represents a huge amount of oil: 124.471 ± 2.926 and 197.598 ± 3.225 g/day, respectively.

In case of ALX animals, the liver enzyme activities increase in the serum, and DNA damage increase in liver cells can probably be explained by increased inflammation, since liver samples obtained from normally fed diabetic animals did not show any significant pathohistological findings (Fig. 4C). Assuming that these changes are caused by inflammation, it can be expected that adding coconut oil to the diet of diabetic animals will reduce it. This actually is the case – the liver enzyme activities in the serum, and increased DNA damage in liver cells were reduced in ALX + VCO animals in comparison to ALX rat group (Table 1). This is another proof that VCO acts as an anti-inflammatory agent, as we already showed through Hp, HMGB1, NF-kB p65, and Nrf2 analysis. Surprisingly, adding coconut oil to the diet of diabetic animals caused almost no liver fat accumulation – micro- and macrovesicular steatosis was found only in one liver sample from ALX + VCO rat group (Fig. 4D). The explanation why liver steatosis is almost absent in diabetic animals fed by coconut oil could be the fact that in diabetes most energy needs are met through lipid catabolism. Paradoxically, diabetes reduces VCO-induced liver steatosis through increased fat burning.

In non-diabetic animals, coconut oil had no impact on the majority of the serum lipid profile parameters. The exception were triglycerides, whose concentration was almost 50% lower in comparison with controls, causing a significant drop in atherogenic index (Table 1). The drop in triglyceride concentration in the serum of rats supplemented with VCO is probably the result of liver steatosis, which causes fat accumulation in the liver, and subsequent fat reduction in serum.

Diabetes itself decreased serum HDLC concentration, and increased serum triglycerides and non-HDLC concentration, causing a rise in the atherogenic index and the TC:HDLC ratio in ALX animals in comparison with controls (Table 1). However, adding coconut oil to diabetic animals caused further increases in serum triglycerides, total cholesterol, high density cholesterol, and non-high density cholesterol concentrations, leading to a drop in the TC:HDLC ratio and an increase in the atherogenic index in comparison to ALX animals (Table 1). These changes clearly indicate intensive lipid catabolism in diabetic animals fed by VCO, supporting our hypothesis about the influence of diabetes on the reduction of liver steatosis in ALX + VCO animals.

The histological analysis of the heart showed no significant pathohistological alteration, indicating a preserved morphology of the heart tissue (Fig. 5A1–D1). The ascending (Fig. 5A2–D2) and abdominal (Fig. 5A3–D3) aorta showed a normal histological structure in all animal groups, without statistical differences in the thickness of medial layer (Fig. 5E). No inflammatory changes were noticed in intimal, medial and adventitial layers, and there were no signs of atherosclerotic development in sub-endothelial part of the blood vessels. These results show that observed changes in inflammation and serum lipids, especially atherogenic index, have not lead to pathological changes in the heart and blood vessels.

4. Conclusion

According to the presented results, it can be concluded that coconut oil exerts distinct effects on glucose and lipid homeostasis in non-diabetic and alloxan-induced diabetic rats. In non-diabetic animals these effects are mostly protective – there is an improvement in insulin sensitivity and the ability to control glycaemia, followed with an almost 50% decline in triglyceride serum. It seems that in diabetic rats coconut oil acts as an anti-inflammatory agent, as it was evaluated by the reduction in liver and serum haptoglobin level, liver NF-kB p65 level, serum ALT and AST concentrations, and liver cells DNA damage in

Table 1
Activities of serum aspartate aminotransferase (AST, U/l), alanine aminotransferase (ALT, U/l) and alkaline phosphatase (AP, U/l), liver comet test (% of DNA in comet tail), fasting serum concentration of triglycerides (TG, mmol/l), total cholesterol (TC, mmol/l), high density cholesterol (HDLC, mmol/l), and non-high density cholesterol (non-HDLC, mmol/l), the TC:HDLC ratio and the Atherogenic index (AI) in rats of control (Con), virgin coconut oil (VCO), alloxan (ALX) and alloxan + virgin coconut oil (ALX + VCO) groups. Data are given as mean ± standard error. Minimal significant level: $p < 0.05$. Significantly different: [a]in respect to Con; [b]in respect to VCO; [c]in respect to ALX.

	Con	VCO	ALX	ALX + VCO
AST	155.63 ± 5.56	240.50 ± 9.52[a]	225.25 ± 9.23[a]	145.50 ± 2.80[bc]
ALT	44.88 ± 2.40	59.38 ± 2.73[a]	114.83 ± 3.43[ab]	88.00 ± 2.75[abc]
AP	60.88 ± 2.48	102.88 ± 2.97[a]	739.25 ± 21.05[ab]	695.83 ± 27.57[ab]
COMET	1.744 ± 0.123	5.706 ± 0.352[a]	13.181 ± 0.690[ab]	8.873 ± 0.570[abc]
TG	1.370 ± 0.028	0.680 ± 0.016[a]	1.890 ± 0.048[ab]	3.997 ± 0.140[abc]
TC	1.850 ± 0.022	1.829 ± 0.041	1.898 ± 0.027	3.188 ± 0.041[abc]
HDLC	1.294 ± 0.0192	1.339 ± 0.023	1.033 ± 0.046[ab]	1.880 ± 0.029[abc]
non-HDLC	0.556 ± 0.010	0.490 ± 0.028	0.865 ± 0.046[ab]	1.308 ± 0.039[abc]
TC:HDLC ratio	1.431 ± 0.010	1.366 ± 0.021	1.854 ± 0.080[ab]	1.697 ± 0.026[abc]
AI	0.025 ± 0.009	−0.295 ± 0.010[a]	0.264 ± 0.025[ab]	0.321 ± 0.011[abc]

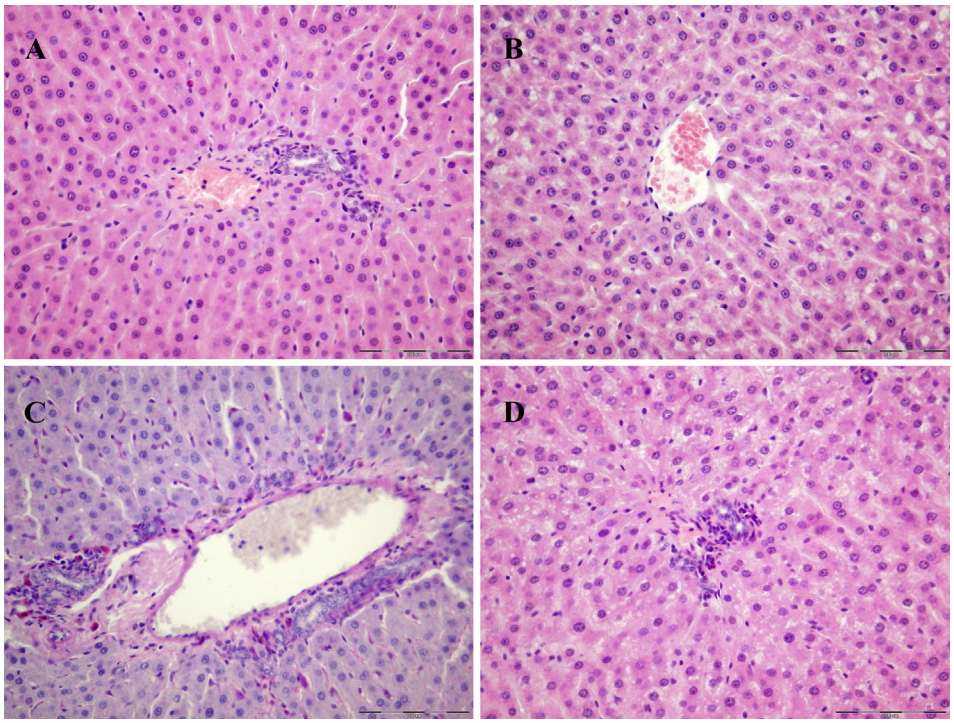
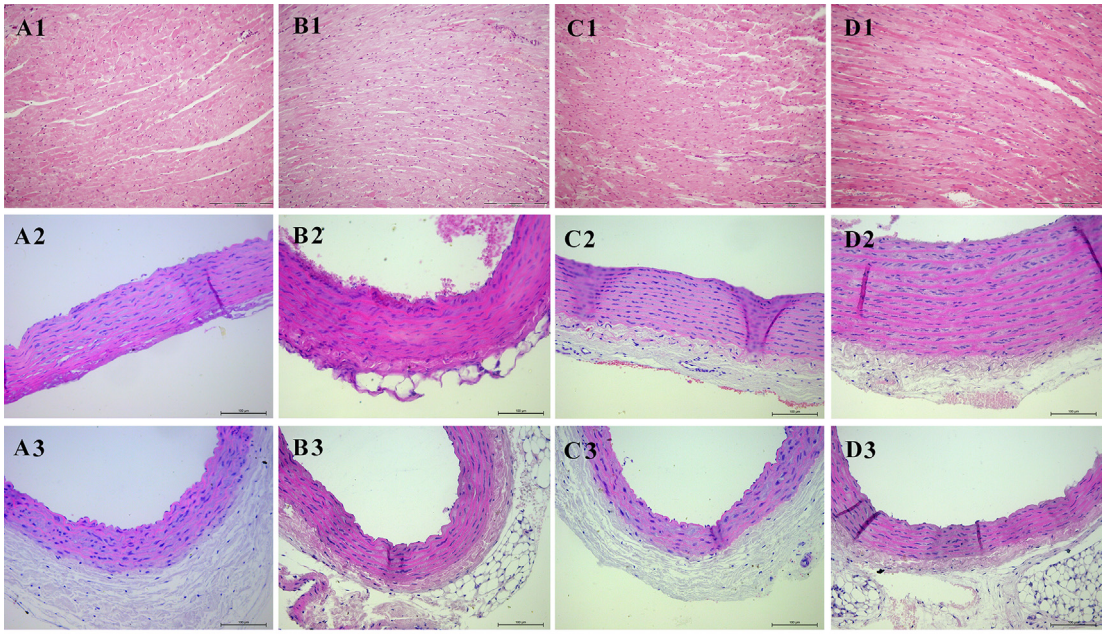


Fig. 4. Liver section stained with haematoxylin and eosin from control (A), virgin coconut oil (B), alloxan (C) and alloxan + virgin coconut oil (D) treated rats. A – normal histological structure of the hepatic lobule in control animals; B – diffuse microvesicular and macrovesicular steatosis present in hepatocytes of the animals from VCO group; lipid accumulation was mostly located in the peripheral hepatocytes; C – ALX group with mononuclear and eosinophilic infiltration in the portal spaces. D – ALX + VCO group with mononuclear and eosinophilic infiltration in the portal spaces and steatosis.

comparison with normally fed diabetic rats. VCO supplementation presents no risk factor for the development of cardiovascular diseases, since it has no impact on heart histology and the ascending and abdominal aorta wall thickening and atherosclerotic plaque development in non-diabetic and diabetic animals. However, in diabetic animals coconut oil causes strong insulin resistance and deteriorates serum lipid profile in every sense, making it clear that the use of coconut oil is contraindicated in case of diabetes. The question is to what extent coconut oil doses used in this experiment are applicable in terms of human use. In this respect, the aim of future research will be to explain the molecular mechanism of VCO-induced insulin resistance in diabetes, and to examine if smaller doses of VCO would have the same



E

	Con	VCO	ALX	ALX+VCO
Ascending aorta	125.07±4.35	137.73±5.84	129.12±5.44	118.68±7.02
Abdominal aorta	87.79±1.99	91.62±1.72	88.60±2.49	84.44±2.02

Fig. 5. Longitudinal section of the left ventricle heart muscle (1), and histological analysis of the ascending (2) and abdominal (3) aorta from control (A), virgin coconut oil (B), alloxan (C) and alloxan + virgin coconut oil (D) treated rats. (E) The tunica media thickness of the ascending and abdominal aorta (μm). Data are given as mean ± standard error.

effects.

5. Ethics statement

All animal procedures were performed in compliance with the ARRIVE guidelines and Directive 2010/63/EU. In accordance to National legislation, all animal procedures were approved by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management, License number 323-07-10153/2016-05/1.

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Declaration of Competing Interest

There is no conflict of interest to declare, either financial or personal, or with other organizations.

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