Peer-reviewed version available at Nutrients 2018, 10, 1547; doi:10.3390/nu10101547

Article

Effects of Unfiltered Coffee and Bioactive Coffee Compounds on the Development of Metabolic Syndrome Components in a High-Fat/High-Fructose-Fed Rat Model

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Abstract: Literature is inconsistent as to how coffee affects the features of the metabolic syndrome (MetS), and which bioactive compounds are responsible for its metabolic effects. We aimed to compare the in-vivo effects of unfiltered coffee with a selected mixture of its compounds on diet-induced MetS. 24 male Sprague-Dawley rats were fed a high-fat (35% W/W) food plus 20% W/W fructose in drinking water for 14 weeks, and were randomized into three groups: control, coffee, or nutraceuticals (5-O-caffeoylquinic acid, caffeic acid, and trigonelline). Coffee or nutraceuticals were provided in drinking water in a dosage equal to 4 cups/day in a human. Compared to the controls, only coffee supplementation decreased total food intake, weight gain, and estimated average plasma glucose. Surrogate measures of insulin resistance (fasting insulin, HOMA-IR, and oral glucose tolerance) were improved at endpoint in the coffee group. Circulating triglyceride levels were also reduced by coffee. Histopathological and quantitative measurements indicated lower grades of liver steatosis after long-term coffee consumption. In conclusion, a combination of phenolic acids and trigonelline was not as effective as coffee per se in improving the components of the MetS. This points to the role of other coffee chemicals and a potential synergism between compounds.

Keywords: Coffee; Insulin Resistance; Metabolic Syndrome X; Non-alcoholic Fatty Liver Disease; Carbon-13 Magnetic Resonance Spectroscopy; Phytotherapy;

1. Introduction

Metabolic syndrome (MetS) encompasses a constellation of cardiometabolic risk factors defined to help to identify people at increased risk of cardiovascular diseases and type-2 diabetes mellitus (T2D). Visceral adiposity and the concomitant insulin resistance (IR) are the driving forces behind the

metabolic derangements of the MetS [1, 2]. Since this syndrome affects 25% of the world's population, an effective preventive measure will confer immense public health benefits.

The potential role of coffee in the prevention of T2D was recently identified through observational research [3]. To understand how coffee affects the risk of T2D, we need to study its effects on T2D risk factors clustered in the definition of the MetS. Epidemiological data show a non-linear reverse relationship between coffee intake and the risk of the MetS [4] and its hepatic component, non-alcoholic fatty liver disease (NAFLD) [5]. NAFLD is a manifestation of ectopic fat deposition which is strongly associated with all features of the MetS [6]. As yet, long-term interventional research on the effects of coffee on different features of the MetS is lacking.

While the coffee beverage is a blend of more than a thousand volatile and non-volatile compounds, only a minority reach bioactive concentrations with moderate daily consumption comprising alkaloids (incl. caffeine and trigonelline), phenolic acids (incl. chlorogenic acids), diterpenes (incl. cafestol and kahweol), and Maillard reaction and degradation products formed during the roasting process (incl. melanoidins and quinides) [7]. However, the enigma still continues as to which compound or combination of compounds explains the preventive potential of coffee on T2D risk factors.

In the present study, we aimed to assess the long-term effects of a realistic dosage of unfiltered coffee on the development of the key features of the MetS and to compare coffee with a combination of its main bioactive compounds. We used a combination rather than single compounds based on the hypothesis that synergy plays an essential role in the biological effects of coffee.

2. Materials and Methods

Animal model. Six-week-old male Sprague Dawley® rats (n=24) were purchased from Taconic A/S (Ejby, Denmark). For the first week, rats were caged individually and allowed to acclimatize with free access to regular rat chow. After acclimatization, ad-libitum high-fat diet (D12492, Research Diets, Inc., New Brunswick, NJ, USA) was introduced and simultaneously, the intervention started. The high-fat diet contained 34.9% W/W fat (60% of total calories), 26.2% W/W protein, and 26.3% W/W carbohydrate. D-(−)-Fructose (Sigma-Aldrich, St. Louis, MO, USA) was also given (~9 gr/day/rat) in drinking water. Adding fructose to a high-fat diet is shown to intensify its deleterious effects on hepatic steatosis and lipid metabolism [8]. High-fat-high-carbohydrate diet-induced models are considered to be the best models of human MetS [9].

Rats were kept under reversed 12-hour light-dark cycles at temperatures of between 21 and 24 °C and 55-70% of relative humidity. Animals were housed and handled by qualified personnel in keeping with the EU and Danish provisions under regular inspections from the Danish Animal Experiments Inspectorate (registration number: 2015-15-0201-00592).

Experimental design and intervention. The experiment consisted of one control and two intervention groups receiving either unfiltered brewed coffee or selected coffee compounds (referred to as the nutraceuticals group) in drinking water for 14 weeks. Eight rats were randomly allocated into each group. Coffee was brewed by a French press coffee maker with boiling water to ground coffee proportion of 180 ml to 8 gr for making 150 ml of coffee brew. Fructose was then added to the beverage and it was diluted with water 2.3 times to make the final drinking solution. On average, each rat received coffee made from ~1.18 gr of ground medium-roast Ethiopian Arabica coffee (Hans & Grethe Coffee - Tea, Aarhus, Denmark) per day. For the nutraceuticals group, we dissolved the total sum of chemicals (24 mg of CGA, 12 mg of CA, and 7 mg of trigonelline per rat per day) in 5 ml of 70% ethanol and added it to the fructose solution. The same amount of ethanol was also given to the other groups. Chlorogenic acid was purchased from MP Biomedicals (Santa Ana, CA, USA) and trigonelline hydrochloride and caffeic acid from Sigma Aldrich (St. Louis, MO, USA). Intervention dosage in both groups was equivalent to 4 cups of coffee in an average adult. We performed the dose conversion according to the body surface area (BSA) normalization method [10] with the following premises: rat BSA=0.06 m2 and human BSA=1.69 m2. BSA was estimated using the equation by Haycock et al. [11]: BSA (m2) = 0.024265 × height0.3964 (cm) × weight0.5378 (kg). We decided to

dispense the drinking solution in fixed amounts every other day to all groups in order to harmonize their fructose intake and intervention dosage.

At baseline, body weight and daily food consumption were measured and a tail blood sample was collected. Weighing of all rats was repeated every week, food consumption monitoring biweekly, and blood sampling monthly. On week 12, nuclear magnetic resonance (MR) imaging was performed (described later in this section). After one week of recovery, rats underwent an oral glucose tolerance test (OGTT) by administering 2 g/kg of D-glucose via gastric gavage and measuring the blood glucose by a FreeStyle Precision glucometer (Abbott GmbH & Co. KG, Wiesbaden, Germany) before and 30, 60, 120, and 180 minutes after the gavage. At the end of the study, rats were deeply anesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital and blood samples were obtained from the retro-orbital plexus and urine samples from the bladder. Liver and pancreas were harvested and fixed by immersion in 4% formaldehyde and stored at 4°C until paraffin embedding (see the histopathological examination section). Rats were then terminated by exsanguination. During the six to eight hours of fasting time before blood samplings, OGTT, and surgery, drinking solution without fructose was given to the animals.

Immunoassay analyses. Glucose, lipoproteins, and alanine aminotransferase (ALT) in plasma and glycated hemoglobin (HbA1C) in whole blood samples (collected in EDTA tubes) were quantified by Cobas c111 analyzer (Roche Diagnostics, Mannheim, Germany). A sensitive rat RIA kit (EMD Millipore, Billerica, MA, USA) was used to measure insulin. NEFA-HR2 assay (Wako Chemicals GmbH, Neuss, Germany) was utilized to determine Non-esterified fatty acids (NEFAs) concentration. Plasma total antioxidant capacity was quantified by a commercial assay kit (MAK 187, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Liver triglycerides content was estimated by cutting around 50 mg of frozen liver tissue per sample on dry ice and obtaining its saponified extract using a published protocol [12]. Triglyceride concentration was then quantified by the Cobas c111 analyzer.

Histological examinations. The right anterior lobe of the liver was dissected and fixed in 10% neutral buffered formalin. Fixed tissues were then embedded in paraffin and cut into 4-mm slices and stained with hematoxylin and eosin, and Masson-trichrome. The blinded examination was performed on a random section from each animal by scanning at low-power and then scrutinizing in detail in five medium-power fields. The sections were photographed under 100× magnification. Steatosis was divided into three types: large-droplet macrovesicular, small-droplet macrovesicular, and microvesicular. Each type was further graded on the scale of 0-3 based on the percentage of hepatocytes containing fat vacuoles: grade 0 <5%, grade 1 = 5-33%, grade 2 = 34-66%, and grade 3 >67%. The sections were additionally evaluated for hepatocyte ballooning, inflammation, and fibrosis.

Hyperpolarized-[1-^{13}C]-pyruvate MR spectroscopy. Rats with a weight range of 400 to 660 grams were scanned in a 3T GE clinical system (GE Healthcare, Milwaukee, WI, USA) equipped with a dual tuned ^{13}C/1H volume rat coil (GE healthcare, Brøndby, DK). Hyperpolarized [1-^{13}C]-pyruvate was prepared and polarized in a SpinLab system (GE Healthcare, Milwaukee, WI, USA) in accordance with the standard protocol [Laustsen 2014]. In brief, rats were anesthetized with sevoflurane (3% sevoflurane in 2 L/min air) and a tail vein catheter (0.4 mm) was inserted for injection of [1-^{13}C]-pyruvate, upon full polarization (>35%) 1.5 mL (37° C, pH 7.4) isotonic [1-^{13}C]-pyruvate solution was injected over 15 s. Anatomical 1H imaging used for positioning the ^{13}C imaging plane a T2-weighted fast spin echo sequence was used in the axial and coronal orientation covering the liver. Following the anatomical scout, an axial oblique slice-selective (10 mm, 10°) ^{13}C-dynamic time series with a repetition time of 1 s (total 120 sec, one image/sec). The sequence was initiated before the injection of [1-^{13}C]-pyruvate. The individual peak areas were fitted using a general linear model fit on the time-domain data, followed by a model-free ratio-metric analysis of the area under the curve (AUC) of product and substrates.

1H MR spectroscopy. Urine samples were thawed at room temperature, vortexed for 30 sec and centrifuged (10,000 rpm for 5 min). A total of 100-400 µL urine was transferred from the supernatant into a 5 mm MR tube (VWR International, PA, USA) and mixed with 100 µl 0.75 M phosphate buffer

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solution containing 0.5% TSP prepared in D2O. All MR spectra were recorded at 298 K on a Bruker Avance 600 spectrometer operating at a 1H MR frequency of 600.13 MHz and equipped with a 5 mm TXI probe (Bruker BioSpin, Rheinstetten, Germany). The Bruker 'zgpr' pulse program was applied as a pre-saturation pulse sequence for water suppression. A total of 64 scans were collected into 32 K data points with a relaxation delay of 2 s. MR spectra were phased and baseline corrected using TopSpin 3.0 software (Bruker BioSpin, Rheinstetten, Germany). The MR peaks were assigned based on the Human Metabolome Database [13] and Chenomx MR Suite (Chenomx Inc., Edmonton, Canada). Selected peaks were integrated relative to the total peak area using Chenomx MR Suite (Chenomx Inc.).

Statistical Methods. Data are presented as mean plus 95% confidence interval (CI) or standard error of the mean. Normality of data was checked using quantile-quantile (q-q) plots and Shapiro-Wilk normality test. Study groups were compared by one-way analysis of variance (ANOVA) followed by group-wise post hoc comparisons. Protected Fisher's least significant difference (LSD) test was used as a valid method for three-group comparisons [14]. Where Levene's test rejected the equality of variances, an F-test from the Welch's test was used instead of the one from one-way ANOVA, and Games-Howell post hoc test was employed as an alternative to the LSD test. Trigonelline in the urine was compared between two intervention groups using independent samples Student's t-test. All the analyses were performed at a two-sided significance level of 0.05 using IBM SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp.). AUC calculations were made using GraphPad Prism for Windows (version 5.01, GraphPad Software, La Jolla California USA). The rate of changes of selected variables was calculated using the slope function of Microsoft Excel 2013 (Microsoft Corp., Redmond, WA).

3. Results

3.1. Food intake and body weight

Figure 1A shows a comparable initial decline in food consumption during the first two weeks due to an adapting response to the shift from normal to high-fat high-fructose (HFHF) diet. The overall food intake during the intervention period (AUC) was lower in the coffee group compared to the control group (post-hoc P=0.023). Total food intake after week two (calculated by the area under the curve) was significantly reduced by coffee consumption as compared with the controls (post-hoc P=0.023). From week five, the body weight of the rats in the coffee group started to split from the control rats with a significantly lower slope of increase (post-hoc P=0.008). Slower weight gain led to significantly lower body weights in the coffee compared to the control group after week 10 (Figure 1B). Pure nutraceuticals mixture failed to replicate the brewed coffee's effects.

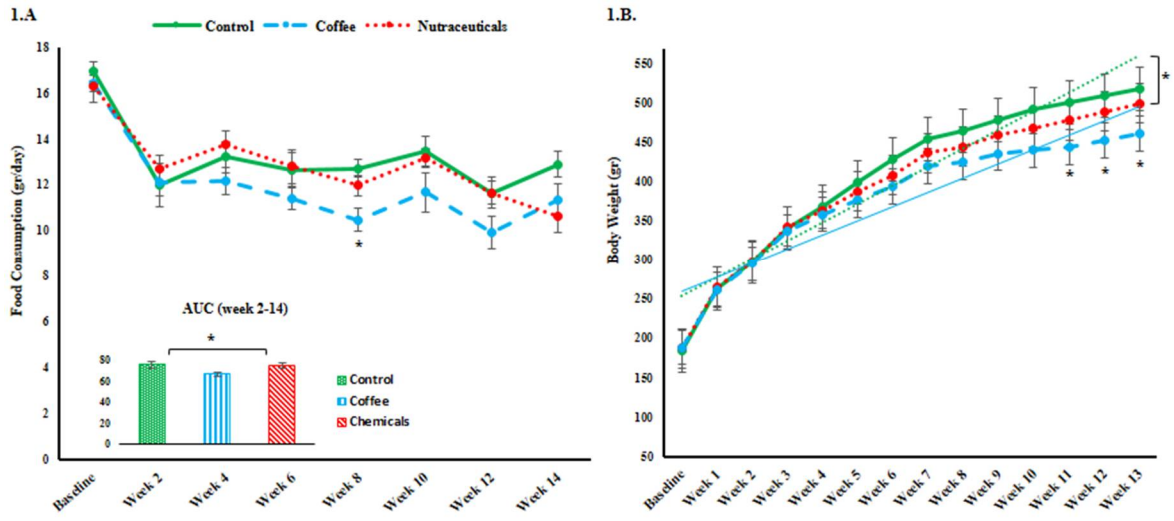

Figure 1. Part A illustrates the mean daily food consumption measured biweekly and the total food consumed between weeks 2 and 14 (as the area under the curve). In part B, the trend of changes in body weight of each group over time is displayed. Straight lines represent the slope of changes in coffee vs the control group; Error bars are standard errors of the mean. Asterisks denote significantly different values from the control group (* P<0.05)

3.2. Plasma biochemistry. Although slightly reduced fasting plasma glucose (FPG) in intervention groups at weeks 10 and 14 did not prove to be significant, long-term glycemic control measured by HbA1C was improved by coffee compared to both control (mean difference: -1.6 mmol/mol, 95% CI: -0.7 to -3.2 mmol/mol) and nutraceuticals groups (Figure 2A). Rats in the coffee group maintained lower fasting insulin levels compared to the other groups all along the intervention period, with decreased AUC of fasting insulin (Figure 2B).

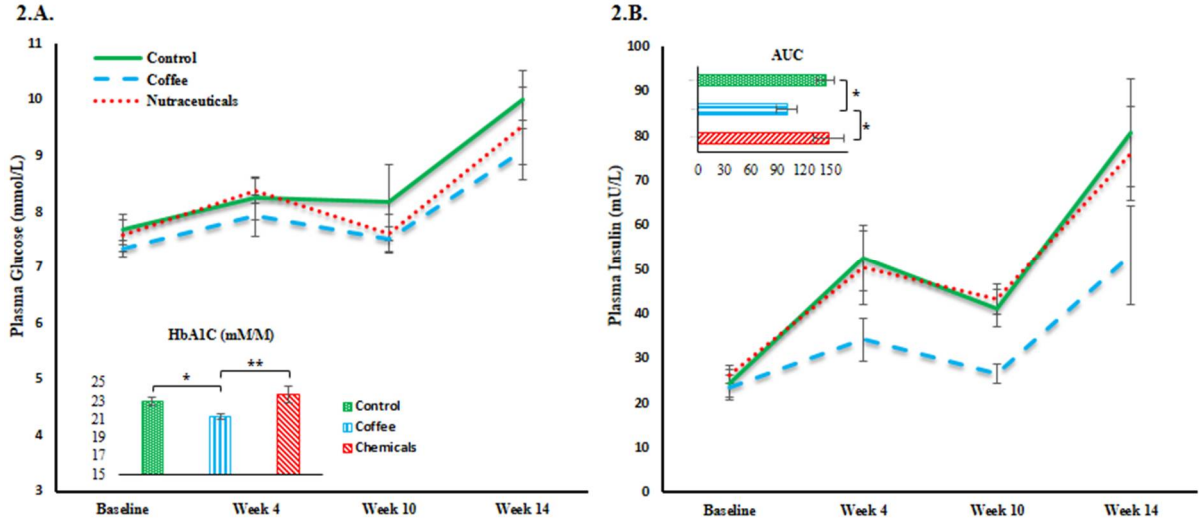

Figure 2. (A) Mean fasting plasma glucose measured monthly are plotted in the line graph and glycated hemoglobin levels measured at endpoint are shown in the bar graph. (B) Fasting plasma insulin levels and a comparison of its area under the curve (AUC) between groups; Error bars are standard errors of the mean. Asterisks denote significantly different values (* P<0.05, ** P<0.01)

IR quantified by HOMA-IR was accordingly mitigated by coffee overall (AUC, F-test P=0.014) and at week 10 (F-test P<0.001) as compared to both control and nutraceuticals groups (P<0.05 in post-hoc comparisons) (Figure 3A). According to the OGTT results displayed in Figure 3B, the plasma glucose peak at 30 minutes was on average 3.0 mmol/L (95% CI: 1.6 – 4.3 mmol/L) lower than that of the control group (P<0.001), and the area under the OGTT curve during the first two hours was significantly lower in the coffee group.

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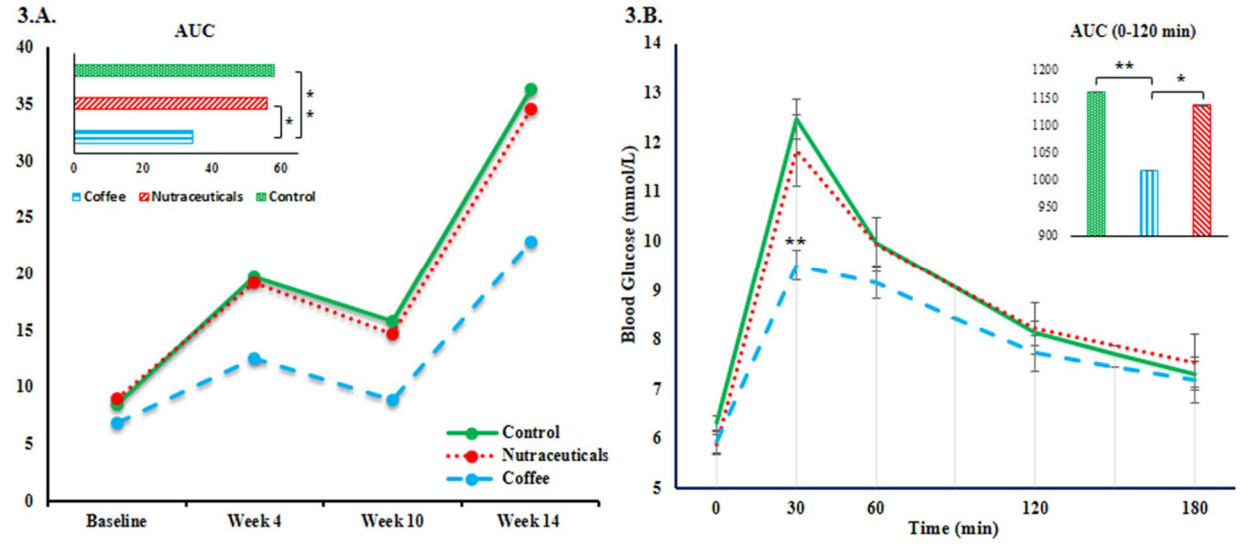

Figure 3. Surrogate measures of insulin resistance: (A) Changes in HOMA-IR in each study group and its area under the curve (AUC); (B) Repeated plasma glucose measurements during an oral glucose tolerance test and the AUC of changes in the first two hours. Error bars are standard errors of the mean. Asterisks denote significantly different values (* P<0.05, ** P<0.01)

Mean plasma lipids levels at endpoint are summarized in the Table. Fasting NEFAs and major lipoprotein values were comparable between groups; however, triglycerides levels were reduced by adding coffee to the HFHF diet. We did not detect any effect of coffee or coffee chemicals on the total plasma antioxidant capacity (Table).

Table. Mean (SEM) value of endpoint immunoassay and 1H-MR spectroscopy measurements. F-test p-values are presented in the right-most column. Significantly different values from the control and from the other intervention group are bolded and marked by superscript symbols

Mean (SEM)	Control	Coffee	Nutraceuticals	Between-group P-Value
Plasma				
Lipids (mmol/L)				
Total Cholesterol	1.650 (0.118)	1.728 (0.099)	1.558 (0.125)	0.604
Non-HDL-C	0.544 (0.045)	0.535 (0.092)	0.488 (0.029)	0.588
LDL-C	0.250 (0.023)	0.336 (0.036)	0.257 (0.011)	0.156
HDL-C	1.106 (0.085)	1.193 (0.098)	1.080 (0.104)	0.690
Triglycerides	0.649 (0.041)	0.380 (0.053)**	0.535 (0.082)	0.010
Nonesterified fatty acids	0.279 (0.039)	0.295 (0.025)	0.288 (0.039)	0.941
Total antioxidant capacity (Trolox equivalent, mmol/L)	102.323 (1.743)	97.228 (2.168)	102.272 (2.344)	0.152
Alanine transaminase (U/L)	18.838 (1.280)	19.875 (1.612)	17.983 (1.061)	0.718
Liver triglycerides content (mmol/L)	2.806 (0.384)	1.379 (0.108)**†	2.386 (0.432)	0.010
Urine (relative units)				
Trigonelline	0.0002 (0.000)	0.038 (0.015)	0.025 (0.019)	0.597¥
Ethanol	1.355 (0.829)	1.378 (0.257)	1.404 (0.262)	0.991

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* significantly different from the control group (* P <0.05, ** P <0.01); † significantly different from the other intervention group († P <0.05); ¥ independent t-test p-value of comparison between two intervention groups

3.3. Hepatic steatosis. While circulating ALT levels were not affected by either of the two interventions, coffee decreased the liver triglycerides content compared to the control rats (P=0.003) (Table). In the microscopic examination of stained liver sections, panacinar steatosis was visible throughout the liver parenchyma in control and nutraceuticals groups, while the cells' morphology was relatively normal with mild steatosis in samples from the coffee group (Figure 4). Steatosis was dominantly of the macrovesicular type with both small and large droplets. On a scale of 0-3, the mean score of steatosis was 2.13 (SD=0.83) in the control, 1.38 (SD=0.74) in the coffee, and 2.00 (SD=0.89) in the nutraceuticals groups, which shows some degrees of variation. Similarly in all groups, infiltration of inflammatory cells, ballooning, and fibrosis were of a minimal level.

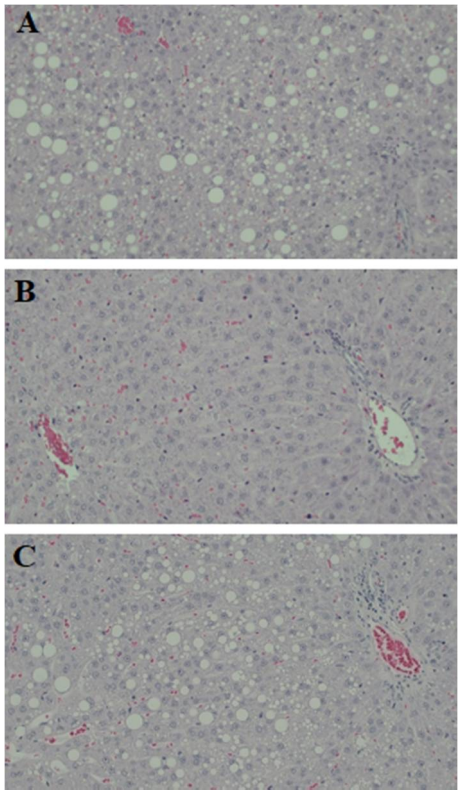

Figure 4. Representative hematoxylin and eosin-stained histological sections of rat liver: (A) Samples from the control group showing marked fatty change. There is mixed small- and large-droplet macrovesicular steatosis affecting most hepatocytes. There is no ballooning, inflammation or fibrosis. (B) Rat liver showing normal morphology with no significant steatosis. (C) Marked steatosis, predominantly large-droplet macrovesicular, in most hepatocytes without ballooning, inflammation or fibrosis

3.4. In-vivo hyperpolarized-[1-13C]-pyruvate metabolic examinations. Four randomly selected rats from each study group were assigned to the MR spectroscopy analysis at intervention week 12. Figure 5A illustrates a typical anatomical image used for positioning the 13C imaging plane. After rapid intravenous injection of hyperpolarized-[1-13C]-pyruvate to fasted animals, signals from hyperpolarized-[1-13C]-pyruvate hydrate, -[1-13C]-lactate, -[1-13C]-alanine, [1-13C]-bicarbonate, and -[13C]-urea were detected in the spectra as shown in a representative example in part B of Figure 5. Signal intensity data were generated for each metabolite by computing AUC of signal intensity curves obtained from a one-minute scanning period (an example in Figure 5C). AUC values are presented as proportional to pyruvate and total carbon. The tendency towards lower mean alanine/pyruvate and alanine/total carbon was not statistically significant. The concentration of other metabolites was similar in all groups as displayed in Figure 5.

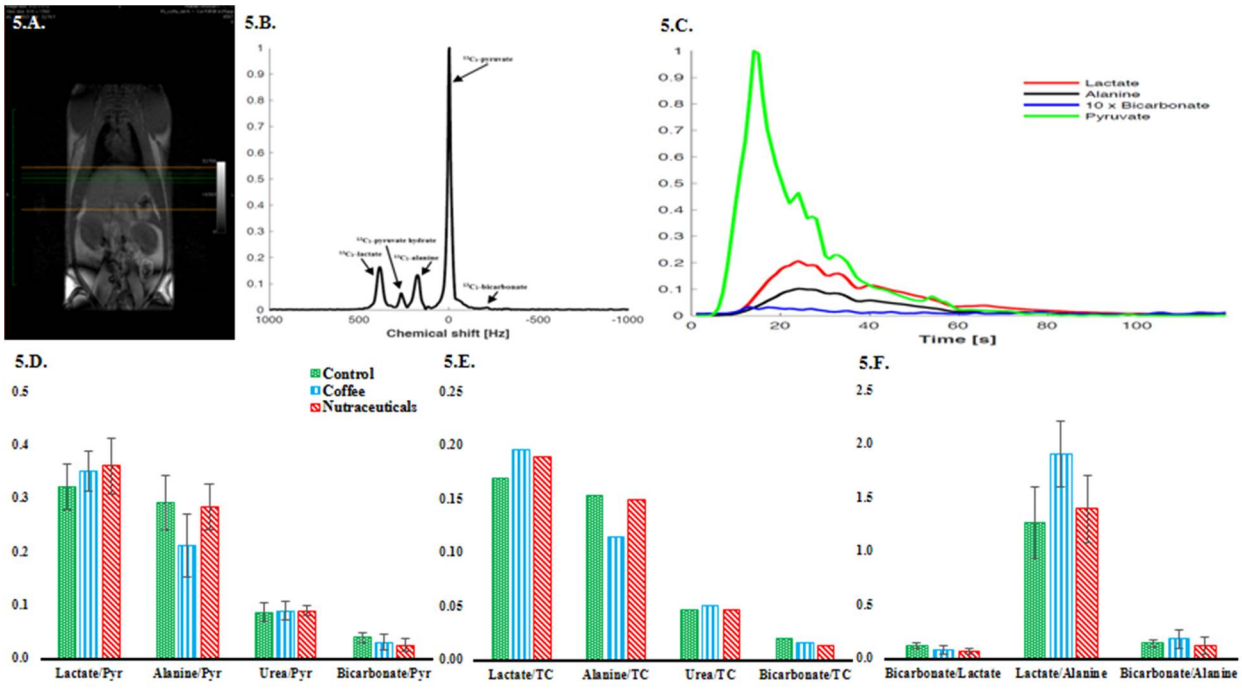

Figure 5. (A) A typical full-torso coronal 1H MR image; (B) A representative 2D 13C sum spectra observed after injecting the hyperpolarized [1-13C]-pyruvate; (C) Changes in the signal intensity of metabolites over the 60 seconds scanning period; (D) Area under the curve of signal intensity-time curves of each metabolite normalized to [1-13C]-pyruvate and (E) total carbon. (F) Signal intensity ratios between pyruvate metabolites

Urine 1H-MR spectroscopy. According to the Table, despite a trend of higher levels in the coffee group, trigonelline excretion in urine was not significantly different between the intervention groups. Our measurements showed closely similar levels of ethanol in the urine samples of all study groups (Table).

4. Discussion

In the present study, we compared the efficacy of unfiltered brewed coffee with compounds from its main chemical groups, i.e. phenolic acids and alkaloids. We hypothesized that these nutraceuticals would replicate, at least partly, the metabolic effects of coffee. Although it is of clinical interest to identify an effective combination of coffee compounds that can be delivered in a dosage form, no research effort has been made to fulfill this purpose.

Among various compounds present in bioactive concentrations in coffee are chlorogenic acids (CGAs), the esters of quinic and trans-cinnamic acids. Among 69 different isomers of CGAs present in coffee [15], 3-, 4-, and 5-O-caffeoylquinic acids (CQAs) are the most abundant. We used 5-CQA in our study in a dosage (24 mg/day/rat) equal to the total CQAs content of 4 cups of brewed medium-roast Ethiopian Arabica coffee (around 155 mg per cup [16]) in an adult. 12 mg/day of caffeic acid was also added to the combination to compensate for the absence of the other groups of phenolic acids. The third constituent of our coffee-based supplement was trigonelline, an alkaloid present in roasted coffee in amounts averaging around 6.2 mg/g of ground beans [17]. The trigonelline present in 4 cups of coffee per day in a human would be equivalent to 7 mg/day in an average rat. Trigonelline is known to exert in-vivo glucose- and lipid-lowering effects [18]. Due to the epidemiological [19, 20] and experimental [21, 22] evidence indicating that both caffeinated and decaffeinated coffee preparations are effective in T2D and MetS prevention, and the uncertainty around the long-term effects of caffeine on insulin sensitivity, we did not incorporate caffeine into our formulation.

Contrary to the nutraceuticals, whole coffee brew consumption diminished the overall food intake and rate and amount of weight gain caused by HFHF food. Cowan et al. mirrored these results with a higher dosage of instant coffee using a similar animal model [23]. Modest weight modifying

effects of coffee have also been observed in some [24, 25] but not all [26, 27] of the previous comparable studies. Caffeine is known to induce weight loss in rodents [28, 29] and help to maintain weight loss in humans [30], but it may not be the only player. CGA-rich green coffee extracts [22, 31] and pure CGAs [32-35] have been proved effective in reducing body weight. Insignificant effects of our phenolic acid-rich chemical mixture may be related to the lower dosage compared to other studies [32, 34, 35] or different route/frequency of administration [33]. It is likely that in moderate dosages the synergy between caffeine and CGAs plays an important role in reducing weight gain. A comprehensive review of the anti-obesity effects of coffee and their proposed mechanisms has recently been published [36].

In our rats, fasting insulin showed an unsteady increase over time with an upsurge between weeks 10 and 14 indicating a delayed development of IR which was alleviated, but not halted, by coffee. This observation, confirmed by a significant reduction in HOMA-IR index, points to insulin-sensitizing effects of coffee and further corroborates our OGTT results. Although the literature is not consistent [23], our findings correspond to some earlier reports [27, 37]. It is puzzling though why the nutraceuticals did not show significant insulin-sensitizing efficacy. The reason might lie in the lower bioavailability of pure chemicals or the lack of other coffee compounds such as lignans and cafestol which were shown to possess insulinotropic and insulin-sensitizing activities [38-40]. Besides, the role of caffeine should not be neglected. Acute caffeine administration decreases insulin sensitivity [41]; however, in a time span of several weeks, this methylxanthine exhibits enhancing effects on glucose disposal rate [26, 29]. Comparable FPG levels in all groups were not surprising based on the knowledge that this index of liver glucose production was either unaltered [24, 25] or increased [23] in the former studies. Nevertheless, lower HbA1C in the coffee group signifies a better long-term glycemic control which is a better predictor of cardiovascular disease and all-cause mortality than the FPG [42].

Noticeably, unfiltered coffee exhibited a significant triglyceride-lowering effect in our study. On the contrary, neither coffee nor nutraceuticals altered the plasma levels of cholesterol and NEFAs after 14 weeks of intervention. Current knowledge suggests that coffee contains compounds with opposing effects on lipoprotein metabolism i.e. diterpenes (cafestol and kahweol) in unfiltered coffee can increase total cholesterol, LDL-C, and triglyceride levels [43], while CGAs and melanoidins show the opposite effects on both triglycerides and LDL-C [44, 45]. In rodents, however, this balance tends to shift towards the lipid-lowering effect since they are relatively resistant to the effects of cafestol [46]. This characteristic may reflect differences in lipoprotein metabolism between rats and humans [47]. An unchanged lipid profile in the nutraceuticals group can be due to the absence of other active substances such as the melanoidins.

Direct measurements showed that coffee administration decreased the hepatic deposition of triglycerides to almost half compare to the control rats. These results were further corroborated by semi-quantitative histological evaluations. Although our model did not display the advanced features of NAFLD or non-alcoholic steatohepatitis, a significant reduction of ectopic lipid deposition provides evidence for the protective properties of coffee against the development and progression of diet-induced NAFLD. Improvement in different aspects of the HF-diet-induced fatty liver was also documented previously in rats [27, 48]. Experimental evidence from human subjects is lacking.

The novel hyperpolarized-13C MR spectroscopy technique enabled us to follow the formation of main pyruvate metabolites in living animals. Since pyruvate is at the intersection of energy metabolism, proportional quantification of its metabolites would provide valuable information about the flux of key enzymes namely pyruvate dehydrogenase complex, alanine aminotransferase, and lactate dehydrogenase [49]. To the best of our knowledge, this technique has never been employed in investigating the effects of coffee or coffee chemicals in living animals. In our experiment, we aimed to explore a possible shift in hepatic carbohydrate metabolism caused by long-term consumption of coffee or its selected compounds. In theory, answering the question that which metabolite conversion rates are likely to be modified by coffee is challenging. However, reversing some of the changes induced by HF diet appears to be a reasonable response. It was shown that six weeks of HF diet can increase alanine/pyruvate and lactate/pyruvate ratios accompanied by a surge

in serum ALT levels [50]. Therefore, the reverse trend of changes in alanine/pyruvate ratio and lack of increase in circulating ALT can be considered metabolically-favorable effects of coffee in our HF-fed model. Non-significant differences between groups were probably due to the small sample size of four rats per group. In a previous study, four weeks of metformin therapy increased hepatic lactate/pyruvate but did not alter bicarbonate/pyruvate ratios in male Wistar rats [51]. We await future studies on the effects of coffee on intracellular carbohydrate metabolism and their relevance to its metabolic effects.

The following limitations have to be taken into consideration when evaluating our results. While it needs to be further confirmed, there are a few clues in the literature that pure CGAs may have lower absorption and different excretion behavior from CGAs from coffee [52]. On the other hand, the absence of standardized degrees of roasting, which affects the CGAs and trigonelline content of coffee, in commercialized products makes equivalency calculations and between-study comparisons unreliable. Next point is that delivering coffee and nutraceuticals in water makes it difficult to deliver the exact dosage and increases the probability of degradation and sedimentation (especially in case of caffeic acid). Finally, poor solubility of caffeic acid in water necessitated the use of ethanol as a solvent. Accordingly, each rat received ~0.6 gram of ethanol per day equal to 1.5 European standard drinks in a human. As described before, urine ethanol levels evidenced equal consumption in all groups, which eliminated the possibility of affecting our results. Ironically, including moderate amounts of ethanol in the rats' regime might have made it more similar to a typical Western diet.

5. Conclusions

Our study on a model of diet-induced MetS showed that a moderate dosage of unfiltered coffee can reduce food intake and weight gain in addition to mitigating average glycemia and IR compared to water. This beverage also diminished circulating triglycerides and their deposition in the liver (steatosis). Our selected combination of coffee nutraceuticals did not replicate any of the metabolic effects observed with coffee. In summary, our findings confirm the previous in-vivo research on the metabolic efficacy of coffee in non-diabetic models and suggest that other compounds than hydroxycinnamic acids and trigonelline are also crucial to the biological effects of coffee. We propose investigating more complex combinations with/without caffeine in higher dosages.

Author Contributions: PSh, KH, SG, and PBJ designed the experiment; PSh and PBJ performed the animal experimentations; CL, HQ, TSN and HSJ performed the MRI experiments and analyzed the data; SJHD performed the histological examinations and contributed tools and reagents; MSS performed the analytical chemistry experiments, contributed reagents and analyzed the data; PSh wrote the manuscript and SG and KH contributed significantly in reviewing the first draft.

Funding: Aarhus University Foundation and the Danish Diabetes Academy supported by the Novo Nordisk Foundation provided funding for this project in form of doctoral fellowships to Pedram Shokouh. Further financial support was provided by a grant from the Innovation Fund Denmark (no: 1308-00028B) and Riisfort Foundation.

Acknowledgments: Authors would like to appreciate Lene Trudsø and Eva Mølgaard Jensen for performing the laboratory tests and providing technical support. This work would have been impossible without skilled assistance from Gitte Eberhard and Sussi Kragh with handling and maintaining the animals. We thank Henrik Vestergaard for his skillful assistance with the MR spectroscopy experiments. Our special gratitude goes to Morten Rahr Clausen for his generous help and guidance with the 1H MR experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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