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Research Paper

Consumption of decaffeinated coffee protects against the development of early non-alcoholic steatohepatitis: Role of intestinal barrier function

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide lacking universally accepted therapies. Studies suggest that coffee consumption is associated with a reduced risk of NAFLD; however, molecular mechanisms and ingredients involved remain to be fully understood. Here, we determined the effects of regular intake of decaffeinated coffee on the development of NAFLD in mice, and molecular mechanisms involved.

Methods: Female C57BL/6J mice (n = 6–7/ group) were pair-fed either a liquid control diet (C) or fat-, fructose- and cholesterol-rich diet (FFC) +/- decaffeinated coffee (DeCaf, 6 g/kg BW) for 4 days or 6 weeks. Indices of liver damage, hepatic inflammation and parameters of insulin resistance and intestinal permeability as well as nitric oxide system were determined.

Results: Early signs of insulin resistance and non-alcoholic steatohepatitis (NASH) found after 6 weeks of FFC feeding were significantly lower in FFC + DeCaf-fed mice when compared to FFC-fed animals. Moreover, elevation of portal endotoxin levels and loss of tight junction proteins in proximal small intestine found in FFC-fed mice were significantly attenuated in FFC + DeCaf-fed animals. These beneficial effects of DeCaf were associated with a protection against the significant induction of inducible NO-synthase protein levels and 3-nitrotyrosine protein adducts found in proximal small intestine of FFC-fed mice. Similar protective effects of DeCaf were also found in mice fed the FFC diet short-term.

Conclusion: Our results suggest that protective effects of DeCaf on the development of NAFLD are at least in part related to maintaining intestinal barrier function.

1. Introduction

It is estimated that non-alcoholic fatty liver disease (NAFLD) affects ~25% of the general global population [1], making NAFLD the most frequently diagnosed liver disease worldwide. Indeed, in the US, NAFLD is by now the second leading cause of liver diseases of adults awaiting liver transplantation [2] and the most rapidly growing cause of hepatocellular carcinoma [3]. Moreover, results of epidemiological studies suggest that liver-specific and overall mortality for patients with NAFLD is higher than in general population and is also associated with

Abbreviations: 3-NT, 3-nitrotyrosine; ANOVA, analysis of variance; Atf, activating transcription factor 6; ALT, alanine transaminase; C, control; Chop, C/EBP homologous protein; DCE, decaffeinated coffee effect; DE, diet effect; DeCaf, decaffeinated coffee; DExDCE, interaction between diet and decaffeinated coffee; Dnajc3, DnaJ homolog subfamily C member 3; Edem1, ER degradation-enhancing alpha-mannosidase-like 1; ER, endoplasmatic reticulum; Grp78, 78 kDa glucose-regulated protein; GTT, glucose tolerance test; FFC, fat-, fructose- and cholesterol-rich diet; Herpud1, homocysteine-responsive endoplasmatic reticulum-resident ubiquitin-like domain member 1 protein; Il, interleukin; iNOS, inducible NO-synthase; Ir, insulin receptor; Irs, insulin receptor substrate; Lbp, lipopolysaccharide binding protein; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; NO, nitric oxide; PCR, polymerase chain reaction; Pdi, protein disulfide isomerase; Ppia, peptidylprolyl isomerase A; Tlr, toll-like receptor; Xbp1, X-box binding protein 1; ZO-1, zonula occludens-1

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impairments of health-related quality of life [4]. NALFD comprises a wide spectrum of alterations ranging from simple steatosis to steatohepatitis (non-alcoholic steatohepatitis, NASH), fibrosis and cirrhosis and even hepatocellular carcinoma [5]. However, the hepatocellular carcinoma is a rare while still serious complication of NAFLD [6]. Despite the alarming increase in prevalence and numbers of patients afflicted with later stages of the disease, molecular mechanisms involved in disease development and even more so progression have not yet been fully elucidated and universally accepted prevention and therapeutic strategies are lacking.

Results of both animal and human studies suggest that general overnutrition and herein particularly over consumption of certain macronutrients like saturated fats and monosaccharides e.g. fructose may be critical contributors to the onset and progression of NAFLD (for overview see [7,8]). Clinical and experimental studies also suggest that alterations at the level of the intestine e.g. alterations of intestinal microbiota composition and intestinal barrier function and subsequently an enhanced translocation of bacterial endotoxin may also be critical in the multifactorial development of NAFLD [9–12]. Indeed, in animal models it was shown that the development of a fat- and/ or sugar- e.g. fructose-induced NAFLD is associated with a loss of tight junction proteins in the proximal small intestine and higher bacterial endotoxin levels in the portal vein [11,13,14]. It was further shown that interventions preventing the loss of tight junction proteins and increases in portal endotoxin levels like oral treatment with antibiotics, bile acids or short chain fatty acids may indeed prevent at least in part the onset and progression of the disease [13,15–17].

Coffee is among the most consumed hot beverages worldwide [18]. Throughout the last decades evidence accumulated that coffee and herein especially filtered coffee may exert several beneficial effects on human health like lowering the risk of cardiovascular mortality and cancer as well as metabolic diseases including liver diseases (for overview see [19]). Indeed, while older studies reported that elevated consumption of unfiltered coffee – probably because of their content of kahweol and cafestol [20] - are associated with an increase of liver enzyme activity in blood [21], more recent studies suggest that the consumption of regular coffee may even be a protective factor for the development of NAFLD and fibrosis [22–24]. It has been proposed that the caffeine content found in coffee may be critical in regards to the beneficial effects of coffee on liver health [25,26], however, as other caffeine-rich beverages lack similar beneficial effects [27] it has been proposed that other compounds like polyphenols e.g. chlorogenic acids or melanoidins may also be involved in the beneficial effects of coffee on the liver (for overview see [26,28]). Despite intense effort to identify compounds and unravel mechanisms involved in the beneficial effects of coffee, to date, neither is fully understood.

Starting from this background the aim of the present study was to determine if decaffeinated coffee protects mice from the development of a high fat, high fructose and high cholesterol diet induced early NAFLD e.g. simple steatosis and steatosis with beginning inflammation and if so, to further determine molecular mechanisms involved.

2. Methods

2.1. Animals and treatment

Female C57BL/6J mice (10 weeks old) obtained from Janvier SAS, Le Genest-Saint-Isle, France, were housed in a specific-pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The local institutional animal care and use committee had approved all procedures before the experiments were carried out. Animals were handled in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All experiments were performed under controlled conditions and mice had free access to tap water at all times. **Long-term experiments**: Mice were adapted to a liquid control diet (C; 69E% carbohydrates, 12E% fat, 19E% protein; Ssniff, Soest, Germany) for 10 days as detailed previously [29]. Animals (n = 6/ group) were then randomly assigned to the following groups: mice fed a fat-, fructose- and cholesterol-rich diet (FFC; 60E% carbohydrates, 25E% fat, 15E% protein with 50% wt/wt fructose and 0.16% wt/wt cholesterol; Ssniff, Soest, Germany), mice fed a FFC diet enriched with commercially available freeze-dried decaffeinated coffee purchased in a local store (FFC + DeCaf, 6 g/kg BW), mice fed C diet or mice fed C diet enriched with the decaffeinated coffee (C + DeCaf, 6 g/kg BW). Concentration of decaffeinated coffee added to the diet was based on previous experiments of others and adapted to our feeding model and research question [30]. This amount of decaffeinated coffee was well tolerated by animals and no adverse effects were observed. Animals were pair-fed these diets for 6 weeks with caloric intake being adjusted daily to the FFC groups and C groups with the lowest caloric intake, respectively. As intake of the different liquid diets was rather similar between paired groups regardless of diet and or addition of decaffeinated coffee, availability of food had not to be limited to the point that mice had no access to food for an extended period of time. Rather, most of the time small amounts of diet were left in all feeding bottles when diets were replenished. Body weight was assessed weekly. After 5 weeks, an oral glucose tolerance test (GTT) was performed after mice were fasted for 6 h as detailed previously [31]. **Short-term experiments:** In line with the long-term feeding experiments, mice were adapted to the consumption of the liquid control diet (C) as detailed above. After adaption, all groups were fed the liquid control diet for four more days with some mice receiving decaffeinated coffee in their diet (6 g/kg BW). Thereafter, animals were assigned to the following groups: control diet (C), control diet enriched with decaffeinated coffee (C + DeCaf), fat-, fructose- and cholesterol-rich diet (FFC) and FFC enriched with decaffeinated coffee (FFC + DeCaf) for four more days. Again, caloric intake was adjusted daily between groups. Experimental setups and composition of diets used are summarized in Supplemental Fig. 1 and Supplemental Table 2. At the end of the trials mice were anesthetized with a ketamine/xylazine mixture (100 mg ketamine/kg BW; 16 mg xylazine/kg BW) via intraperitoneal injection. Blood was collected from portal vein just prior to killing and liver as well as intestinal tissue samples were collected and snap-frozen or fixed in neutral-buffered formalin.

2.2. Histological evaluation of liver sections and hepatic lipid accumulation

To evaluate status of liver damage paraffin-embedded liver sections (4 μm) were stained with hematoxylin and eosin (Sigma-Aldrich, Steinheim, Germany) and NAFLD Activity Score (NAS) was used for scoring liver histology as previously detailed [14]. Using a commercially available kit, number of neutrophil granulocytes in liver sections was assessed (Naphthol AS-D Chloroacetate (Specific Esterase) Kit; Sigma-Aldrich, Steinheim, Germany) as described before [32]. Triglyceride concentration in liver homogenates was determined as previously described [32].

2.3. Blood parameters of liver damage and endotoxin measurement

Alanine transaminase (ALT) activity in plasma of mice was measured in a routine laboratory (Friedrich-Alexander University Erlangen-Nürnberg, Germany). Bacterial endotoxin levels in portal plasma were determined using a limulus amebocyte lysate assay (Charles River, Ecully, France) as reported in detail previously [31]. Recovery rates ranged from ~ 83% to ~ 122%

2.4. Immunohistochemical staining of intestinal tissue

Using immunohistochemical staining concentration of 3-nitrotyrosine (3-NT), inducible NO-synthase (iNOS), occludin and zonula occludens-1 (ZO-1) in proximal small intestinal tissue were evaluated as

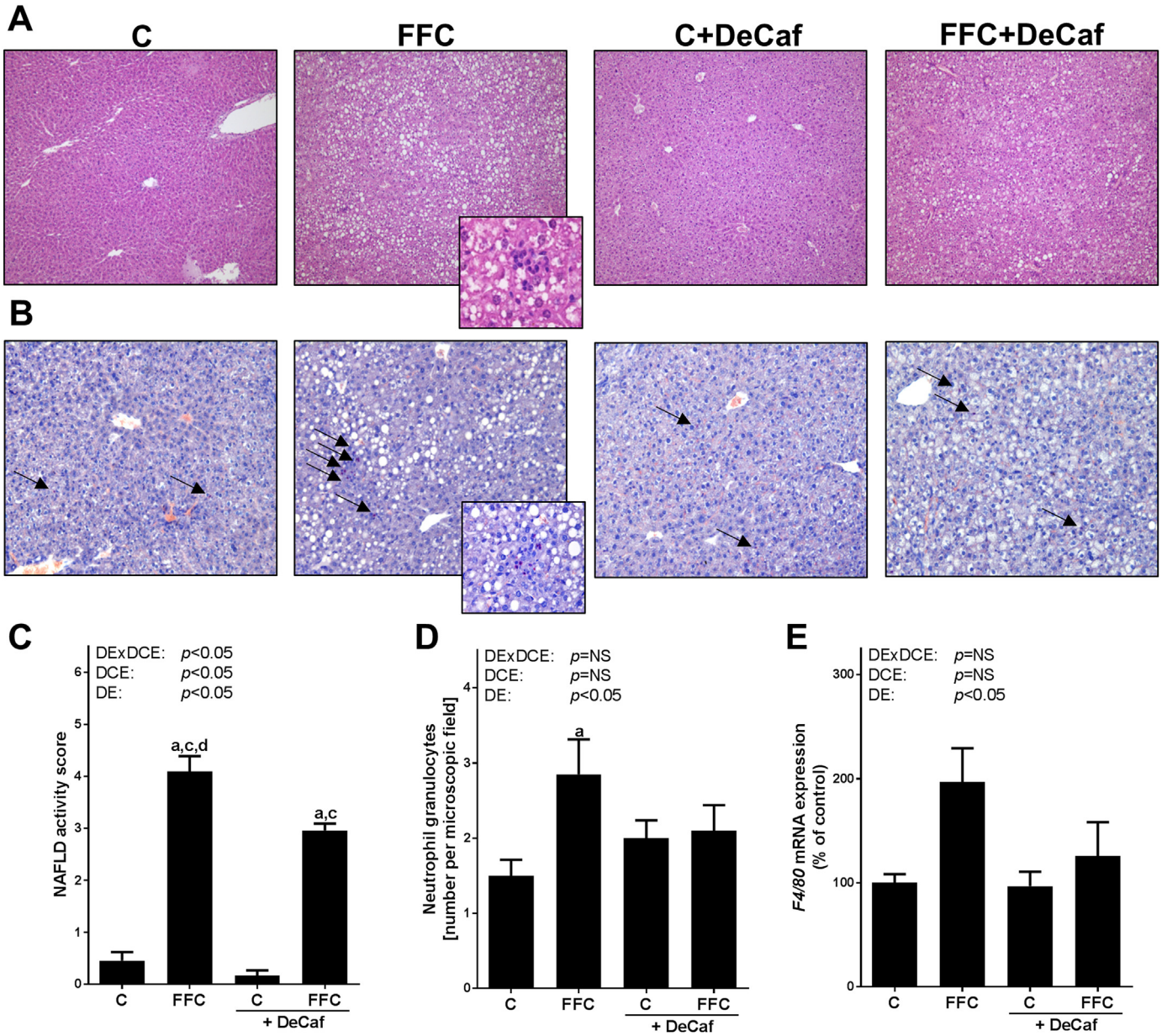

Fig. 1. Markers of liver damage and inflammation in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment. Representative pictures of (A) hematoxylin and eosin staining (200×, 630×) as well as (B) neutrophil granulocytes (200×, 630×) in liver tissue. (C) Evaluation via NAFLD activity score, (D) number of neutrophil granulocytes and (E) *F4/80* mRNA expression in hepatic tissue of mice. Values are means ± standard error of means. C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect, DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; NS: not significant. $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee; $^{d}p < 0.05$ compared with mice fed a FFC diet enriched with decaffeinated coffee.

detailed previously [14,32,33]. In brief, just prior to incubation with primary specific polyclonal antibodies (3-NT: Santa Cruz Biotechnology, Dallas, TX, USA; iNOS: Thermo Fisher Scientific, Waltham, MA, USA; occludin and ZO-1: Invitrogen, Carlsbad, CA, USA), deparaffinated tissue sections were incubated with protease (occludin, ZO-1) or heated in mono-sodium citrate buffer and blocked using bovine serum albumin solution (3-NT, iNOS). Sections were than incubated with peroxidase-linked secondary antibodies and diaminobenzidine (Peroxidase Envision Kit, DAKO, Hamburg, Germany). Staining was evaluated using analysis system (Leica Applications Suite, Leica, Wetzlar, Germany) integrated in a microscope (Leica DM6 B, Leica, Wetzlar, Germany) as described previously [32,33].

2.5. RNA isolation and real-time RT-PCR

Expression of genes listed in Supplemental Table 1 in liver tissue of mice were determined using RNA extracted from tissue with Trizol (peqGOLD Trifast, Peqlab, Erlangen, Germany). To assess mRNA expression in liver tissue, cDNA synthetized with reverse transcription system obtained from Promega GmbH (Madison, WI, USA) was used in real-time polymerase chain reaction (PCR) as described in detail before and normalized to 18S ([34], primer sequences are shown in Supplemental Table 1). To determine mRNA expression in small intestinal tissue, SensiFast SYBR No-ROX Kit in tandem with SensiFast cDNA was used for real-time PCR and normalized to peptidylprolyl isomerase A (Ppia) (Bioline, London, UK) [35].

2.6. Western blot

Protein was isolated from proximal small intestinal tissue using RIPA buffer containing an antiprotease cocktail (Sigma Aldrich, Steinheim, Germany) as previously described [32]. Thirty μg protein were separated in a polyacrylamide gel and transferring on a nitrocellulose membrane or polyvinylidene difluride membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, membranes were incubated overnight with primary antibody (3-NT, activating transcription factor 6 (ATF6), C/EBP homologous protein (CHOP), 78 kDa glucose-regulated protein (GRP78), protein disulfide isomerase (PDI), β-ACTIN) followed by an incubation with the appropriate secondary antibody and bands were detected using Pierce ECL Western Blotting Substrate or Super Signal West Dura kit (both Thermo Fisher Scientific, Waltham, MA, USA) [32,35]. Densitometric analysis of bands was performed using Image J software.

2.7. Statistical analysis

All data are presented as means ± standard error of means (SEM). Statistical Analysis was performed using PRISM (Version 7.03, GraphPad Software, Inc.). Data were log-transformed if Bartlett´s test was unequal. Outliers were determined using Grubb´s test. A two-factorial analysis of variance (ANOVA) was used to determine statistical differences between groups followed by Tukey´s post hoc test. $P < 0.05$ was defined to be significant.

3. Results

3.1. Markers of hepatic fat accumulation and inflammation as well as glucose metabolism

Neither C nor C + DeCaf-fed mice showed any signs of liver damage as determined by NAS and ALT plasma activity or any other adverse effects. Caloric intake of FFC-fed groups per se was significantly higher than in C-fed groups while body weight gain between C- and FFC-fed mice was similar regardless of additional treatments. Despite not developing overweight or obesity and in line with previous findings using similar kind of feeding model [29,34,36], FFC-fed mice developed early signs of NASH with massive macrovesicular fat accumulation and marked inflammatory alterations after 6 weeks (see Fig. 1, Table 1). While body weight gain, total caloric intake and liver to body weight ratio as well as ALT activity in plasma were similar between FFC- and FFC + DeCaf-fed mice, liver damage as assessed by NAS was significantly lower in FFC + DeCaf-fed animals when compared to FFC-fed animals. As data varied considerable in some groups, only ALT activity in FFC + DeCaf-fed mice was significantly higher than in C-fed groups (Table 1). In line with these findings number of neutrophil granulocytes in liver tissue and expression of the *F4/80* mRNA were also both almost at the level of controls in livers of FFC + DeCaf-fed animals while both markers were higher in livers of FFC-fed mice when compared to controls ($p < 0.05$ for neutrophil granulocytes and $p = 0.09$ for *F4/80* mRNA expression) (Fig. 1). Expression of *interleukin 1b (Il1b)* in liver tissue was significantly higher in livers of FFC-fed mice than in livers of FFC + DeCaf- and C + DeCaf-fed animals ($p < 0.05$ for both) as well as considerable higher compared to C-fed mice ($p = 0.05$) (Fig. 2). *Interleukin 6 (Il6)* mRNA expression was higher in livers of FFC-fed animals when compared to C + DeCaf-fed mice ($p = 0.11$); however, as data varied considerable in some groups, expression of *Il6* did not differ between C- and FFC-fed animals (Fig. 2).

While fasting glucose levels were similar between groups, area under the curve (AUC) of GTT was markedly higher in FFC-fed animals than in C-fed and C + DeCaf-fed animals ($p < 0.05$ for C vs. FFC; $p = 0.06$ for C + DeCaf vs FFC) (see Fig. 3). AUC of GTT from FFC + DeCaf-fed mice did not differ from other groups. In line with these findings, mRNA expression of *insulin receptor* (*Ir)* in liver tissue at the end of the trial was significantly higher in livers of FFC-fed mice than in all other groups. However, neither mRNA expression of *insulin receptor substrate 1 (Irs1)* nor *Irs2* differed between groups.

3.2. Markers of intestinal permeability and toll-like receptor-4 in proximal intestine and liver

Expression of *toll-like receptor 4 (Tlr4)* mRNA was significantly higher in livers of FFC-fed mice when compared to both control groups, while mRNA expression of *Tlr4* mRNA in FFC + DeCaf-fed animals neither differed from C-, C + DeCaf- or FFC + DeCaf-fed animals (Fig. 2). Expression of *lipopolysaccharide binding protein* (*Lbp)* was also significantly higher in livers of FFC-fed mice than in C-fed and FFC + DeCaf-fed animals while mRNA expression of *Lbp* in livers of FFC + DeCaf-fed animals did not differ from that of controls (Fig. 2). In line with these findings, endotoxin levels in portal plasma were also significantly higher in FFC-fed animals than in both control groups ($p < 0.05$) and FFC + DeCaf-fed mice (FFC vs. FFC + DeCaf $p = 0.05$, Fig. 4). Furthermore, protein concentration of the tight junction protein ZO-1 suggested to be indicative of paracellular barrier integrity [37], was significantly lower in proximal small intestine of FFC-fed groups compared to all other groups ($p < 0.05$ for all, Fig. 4). Occludin protein levels in proximal small intestine were also lower in FFC-fed mice than in all other groups; however, due to the high variability within groups differences reach only the level of significance for FFC- vs. FFC + DeCaf-fed and FFC- vs. C-fed mice ($p < 0.05$).

Table 1
Caloric intake, body weight and markers of liver damage in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment.

Parameter	Groups				*p*-Value		
	C	FFC	C + DeCaf	FFC + DeCaf	DExDCE	DCE	DE
Caloric intake [kcal/mouse/d]	9.2 ± 0.1	11.1 ± 0.2[a,c]	9.2 ± 0.2	11.1 ± 0.2[a,c]	NS	NS	< 0.05
Body weight [g]	22.3 ± 0.4	22.9 ± 0.3	23.0 ± 0.5	23.3 ± 0.3	NS	NS	NS
Weight gain [g]	2.8 ± 0.4	2.9 ± 0.3	3.0 ± 0.5	3.7 ± 0.3	NS	NS	NS
Liver weight [g]	1.0 ± 0.02	1.5 ± 0.04[a,c]	1.1 ± 0.03	1.6 ± 0.05[a,c]	NS	< 0.05	< 0.05
Liver: body weight ratio [%]	4.6 ± 0.2	6.7 ± 0.2[a,c]	4.9 ± 0.1	6.7 ± 0.2[a,c]	NS	NS	< 0.05
Plasma ALT [U/l]	19.0 ± 1.7	41.2 ± 8.9	20.4 ± 5.1	85.6 ± 22[a,c]	NS	NS	< 0.05
Steatosis[#]	0.5 ± 0.2	2.6 ± 0.2[a,c]	0.2 ± 0.1	2.4 ± 0.2[a,c]	NS	NS	< 0.05
Inflammation[#]	n.d.	1.2 ± 0.3	n.d.	0.5 ± 0.2	–	–	–

Values are means ± standard error of means. ALT: alanine transaminase; C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect; DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; n.d.: not detected; NS: not significant. [a]$p < 0.05$ compared with mice fed a control diet; [c]$p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee; [#]NAFLD Activity Score.

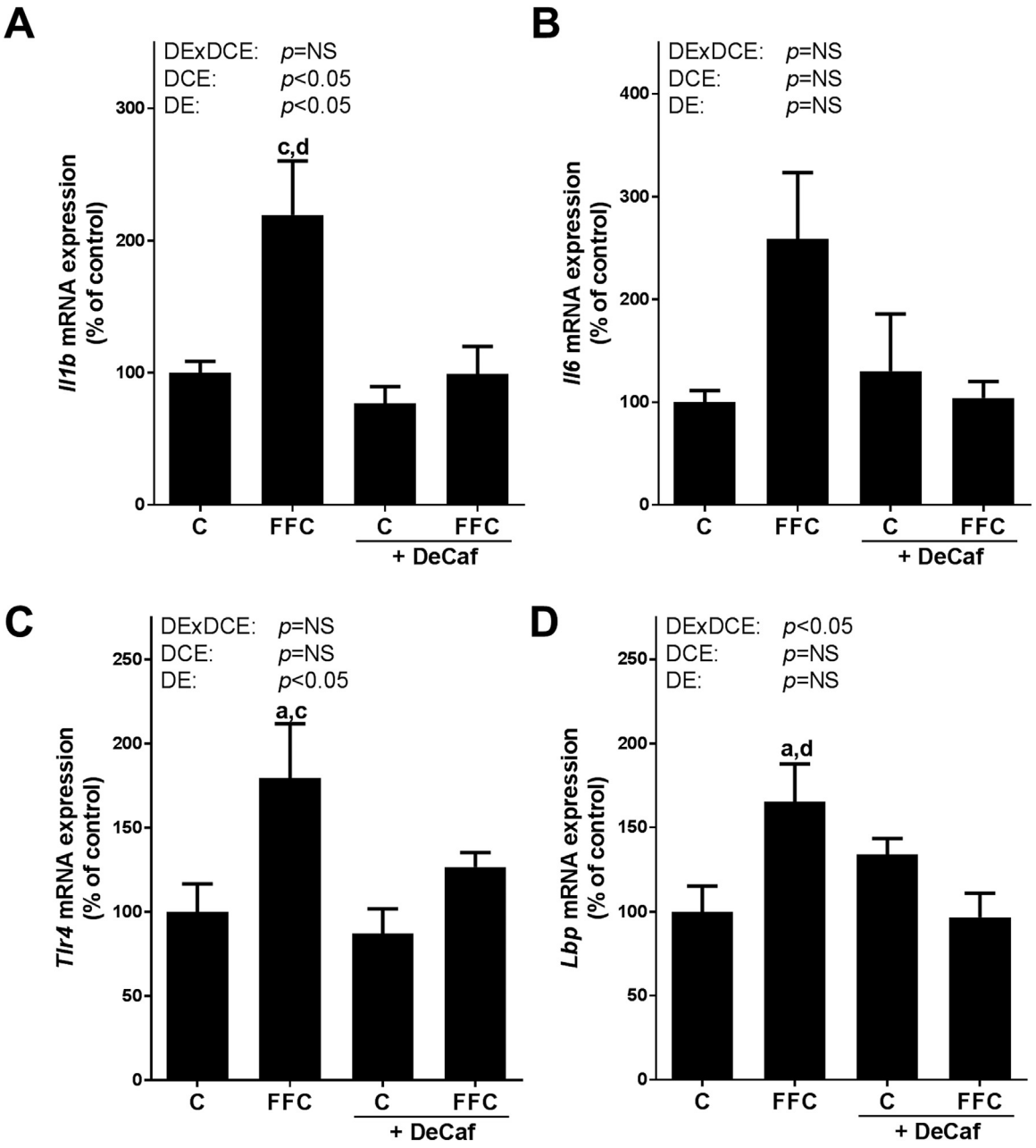

Fig. 2. Markers of inflammation and toll-like receptor 4 signaling cascade in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment. (A) *Il1b*, (B) *Il6*, (C) *Tlr4* and (D) *Lbp* mRNA expression in liver tissue. Values are means ± standard error of means. C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect, DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; Il: interleukin; Lbp: lipopolysaccharide binding protein; NS: not significant; Tlr: toll-like receptor. $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee; $^{d}p < 0.05$ compared with mice fed a FFC diet enriched with decaffeinated coffee.

3.3. Markers of ER stress in proximal small intestine

Expression of markers of ER stress in proximal small intestine between C-fed mice and FFC-fed mice did not differ at mRNA or protein level. (see Table 2, Supplemental Fig. 2). However, *Xbp1* as well as *Edem1* were significantly induced in C + DeCaf-fed mice compared to control group (see Table 2), whereas between FFC- and FFC + DeCaf-fed mice these markers were similar.

3.4. Markers of nitric oxide in proximal small intestine

Protein concentration of 3-NT and iNOS in proximal small intestine was significantly induced in FFC-fed mice compared to both control groups ($p < 0.05$), while protein concentration of 3-NT and iNOS in FFC + DeCaf-fed mice did not differ from all other groups (Fig. 5). In line with these findings, Western blot analysis of 3-NT protein adducts also suggested that in proximal small intestine of FFC-fed mice 3-NT protein adduct concentration is markedly higher than in all other groups; however, due to a lack of protein lysate only n = 3 mice/groups were analyzed. Representative blot is shown in Fig. 5.

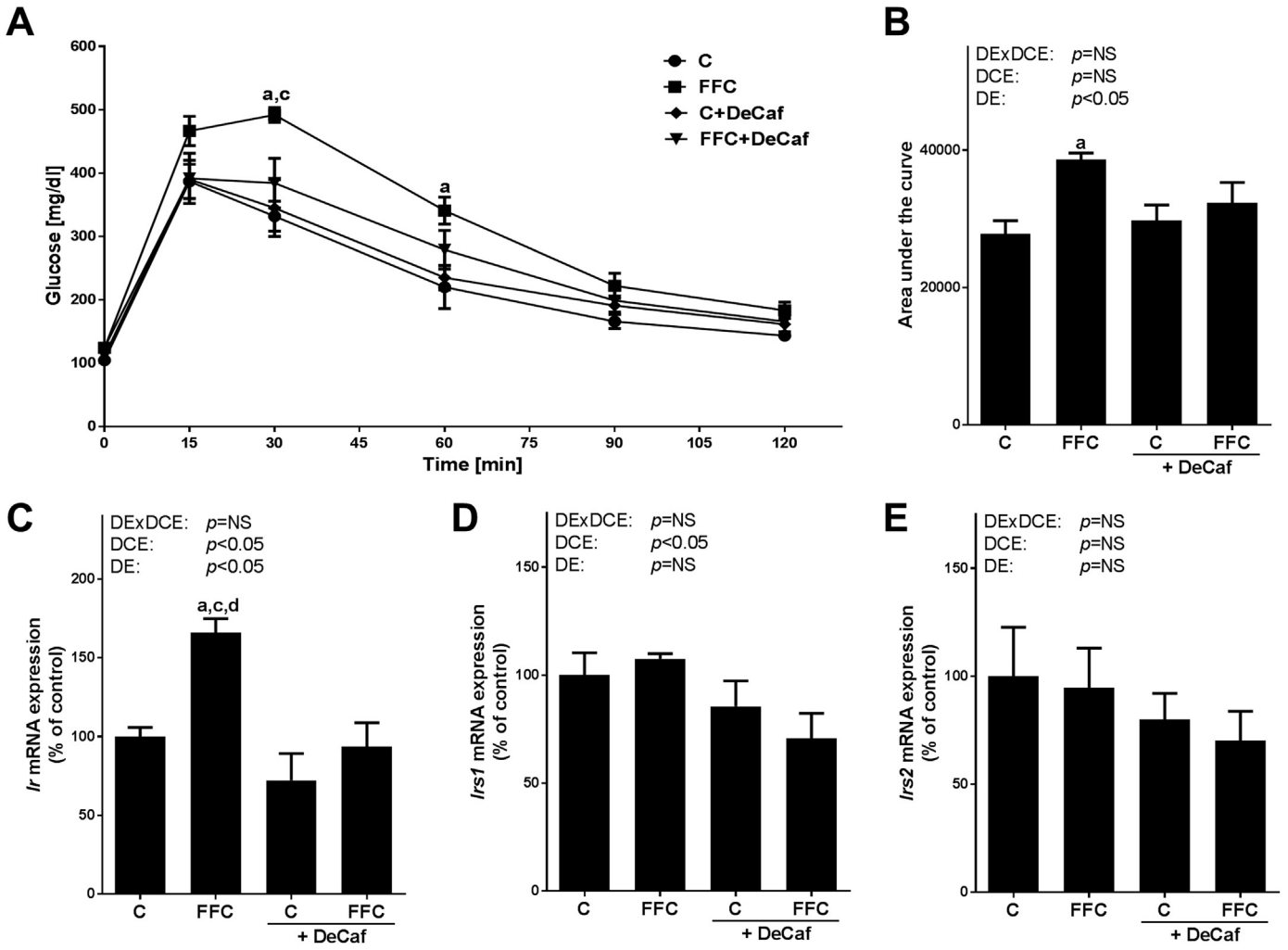

Fig. 3. Markers of glucose metabolism in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment. (A) Glucose levels during glucose tolerance test and (B) area under the curve. (C) *Ir*, (D) *Irs1* and (E) *Irs2* mRNA expression in liver tissue. Values are means ± standard error of means. C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect, DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; Ir: insulin receptor; Irs: insulin receptor substrate; NS: not significant. $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee; $^{d}p < 0.05$ compared with mice fed a FFC diet enriched with decaffeinated coffee.

3.5. Effect of decaffeinated coffee on the early onset of steatosis and intestinal barrier

To determine if decaffeinated coffee protects animals from early signs of NAFLD e.g. fat accumulation, mice were fed a FFC diet +/- DeCaf or C diet +/- DeCaf for four days. In line with previous findings of our group [15], and despite similar caloric intake and body weight among C- and FFC-fed groups (Table 3), FFC-fed mice developed massive microvesicular fat accumulation as also seen by triglyceride concentration in liver tissue and NAS (see Fig. 6). In contrast, fat accumulation was significantly lower in FFC + DeCaf-fed animals when compared to FFC-fed animals. Still, NAS was significantly higher in FFC + DeCaf-fed mice than in their respective control group. In line with the findings in the long-term feeding experiments in the present study, the protective effects of DeCaf were again associated with a protection against the increase in portal endotoxin levels found in FFC-fed animals ($p < 0.05$ for FFC vs. all other groups) (Fig. 6).

4. Discussion

4.1. Decaffeinated coffee consumption protected mice from the development of NAFLD and insulin resistance

Results of several epidemiological studies suggest that coffee intake and herein especially a high coffee consumption (> 3 cups) may lower the risk of fibrosis in NAFLD while data for earlier stages of the disease are still inconsistent (for overview see [38]). Despite intense research, the identification of compounds and molecular mechanisms involved is not fully understood (for overview see [28]). Here, a liquid fat-, fructose- and cholesterol-rich dietary pair feeding model shown before to induce “normal-weight” NAFLD e.g. leading to the development of non-alcoholic steatohepatitis over time without animals developing overweight or even obesity [31] was used to study the effects of decaffeinated coffee on the development of NAFLD. Indeed, in the present study, regardless of diet fed or additional treatments animals stayed normal weight. The lack of exceptional body weight gain in the long-term FFC-fed mice consuming on average ~+ 1.9 kcal/mouse when compared to C-fed animals might have resulted from a greater physical activity or increased energy expenditure. In support of the latter, it was

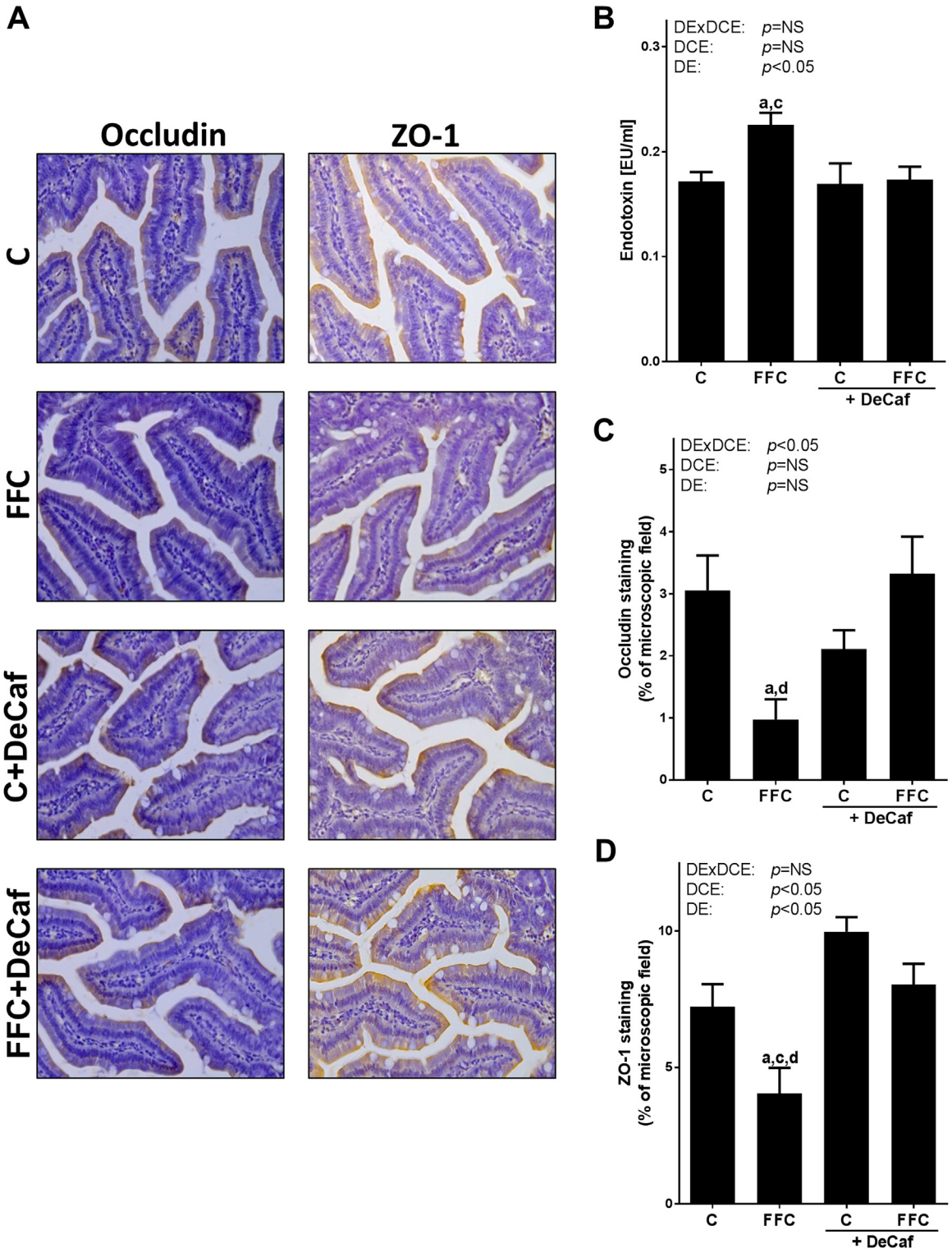

Fig. 4. Markers of intestinal permeability in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment. (A) Representative pictures (400×) of occludin and ZO-1 protein staining in proximal small intestine and (B) portal endotoxin concentration. Densitometric analysis of (C) occludin and (D) ZO-1 protein staining. Values are means ± standard error of means. C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect, DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; NS: not significant; ZO-1: zonula occludens-1 $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee; $^{d}p < 0.05$ compared with mice fed a FFC diet enriched with decaffeinated coffee.

demonstrated before that chronic intake of fructose in C57Bl/6 mice was not associated with increased overall body weight gain but rather elevated heat production and fat mass [39]. Using this feeding model, it was shown in the present study that decaffeinated coffee protects mice not only from the very early phases of the disease e.g. fat accumulation but also attenuates inflammatory alterations (e.g. development of

Table 2
Intestinal ER-stress markers in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment.

Parameter	Groups				*p*-Value		
	C	FFC	C + DeCaf	FFC + DeCaf	DExDCE	DCE	DE
***Atf4* mRNA**[#]	100 ± 16	174 ± 29	177 ± 31	136 ± 16	< 0.05	NS	NS
***Atf6* mRNA**[#]	100 ± 15	130 ± 17	154 ± 2.1	130 ± 19	NS	NS	NS
***Dnajc3* mRNA**[#]	100 ± 13	110 ± 20	115 ± 16	114 ± 19	NS	NS	NS
***Edem1* mRNA**[#]	100 ± 23	131 ± 5.3	171 ± 21[a]	114 ± 12	< 0.05	NS	NS
***Grp78* mRNA**[#]	100 ± 17	145 ± 27	101 ± 4.6	114 ± 21	NS	NS	NS
***Herpud1* mRNA**[#]	100 ± 20	172 ± 39	127 ± 8.2	108 ± 21	NS	NS	NS
***Pdi4* mRNA**[#]	100 ± 14	96.0 ± 14	76.8 ± 7.7	83.3 ± 9.4	NS	NS	NS
***Xbp1* mRNA**[#]	100 ± 21	126 ± 11	182 ± 21[a]	134 ± 19	NS	< 0.05	NS
ATF6/β-ACTIN[§]	1.2 ± 0.2	1.3 ± 0.3	1.6 ± 0.3	1.8 ± 0.5	NS	NS	NS
CHOP/β-ACTIN[§]	1.1 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.2	NS	NS	NS
GRP78/β-ACTIN[§]	0.18 ± 0.04	0.16 ± 0.03	0.18 ± 0.03	0.16 ± 0.01	NS	NS	NS
PDI/β-ACTIN[§]	0.58 ± 0.09	0.54 ± 0.05	0.64 ± 0.15	0.61 ± 0.05	NS	NS	NS

Values are means ± standard error of means. Atf: activating transcription factor 6; C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; Chop: C/EBP homologous protein; DCE: decaffeinated coffee effect; DE: diet effect; DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; Dnajc3: DnaJ homolog subfamily C member 3; Edem1: ER degradation-enhancing alpha-mannosidase-like 1; ER: endoplasmatic reticulum; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; Grp78: 78 kDa glucose-regulated protein; Herpud1: Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein; NS: not significant; Pdi: protein disulfide isomerase; Xbp1: X-box binding protein 1. [a]$p < 0.05$ compared with mice fed a control diet; [#]results presented as % of control; [§]densitometric analysis of western blot bands referred to β-ACTIN.

inflammatory foci, infiltration of neutrophils, induction of proinflammatory cytokines) and insulin resistance associated with later stages of the disease. However, DeCaf had no effect on ALT activity in plasma. Indeed it has been suggested that ALT activity may not be indicative of disease severity [40]. It was demonstrated that patients with similar severity of NASH showed various levels of ALT and that insulin resistance in adipose tissue and high liver triglycerides are major factors in elevation of ALT, rather than hepatic insulin resistance [40]. Despite the lacking effect on ALT activity of the present study, results are in line with those of others showing that coffee regardless of its caffeine content may protect rodents at least in part from the development of diet-induced steatosis and steatohepatitis [41–43] as well as impairments of glucose tolerance [41]. The apparent discrepancy between glucose tolerance, fasting glucose levels and genes involved in insulin signaling in liver tissue may have resulted from the fact that weight gain of FFC-fed mice was almost similar to that of controls. Indeed, results of Jensen et al. suggest that in "normal-weight" mice fed a high fat diet containing different amounts of sucrose, GTT is impaired even in the absence of altered fasting glucose levels or insulin sensitivity [44]. Our findings are also in line with others showing that coffee may improve glucose tolerance in dietary models of insulin resistance and that this seems not only to depend upon its caffeine content [41,45]. Indeed, recently it was shown that coffee compounds like caffeic acid, trigonelline and cafestol may protect mice from a high fat diet induced hyperinsulinemia. Interestingly, similar to the findings in the present study, fasting glucose levels were not affected [46]. Contrasting the findings of others, weight gain of FFC-fed mice in the present study was similar between groups. Previous studies of others suggested that male rats and mice fed a high fat diet gained less weight when being fed coffee regardless of caffeine content and despite similar caloric intake [47,48]. Differences between these studies and the present study might have resulted from differences in diets e.g. pelleted high fat vs. liquid fat-, fructose- and cholesterol-rich diet in the present study, gender (male vs. female mice) or the application (drinking water vs. mixed in a liquid diet in the present study). Taken together, results of the present study suggest that short and long-term consumption of DeCaf exerts protective effects on the development of NAFLD and that this is associated with improved glucose tolerance. However, our results also suggest that the protective effects differ between the very early phase e.g. lower lipid accumulation and later phases of the disease e.g. less inflammation while limited effects on fat accumulation further suggesting that DeCaf may only effect specific pathways involved in the disease shown repeatedly to be multifactorial (for overview see [49]). Indeed, it may be that DeCaf slows the progression of the disease rather than preventing it. This needs to be assessed in future studies employing long-term feeding experiments.

4.2. The protective effects of decaffeinated coffee on the development of NAFLD and insulin resistance are associated with a protection of intestinal barrier function

During the last years, data suggesting that alterations at the level of the gut e.g. changes of intestinal microbiota and barrier function as well as increased translocation of bacterial endotoxin may be among the critical factors contributing to the onset and progression of NAFLD [9,12,50]. In the present study, the protective effects of DeCaf were associated with a protection against the loss of tight junction proteins in small intestinal tissue and the increase of bacterial endotoxin levels in portal blood found in animals only fed the FFC diet. Interestingly, these effects were not only found when animals consumed DeCaf chronically but also after a short-term intake. Furthermore, *Tlr4* and *Lbp* mRNA expressions in liver tissue, which have been shown to be critical in mediating the effects of bacterial endotoxin in settings of NAFLD [51,52], were almost at the level of controls in livers of FFC-fed mice concomitantly fed DeCaf. Results of our study are in line with those of others showing that the protective effects of an extended intake of decaffeinated coffee on the development of NAFLD in rats were associated with a protection against the loss of intestinal tight junction proteins [48]. Furthermore, results of studies in healthy rodents and humans suggest that the consumption of coffee may alter microbiota composition in colon and feces within days [53,54]. It was further shown that in settings of high fat diet beneficial effects of caffeinated coffee on the liver fat content and body weight were associated with changes on fecal bacterial composition and increased levels of circulating short-chained fatty acids [55]. However, while all of these studies suggest that caffeinated coffee intake is associated with changes of bacterial composition in distal parts of the gastrointestinal tract, the question if intestinal barrier function and even more so bacterial endotoxin levels were altered remained unanswered. Indeed, the beneficial effects of fecal microbiota transfer on high fat diet induced NAFLD in mice were reported to be related to "normalization" of tight junction protein levels and lowered serum endotoxin level [56]. However, if the beneficial effects of decaffeinated coffee on tight junction proteins in small intestine and bacterial endotoxin levels in portal blood found in

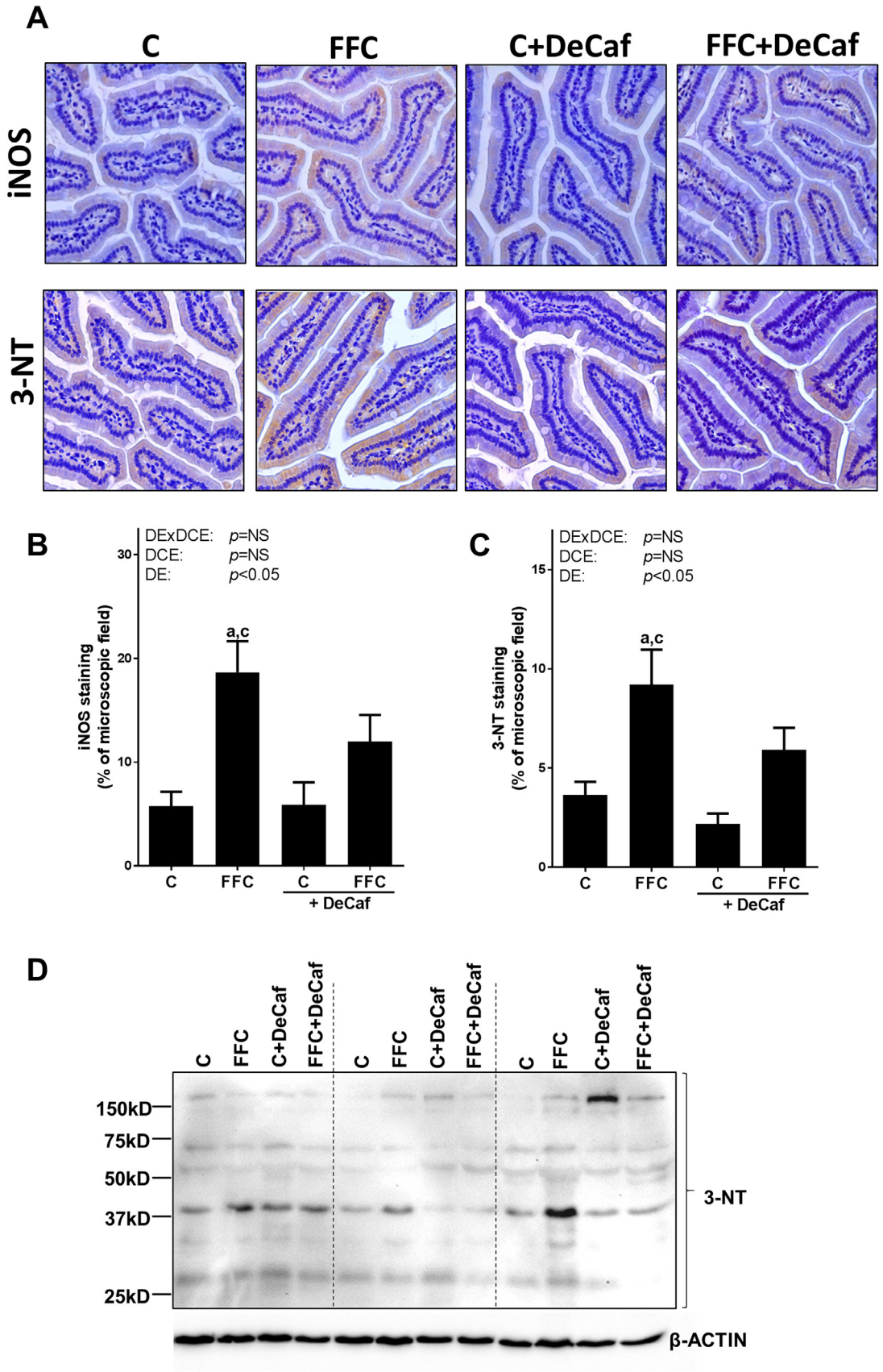

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Fig. 5. Protein levels of inducible NO-synthase and 3-nitrotyrosin protein adducts in proximal small intestine of mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment. (A) Representative pictures (400×) and densitometric analysis of (B) iNOS and (C) 3-NT protein staining. (D) Representative Western blot of 3-NT protein adducts and β-ACTIN. Values are means ± standard error of means. 3-NT: 3-nitrotyrosine; C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect, DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; iNOS: inducible NO-synthase; NS: not significant. $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee.

the present study were related to changes of intestinal microbiota composition in small intestine remains to be determined. While this is a major shortcoming of the present study, our data still suggest that chronic and short-term intake of decaffeinated coffee might affect intestinal barrier function and subsequently translocation of bacterial endotoxin (Fig. 7).

4.3. The protective effects of decaffeinated coffee consumption on intestinal barrier function are not associated with altered ER stress markers in proximal small intestine but a protection against NO stress

To date, molecular mechanisms involved in impairments of intestinal barrier function found in settings of NAFLD are not fully understood. Results of several studies suggest that ER hepatic stress emerged as a critical contributor to the development of NAFLD [57,58] and may also be critical in intestinal barrier dysfunction (for overview see [59]). Furthermore, it has been suggested that coffee with and without caffeine may affect ER stress [42,60]. In the present study, protective effects of DeCaf on the development of NAFLD and the loss of tight junction proteins in small intestine was not associated with changes of markers of ER stress. Rather, markers of ER stress determined were similar between controls and FFC-fed mice regardless of additional treatments. However, both *Edem1* and *Xbp1* mRNA expression were significantly increased in small intestinal tissue of control mice fed DeCaf when compared to C-fed animals. Somewhat in line with these findings, Salomone et al. showed that decaffeinated coffee induced the expression of chaperones in liver tissue probably to ensure correct protein folding [42]. Reasons for the lack of expression of these genes in FFC + DeCaf-fed mice remains to be determined, including alterations in calcium homeostasis, which is known to regulate ER stress response [61].

Results of several studies suggest that nitric oxide (NO) produced via iNOS resulting in reactive NO oxidation intermediates may also play a critical role in the loss of intestinal barrier function (or overview see [62]). In the present study the loss of tight junction proteins in proximal small intestine and elevated bacterial endotoxin levels in portal plasma found in FFC-fed mice were associated with increased iNOS protein and 3-NT protein adduct concentrations in proximal small intestine. These alterations were almost completely attenuated in small intestinal tissue of FFC-fed mice enriched with DeCaf. *In vitro* studies of other groups have shown that components of coffee, like kahweol or chlorogenic acid can suppress iNOS expression [63,64]. If these coffee compounds were also involved in the suppressing effects of DeCaf on iNOS in the present study as well as molecular mechanisms involved needs to be determined in future studies.

When interpreting data of the present study it needs to be kept in mind that decaffeinated coffee contains small amounts of caffeine (Germany: max. 0.1% [65]). Accordingly the doses of dried coffee powder fed to animals in the present study would correspond to a caffeine intake of 6 mg/kg BW/day in a mouse or 420 mg/day for a normal weight 70 kg man. While this amount of caffeine is in accordance with the maximum daily dose thought to be not harmful for non-pregnant adults (recommend daily intake < 400 mg/day (EFSA), [66]), studies showing a protective effect of caffeine on the development of NAFLD in rodents used markedly higher concentrations (20–400 mg/kg BW/day; [25,67–69]) further suggesting that effects found in the present study may not solely have resulted from caffeine remnants found after decaffeination. Indeed, in recent years data accumulated suggesting that polyphenol and melanoidins formed during roasting may also impact health effects of coffee brew [70]. For instance Vitaglione et al. reported that the effects of both polyphenols and melanoidins extracted from decaffeinated coffee were almost comparable to the protective effects of whole decaffeinated coffee on the development of a high fat diet induced NAFLD [43].

5. Conclusion

In line with the results of other groups [41–43,46] data presented here suggests that coffee protects the liver of mice from the development of early stages of NAFLD and that these protective effects may be, at least in part, independent of caffeine. Our results also suggest that the beneficial effects of coffee on the development of the disease in mice may result from a protection against the development of intestinal barrier dysfunction and the induction of intestinal iNOS already found in the very early onset of the disease e.g. within days of changing control diet to a fat-, fructose- and cholesterol-rich diet. However, if similar effects on intestinal barrier function and translocation of bacterial endotoxin are also found in humans consuming coffee and if this then is causally involved in the beneficial effects of coffee on the development of NAFLD reported in meta-analysis [24,38] remains to be

Table 3
Caloric intake, body weight and markers of liver damage in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment for four days.

Parameter	Groups				*p*-Value		
	C	FFC	C + DeCaf	FFC + DeCaf	DExDCE	DCE	DE
Caloric intake [kcal/mouse/d]	9.2 ± 0.4	9.5 ± 0.6	8.8 ± 0.2	9.7 ± 0.5	NS	NS	NS
Body weight [g]	19.1 ± 0.4	19.3 ± 0.4	19.3 ± 0.4	19.3 ± 0.3	NS	NS	NS
Weight gain [g]	1.1 ± 0.2	1.4 ± 0.1	1.9 ± 0.2	1.7 ± 0.3	NS	< 0.05	NS
Liver weight [g]	0.9 ± 0.03	1.2 ± 0.05[a,c]	1.0 ± 0.04	1.1 ± 0.05[a]	NS	NS	< 0.05
Liver: body weight ratio [%]	4.8 ± 0.2	6.1 ± 0.2[a,c]	5.1 ± 0.2	5.9 ± 0.3[a,c]	NS	NS	< 0.05
Plasma ALT [U/l]	21.1 ± 2.6	16.1 ± 1.4	16.5 ± 1.9	15.2 ± 1.7	NS	NS	NS

Values are means ± standard error of means. ALT: alanine transaminase; C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect; DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; NS: not significant. $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee.

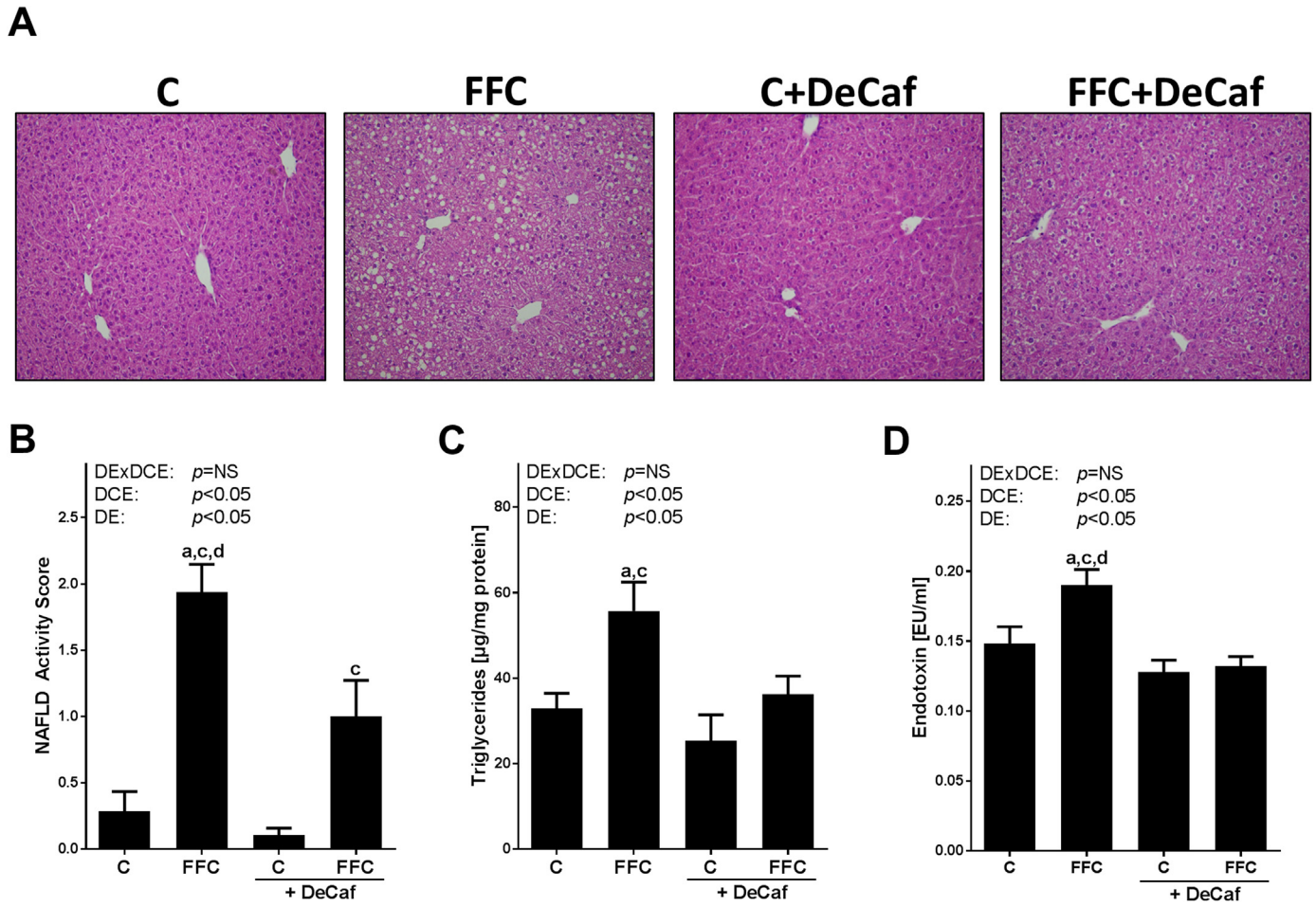

Fig. 6. Markers of liver damage and portal endotoxin levels in mice fed a control or fat-, fructose- and cholesterol-rich (FFC) diet ± decaffeinated coffee (DeCaf) enrichment for four days. (A) Representative pictures of hematoxylin and eosin staining (200×) of liver tissue and (B) evaluation via NAFLD activity score. (C) Triglyceride concentration of liver tissue and (D) portal endotoxin concentration. Values are means ± standard error of means. C: control diet; C + DeCaf: control diet enriched with decaffeinated coffee; DCE: decaffeinated coffee effect; DE: diet effect, DeCaf: decaffeinated coffee; DExDCE: interaction between diet and decaffeinated coffee; FFC: fat-, fructose- and cholesterol-rich diet; FFC + DeCaf: fat-, fructose- and cholesterol-rich diet enriched with decaffeinated coffee; NS: not significant. $^{a}p < 0.05$ compared with mice fed a control diet; $^{c}p < 0.05$ compared with mice fed a control diet enriched with decaffeinated coffee; $^{d}p < 0.05$ compared with mice fed a FFC diet enriched with decaffeinated coffee.

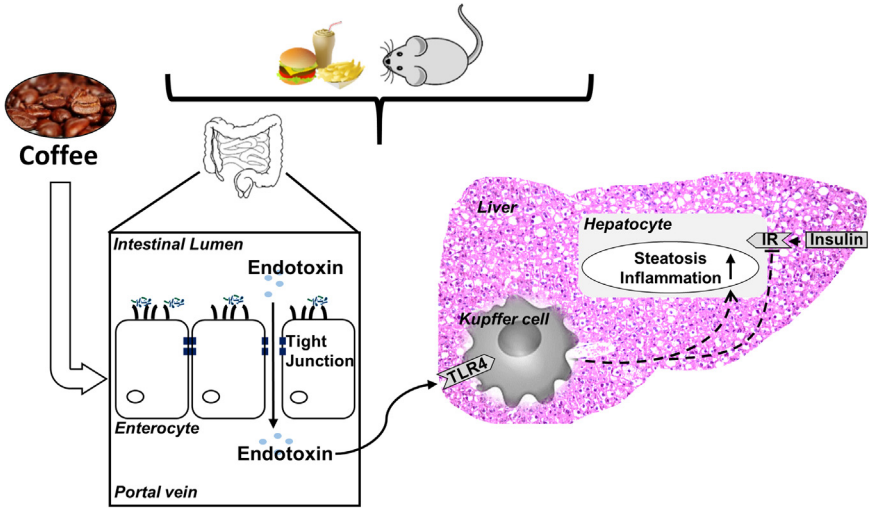

Fig. 7. Graphical summary of the protective effect of decaffeinated coffee on development of early non-alcoholic steatohepatitis in female mice. Coffee is able to protect mice from development of early stages of NASH and may result from the protective effect of coffee on impaired intestinal barrier function. IR: insulin receptor; NASH: non-alcoholic steatohepatitis; TLR4: toll-like receptor 4.

determined.

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Conflicts of interest

IB received financial support from Yakult Ltd. for another, unrelated research project. All other authors declare no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.101092

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