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TITLE PAGE

TITLE: EZETIMIBE ENHANCES MACROPHAGE TO FECES REVERSE CHOLESTEROL TRANSPORT IN GOLDEN SYRIAN HAMSTERS FED A HIGH CHOLESTEROL DIET.

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RUNNING TITLE: EFFECT OF EZETIMIBE ON REVERSE CHOLESTEROL TRANSPORT IN HAMSTER

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Nonstandard abbreviations used: AUC, area under the curve; HF, high cholesterol diet; IPGTT, intra-peritoneal glucose tolerance test; SR-B1, scavenger receptor type B class 1; SCD1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; NPC1L1, Niemann-Pick C1 Like 1; HDL, high density lipoprotein; LDL, low density lipoprotein, VLDL, very Low Density Lipoprotein.

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Abstract

The aim of this work was to evaluate reverse cholesterol transport (RCT) in hamster, animal model expressing CETP under a high cholesterol diet (HF) supplemented with Ezetimibe using primary labelled macrophages. We studied three groups of hamsters (n=8/group) for 4 weeks: 1) chow diet group: Chow, 2) High cholesterol diet group: HF and 3) HF group supplemented with 0.01% of ezetimibe: HF+0.01%Ezet. Following intraperitoneal injection of ^{3}H-cholesterol-labelled hamster primary macrophages, we measured the in vivo macrophage-to-feces RCT.

HF group exhibited an increase of triglycerides (TG), cholesterol, glucose in plasma and higher TG and cholesterol content in liver ($p<0.01$) compared to Chow group. Ezetimibe induced a significant decrease in plasma cholesterol with a lower LDL and VLDL cholesterol ($p<0.001$) and in liver cholesterol ($p<0.001$) and TG ($p<0.01$) content compared to HF. In vivo RCT essay showed an increase of tracer level in plasma and liver ($p<0.05$) but not in feces in HF compared to Chow group. The amount of labelled total sterol and cholesterol in liver and feces was significantly reduced ($p<0.05$) and increased ($p=0.05$) respectively with Ezetimibe treatment. No significant increase was obtained for labelled feces bile acids in HF+0.01%Ezet compared to HF. Ezetimibe decreased SCD1 gene expression and increased SR-B1 ($p<0.05$) in liver but did not affect NPC1L1 nor ABCG5 and ABCG8 expression in jejunum. In conclusion, ezetimibe exhibited an atheroprotective effect by enhancing RCT in hamster and decreasing LDL cholesterol. Ours findings showed also a hepatoprotective effect of ezetimibe by decreasing hepatic fat content.

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Significant statement:

This work was assessed to determine the effect of ezetimibe treatment on high cholesterol diet induced disturbances and especially the effect on reverse cholesterol transport in animal model with CETP activity and using labelled primary hamster macrophages. We were able to demonstrate that ezetimibe exhibited an atheroprotective effect by enhancing RCT and by decreasing LDL cholesterol in hamster. We showed also a hepatoprotective effect of ezetimibe by decreasing hepatic fat content.

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Introduction

Metabolic syndrome is cluster of metabolic disturbances that increase cardiovascular disease. Dyslipidemia is a common disturbance in metabolic syndrome (Ginsberg et al., 2006). Cholesterol homeostasis is a balance of cholesterol synthesis, intestinal absorption, and bile acids elimination. The current treatment for hypercholesterolemia is statins, which lower *the* hepatic cholesterol synthesis by inhibiting HMGCoA enzyme activity, and then accelerates hepatic LDL uptake. Active hepatic synthesis of cholesterol and LDL uptake are also modulated by the amount of absorbed cholesterol in the small intestine. Ezetimibe known to inhibit cholesterol absorption via Niemann-Pick C1 Like 1 (NPC1L1) mediated cholesterol absorption at the brush border of the intestine (Altmann et al., 2004). NPC1L1 has been identified as the main transporter of intestinal cholesterol and has been shown to be a target of ezetimibe (Garcia-Calvo et al., 2005). Ezetime is an effective lowering lipid drugs (Jacobson et al. 20015). A large clinical study in individuals aged ≥75 years with elevated LDL-C showed that ezetimibe prevented cardiovascular events by decreasing LDL cholesterol (Ouchi et al., 2019).

Ezetimibe enhances macrophage-to-feces reverse cholesterol transport (RCT) in mice as shown by in vivo RCT experiments (Briand, 2010; Wang et al., 2014). In the present study, hamsters have been utilized as an alternate model with lipoprotein metabolism close to humans to determine the impact of ezetimibe treatment on the efficacy of RCT. Contrarily to more classical protocols using murine cells (Castro-Perez et al., 2011; Tchoua et al., 2008), we have studied (Kasbi Chadli et al., 2013) the RCT in hamster using labelled primary hamster macrophages. The use of primary hamster macrophages avoids the cross-species inflammatory reaction which could potentially lead to an immunological targeted destruction of macrophages by using J774 murine cells. Moreover, these cells does not express

apolipoprotein E (apoE) gene (Bernard et al., 1991) involved in the removal of excess cholesterol from macrophage foam cells (Mazzone and Reardon, 1994). Few studies with regards to ezetimibe has been conducted using hamster, only one of them focused on the cholesterol flux from peripheral cells to the liver to further excretion into bile and feces (Uto-Kondo et al., 2014) using labelled primary hamster macrophages. Hamster, unlike mice, is a model that expressed cholesterol ester transfer protein (CETP) which plays an important role in RCT. It is established plasma cholesterol concentrations and cholesterol distribution among lipoproteins are affected by ezetimibe in hamster (van Heek et al., 2001). It has never been studied whether these changes in the metabolism lipoproteins induced by ezetimibe observed in hamster affect lipid and glucose metabolic disturbances.

To address this question we characterized the effects of a high cholesterol diet supplemented with 0.01% ezetimibe in Golden Syrian hamsters and assessed in vivo macrophage-to-feces RCT using labelled primary macrophages as well as the effect of ezetimibe on lipid and glucose metabolic disturbances.

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Material and methods

Ethics statement:

The experimental protocol was approved by the local Animal Used and Care Advisory Committee (Bretagne-Pays de la Loire committee) and was adhered to European Union guidelines. Animals were sacrificed by intra-cardiac injection of lethal dose of pentobarbital. All animal trial was carried out under isofluran anesthesia.

Animals and diets

Male hamsters (R. Janvier, France) were housed in plastic cages (2 hamsters/cage) containing wood shavings and maintained in a room with a 12-hour light cycle with free access to food and water. Animals (82-99g) were adapted to these conditions and fed a rodent chow diet (A04 rodent diet, Safe Diets, France) for 1 week. This rodent chow diet was defined as the control diet.

Hamsters were then kept on the control diet (n=8), diet rich in cholesterol (0.3% of cholesterol) (n=8) or high cholesterol diet supplemented with 0.01% Ezetimibe (n=8) during 4 weeks. The groups with high cholesterol diet received 10% fructose in drinking water. Hamsters were fasted overnight and slight isoflurane anaesthesia was used to collect blood by retro-orbital bleeding to measure plasma lipids. After sacrifice, hamster were exsanguinate and liver was harvested, flash-frozen in liquid nitrogen and stored at -80°C until analysis. Blood glucose was monitored using a glucometer (Roche Diagnostics, France). Three other separate sets of hamsters fed the chow, high cholesterol or high cholesterol+ezetimibe diet over 4 weeks were used for determination of in vivo triglyceride production (n=8 per group) and in vivo macrophage-to-feces RCT (n=8 per group), as described below.

Biochemical analysis

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To determine plasma lipids, hamsters were fasted overnight (18) and blood was collected by retro-orbital under isofluran anesthesia. Blood was then centrifugated ((4°C, 10 min, 3000 g) to separate plasma. Triglyceride and total cholesterol were measured using commercial kits (Biomerieux, France). Lipoproteins were separated using fast protein liquid chromatography (AKTA FPLC SYSTEM, GE Healthcare, USA). Cholesterol ester transfer protein (CETP) activity was assayed by a commercial kit (RoarBiomedical, NY, USA). Evaluation of Lecithin cholesterol acyl transferase (LCAT) activity was performed by the measurement of the decrease of free cholesterol after 1 hour of plasma incubation at 37°C. This decrease is expressed as the percentage of free cholesterol transformed into cholesteryl ester by hour (Nagasaki and Akanuma, 1977).

Intraperitoneal glucose tolerance test (IPGTT)

An intraperitoneal glucose tolerance test was performed at 09.00 hours after being unfed for 18 h. Eight hamsters of each group received 2 g of glucose per kg BW intraperitoneally. Blood samples were obtained from tail vein at 0 (fasting), 15, 20, 30, 60 and 120 min and glycaemia was measured immediately using fresh blood (glucometer: Accu-Chek active; Roche Diagnostics, Mannhein, Germany). Areas under the curves (AUC) were calculated by the trapezoid rule from the glucose measurements at 0 (fasting), 15, 30, 60, 120 and 180 min (mg x min/dl).

Measurement of in vivo hepatic secretion of triglycerides (TG):

The effect of Ezetimibe on the hepatic TG secretion was determined using Tyloxapol, a nonionic surfactant (Triton WR1339) that inhibits lipoprotein lipase (Schotz et al., 1957). Hamsters were anesthetized with isofluran and blood samples were collected from the eye orbital venous plexus. Blood samples were taken at baseline, just before peritoneal injection of Tyloxapol and then, at 30 min (t30), 2h (t120) and 3h (t180). The first 30 min after Tyloxapol injection are required to detergent equilibration and initiation of lipoprotein

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accumulation (Millar et al., 2002). Plasma TG accumulation from t30 to t180 after Tyloxapol injection is proportional to VLDL TG secretion (Siri et al., 2001). Hepatic TG production rate for individual hamsters was therefore calculated using linear increment between 30 min and 3h.

Isolation of peritoneal-elicited macrophages

Isolation of peritoneal macrophages was described in our previous study (Treguier et al., 2011). briefly hamsters were injected intraperitoneally (IP) with 3% Brewer thioglycollate medium (Sigma). Three days after injection, hamsters were sacrificed, macrophages were collected by peritoneal washing with ice-cold PBS and then centrifuged. The macrophages were suspended in 5 mL of red blood cell lysis buffer and incubated in ice during 15 minutes. After centrifugation the macrophages were collected and suspended in RPMI culture medium supplemented with 10% FBS (Gibco). The cells were then plated in flasks in RPMI complete medium and allowed to adhere, non adherent cells were removed

In vivo macrophage-to-feces reverse cholesterol transport

Hamster macrophages were radiolabeled as described previously (Treguier et al., 2011). Briefly, macrophages were loaded with ^{3}H-cholesterol labeled acetylated LDL. Cells were pelleted by centrifugation and suspended in PBS before hamsters injection (n=6/group). Hamsters were then housed in individual cage. Blood was drawn at 48 and 72 hours and radioactivity was counted in a liquid scintillation counter. After hamster sacrifice, liver was harvested to assess the radioactivity up-take.

The total feces collected over 72 hour water was homogenized to extract the ^{3}H-cholesterol and ^{3}H-bile acid fractions as we have previously described (Treguier et al., 2011). The extracts were treated to determine radioactivity. Results were expressed as a percent of the

radioactivity injected recovered in plasma, liver and feces. The volume of plasma was estimated as 3.5% of the body weight.

RNA extraction and gene expression analysis

After homogenization of liver samples, mRNA was extracted using Trizol reagent (Invitrogen, France). RT-PCR analysis was assessed as follows: 1 μg of total RNA was reverse-transcribed using MML-V reverse transcriptase (Promega, France). Real time quantitative PCR was performed on the 7000 Sequence Detection System with SYBR green MESAGREEN Master Mix Plus (Eurogentec, Angers, France). All reactions were duplicates. Cyclophilin was used as a reference. The expression of CYP7A1,SR-B1, LDLr and ABCA1 was measured in liver (8 hamsters/group). The expression of NPC1L1, ABCG5 and ABCG8 was measured in intestine. Primers sequences are available on request.

Determination of hepatic lipid content:

Lipids were extracted from 30 mg of hepatic tissue using Folch method (Folch et al., 1957). The tissue was homogenized with chloroform/methanol (2/1), and then centrifuged to recover the liquid phase. The solvent was washed with saline solution and centrifuged. The chloroform phase was evaporated and lipids were suspended in ethanol. Triglyceride and cholesterol concentration were assessed using commercial kits (TG: PAP 150, cholesterol RTU; Biomérieux, Marcy-l'Etoile, France) (n=8/group).

Statistical analysis

The statistical significance of differences between groups was evaluated using ANOVA test followed by 2 tailed student's t test for unpaired comparisons. $P<0.05$ was considered significant. Values are presented as mean ± SEM.

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Results

Ezetimibe did not affect weight and fasted blood glucose and glucose tolerance but it decreased plasma cholesterol by decreasing VLDL and LDL cholesterol:

No change was observed for body weight among groups. Blood fasted glucose was higher in groups under high cholesterol diet compared to chow control group with no difference between HF and HF+0.01%Ezet (Table 1). The measurement of area under curves after IPGTT did not show any difference in glucose tolerance among the three groups (data not shown).

High cholesterol diet increased significantly plasma cholesterol ($p<0.001$), triglycerides (TG) ($p<0.001$) and LCAT activity ($p<0.05$). Ezetimibe supplementation did not affect TG concentration and LCAT activity but lowered significantly plasma cholesterol concentration in HF+0.01%Ezet compared to HF ($p<0.005$) (table 2). The cholesterol lipoprotein profile showed a significant decrease in VLDL ($p<0.05$) and LDL ($p<0.001$) cholesterol in HF+0.01%Ezet compared to HF ($p<0.005$) (figure 1). No effect was observed on CETP activity (table 1).

Ezetimibe lowered hepatic lipid accumulation but did not affect hepatic VLDL TG secretion:

While the total liver weight was significantly higher in HF compared to Chow group ($p<0.01$) this parameter is normalized in HF+0.01%Ezet group compared to HF group ($p<0.005$). The measurement of lipid content in liver showed a significant increase in TG and cholesterol in HF group compared to Chow group ($p<0.005$). Ezetimibe treatment cancelled the cholesterol accumulation in liver ($p<0.001$) and decreased TG content in liver in HF + 0.01%Eze group compared to HF group ($p<0.01$, figure 2). The measure of hepatic TG secretion did not show a significant difference among groups even between HF and Chow group (figure 3).

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Ezetimibe promoted fecal cholesterol excretion without effect on bile acids excretion

To assess whether RCT is affected in vivo, control chow, HF and HF+0.01%Ezet hamsters were injected with ^{3}H-cholesterol labelled hamster primary macrophages. Figure 4A showed higher plasma ^{3}H-tracer appearance at 24h in HF compared to control Chow group. At 72h, plasma ^{3}H-tracer appearance was lower in HF+0.01%Ezet group compared to Chow group ($p=0.02$). No change was observed at 48h after macrophage injection among the three groups (Figure 4A).

Tracer recovery in liver was higher in HF group compared to control chow diet ($p<0.05$) while HF+0.01%Ezet group exhibited less ^{3}H-cholesterol accumulation compared to HF ($P=0.05$) (figure 4 B)

No difference was measured between HF and Control for fecal cholesterol and bile acids excretion (Figure 4C). A higher ^{3}H-tracer was measured in fecal total sterols in HF+0.01%Ezet compared to both HF and control chow diet ($p<0.05$). This increase in total sterol excretion was mainly related to an increase in fecal cholesterol since no significant effect of Ezetimibe on tracer recovery in fecal bile acids was observed among groups (Figure 4C)

Cholesterol metabolism gene expression is affected by Ezetimibe treatment

In liver the measurement of gene expression showed a decrease in gene expression of LDLr and ABCA1 in HF and HF+0.01%Ezet groups compared to chow control ($p<0.05$). The gene expression of SR-B1 was lower in HF compared to control ($p<0.05$). Treatment with Ezetimibe normalized the expression of SR-B1 ($p<0.01$) without affecting LDLr and ABCA1 (Figure 5A).

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SCD1 and CYP7A1 gene expression was higher in HF group compared to Chow group ($p<0.001$). Treatment with Ezetimibe decreased significantly SCD1 ($p<0.005$) expression and had no effect on CYP7A1 (Figure 5A). In intestine NPC1L1, ABCG5 and ABCG8 expression did not differ among the three groups (Figure 5B).

Discussion:

The process of reverse cholesterol transport (RCT) consists of the delivery of cholesterol from peripheral tissues to the liver, followed by its secretion into the bile, and subsequently its final elimination in the feces. RCT in cholesterol-loaded macrophages from atherosclerotic plaques plays a critical role in disease prevention by mediating the transfer of this cholesterol to the liver (or intestine) for disposal (Cuchel and Rader, 2006). It was reported that the absorption of cholesterol from the intestine is an important determinant of macrophage-to-feces RCT (Briand et al., 2009; Sehayek and Hazen, 2008).

The present study investigated ~~for the first time~~ the effect of Ezetimibe on reverse cholesterol transport in hamster, animal model with cholesterol metabolism close to that of human, by using primary labelled macrophages. The results demonstrated that Ezetimibe treatment significantly reduced the plasma cholesterol by decreasing VLDL and LDL cholesterol and promoted fecal cholesterol excretion in hamster fed with high- cholesterol diet compared to control hamsters. Moreover Ezetimibe decreased triglycerides and cholesterol content in liver indicating favorable effects on liver steatosis. This result was associated with a significant reduction in mRNA levels of SCD1 in the liver. Ezetimibe treatment did not improve higher glycemia induced by high cholesterol diet.

In our study high cholesterol diet associated with fructose in drinking water increased fasting blood glucose in HF and HF+0.01%Ezet groups compared to Control and no difference was observed in presence of Ezetimibe treatment. This result was also obtained in other study in

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hamster feeding high fat diet and supplemented with Ezetimibe where Ezetimibe improved lipid profile without affecting plasma glucose (van Heek et al., 2001). In contrast several studies using Ezetimibe described a lower fasting blood glucose associated with better glucose tolerance in hamster (Naples et al., 2012), in mice (Chang et al., 2015; Wang et al., 2014), in rat (Deushi et al., 2007) and in human (Park et al., 2011). This effect was related to the stimulation of glucagon like peptide 1 by Ezetimbe (Chang et al., 2015) or the improvement of lipid profile and hepatic insulin sensitivity (Deushi et al., 2007; Park et al., 2011). In a study focusing on obesity and diabetes, Ezetimibe treatment or NPC1L1 deficiency was shown to attenuate weight gain and insulin resistance in mice fed a high fat diet (Labonte et al., 2008). In our study, the use of high cholesterol and high fructose diet could explain the lack of effect on glucose metabolism compared to studies using only high fat diet.

Interestingly, ezetimibe also improved hepatic lipid accumulation and systemic dyslipidemia induced by high cholesterol diet. This result is consistent with others where Ezetimibe treatment improved hepatic steatosis in leptin receptor-deficient Zucker obese rats (Deushi et al., 2007; Nomura et al., 2009). Ezetimibe also reduced hepatic steatosis in mice on the methionine choline-deficient diet (Assy et al., 2006) or diets containing high amounts of cholesterol (Nozaki et al., 2009; Zheng et al., 2008). In another study focusing on the role of NPC1L1 in protein trafficking, NPC1L1 knockout mice were shown to be resistant to hepatic steatosis induced by a lithogenic diet that contains high amounts of bile acids plus cholesterol and fat (Davies et al., 2005). Fat content of the liver reflects the balance between non-esterified fatty acids flux, fatty acid oxidation, de novo lipogenesis and VLDL secretion. Taking into consideration its primary effect, ezetimibe seemed to ameliorate liver steatosis by decreasing lipid absorption and subsequently decreased lipoprotein synthesis. On the other hand, in our study hepatic TG secretion was not affected by ezetimibe treatment despite the

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decrease in hepatic SCD1 gene expression observed in treated hamsters. This result is in accordance with a study in mice (Wang et al., 2014) and in obese rat (Deushi et al., 2007) treated with Ezetimibe which indicates that ezetimibe may affect clearance or uptake but not secretion of triglyceride (MacArthur et al., 2007).

In our study as in others, Ezetimibe promoted macrophage reverse cholesterol transport. We showed that using NPC1L1 inhibitor resulted in an increase in fecal cholesterol excretion. In this study we showed that Ezetimibe treatment decreased plasma cholesterol by decreasing VLDL and LDL cholesterol without affecting HDL cholesterol. These changes were associated with an increase in hepatic SR-B1 gene expression indicating a possible higher selective uptake of HDL cholesterol. This result was not associated with change in ABCA1 gene expression nor in CETP and LCAT activity suggesting that although Ezetimibe treatment increased significantly SR-B1 expression it did not affect HDL formation. Study in mice indicated a similar result with no change in HDL cholesterol (Briand et al., 2009). About non-HDL cholesterol metabolism, Ezetimibe decreased LDL and VLDL plasma cholesterol, but no change was observed in LDLr gene expression and as mentioned above no effect was observed in CETP activity. Apolipoprotein B100 kinetic studies in men with primary hypercholesterolemia revealed that Ezetimibe decreased LDL cholesterol, primarily through enhanced catabolic rates of VLDL, intermediate-density lipoprotein (IDL), and LDL, which is consistent with an up-regulation of hepatic LDL receptor activity (Tremblay et al., 2006). These data suggested that Ezetimibe acting mainly in intestine by inhibiting cholesterol absorption leading to more fecal cholesterol excretion. Higher fecal cholesterol excretion was also showed (Siri et al., 2001) in hamster under high cholesterol diet treated with ezetimibe (Uto-Kondo et al., 2014). In this later study bile duct ligation in hamsters abolished the enhancement of fecal excretion of macrophage derived ^{3}H-cholesterol suggesting that Ezetimibe promotes RCT by the inhibition of the cholesterol absorption which mainly derived

from bile under Ezetimibe treatment (Uto-Kondo et al., 2014). In our study, the labelled cholesterol recovered in the feces was significantly increased while no change occurred in the bile acids fraction. This result was associated with no change in hepatic CYP7A1 gene expression indicating that Ezetimibe did not affect the conversion of cholesterol into bile acids. Our results are in accordance with those observed in mice (Briand et al., 2009) and hamster (Uto-Kondo et al., 2014).

As in our study, Repa et al reported that NPC1L1 expression was lower in the intestine of mice fed a cholesterol-enriched diet compared with those on chow one (Repa et al., 2004), in contrast, Field et al. showed that intestinal NPC1L1 expression in hamsters was much less sensitive to a cholesterol-enriched diet (Field et al., 2004). In the present study Ezetimibe did not affect ABCA1, ABCG5, ABCG8 and NPC1L1 relative gene expression; similarly a study in hamster measured protein expression of ABCG5, ABCG8, and NPC1L1 showed no change with ezetimibe treatment (During et al., 2005; Naples et al., 2012), While these genes were described to be down-regulated by Ezetimibe in caco 2 cells (During et al., 2005). A recent study showed that Ezetimibe may not only prevent atherosclerosis by inhibiting cholesterol absorption in the intestine, but also by increasing the expression of Apolipoprotein A-II in hepatocytes (Yan et al., 2019). Study in Human showed in accordance with this hypothesis that Ezetimibe increased the efficiency of reverse cholesterol transport from rapidly mixing plasma and tissue cholesterol pools into the stool (Lin et al., 2017). Although we did not showed a change in ABCG5, ABCG8 or in NPC1L1 expression we cannot exclude a potential effect of Ezetimibe on transintestinal cholesterol excretion (TICE). The atheroprotective effect of Ezetimibe may result in stimulation in, at least, one of TICE pathways, i- excretion into the intestinal lumen mainly via ATP-binding cassette (ABC) G5/8, ii- cholesterol absorption from the intestine by Niemann-Pick C1 like protein 1, iii- the uptake of plasma lipoproteins by enterocytes at the basolateral membrane, or iv- the excretion of cholesterol in chylomicrons

into the lymph (Reeskamp et al., 2018). The mechanism by which Ezetimibe controlled cholesterol absorption and especially how it affects the expression of genes involved in cholesterol absorption remain not clear and the mechanism implying the role of ezetimibe as NPC1L1 blocker remain the most plausible.

In conclusion, ezetimibe exhibited an atheroprotective effect by enhancing RCT in hamster and decreasing LDL cholesterol. Ours findings showed also a hepatoprotective effect of ezetimibe by decreasing hepatic fat content. Despite these interesting observations, the mechanism by which NPC1L1 deficiency or Ezetimibe treatment alleviates hepatic steatosis, insulin resistance, and obesity remain largely unexplored and need more investigation in animal model and in human.

Contribution's authors :

Participated in research design: KO, ST, FB.

Conducted experiments and performed data analysis: MT; FKC.

Wrote or contributed to the writing of the manuscript: FKC, MT and KO.

References

Altmann, S.W., Davis, H.R., Jr., Zhu, L.J., Yao, X., Hoos, L.M., Tetzloff, G., Iyer, S.P., Maguire, M., Golovko, A., et al., 2004. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science 303:1201-4.

Assy, N., Grozovski, M., Bersudsky, I., Szvalb, S., Hussein, O., 2006. Effect of insulin-sensitizing agents in combination with ezetimibe, and valsartan in rats with non-alcoholic fatty liver disease. World J Gastroenterol 12:4369-76.

Bernard, D.W., Rodriguez, A., Rothblat, G.H., Glick, J.M., 1991. cAMP stimulates cholesteryl ester clearance to high density lipoproteins in J7774 macrophages. J Biol Chem 266:710-6.

Briand, F., 2010. The use of dyslipidemic hamsters to evaluate drug-induced alterations in reverse cholesterol transport. Curr Opin Investig Drugs 11:289-97.

Briand, F., Naik, S.U., Fuki, I., Millar, J.S., Macphee, C., Walker, M., Billheimer, J., Rothblat, G., Rader, D.J., 2009. Both the peroxisome proliferator-activated receptor delta agonist, GW0742, and ezetimibe promote reverse cholesterol transport in mice by reducing intestinal reabsorption of HDL-derived cholesterol. Clin Transl Sci 2:127-33.

Castro-Perez, J., Briand, F., Gagen, K., Wang, S.P., Chen, Y., McLaren, D.G., Shah, V., Vreeken, R.J., Hankemeier, T., et al., 2011. Anacetrapib promotes reverse cholesterol transport and bulk cholesterol excretion in Syrian golden hamsters. J Lipid Res 52:1965-73.

Chang, E., Kim, L., Choi, J.M., Park, S.E., Rhee, E.J., Lee, W.Y., Oh, K.W., Park, S.W., Park, D.I., et al., 2015. Ezetimibe Stimulates Intestinal Glucagon-Like Peptide 1 Secretion Via the MEK/ERK Pathway Rather Than Dipeptidyl Peptidase 4 Inhibition. Metabolism.

Cuchel, M., Rader, D.J., 2006. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation 113:2548-55.

Davies, J.P., Scott, C., Oishi, K., Liapis, A., Ioannou, Y.A., 2005. Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. J Biol Chem 280:12710-20.

Deushi, M., Nomura, M., Kawakami, A., Haraguchi, M., Ito, M., Okazaki, M., Ishii, H., Yoshida, M., 2007. Ezetimibe improves liver steatosis and insulin resistance in obese rat model of metabolic syndrome. FEBS Lett 581:5664-70.

During, A., Dawson, H.D., Harrison, E.H., 2005. Carotenoid transport is decreased and expression of the lipid transporters SR-BI, NPC1L1, and ABCA1 is downregulated in Caco-2 cells treated with ezetimibe. J Nutr 135:2305-12.

Field, F.J., Born, E., Mathur, S.N., 2004. Stanol esters decrease plasma cholesterol independently of intestinal ABC sterol transporters and Niemann-Pick C1-like 1 protein gene expression. J Lipid Res 45:2252-9.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509.

Garcia-Calvo, M., Lisnock, J., Bull, H.G., Hawes, B.E., Burnett, D.A., Braun, M.P., Crona, J.H., Davis, H.R., Jr., Dean, D.C., et al., 2005. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci U S A 102:8132-7.

Ginsberg, H.N., Zhang, Y.L., Hernandez-Ono, A., 2006. Metabolic syndrome: focus on dyslipidemia. Obesity (Silver Spring) 14 Suppl 1:41S-49S.

Jacobson TA, Ito MK, Maki KC, Orringer CE, Bays HE, Jones PH, et al.2015 National lipid association recommendations for patient-centered management of dyslipidemia: part 1- full report. Journal of Clinical Lipidology ;9(2):129-69

Kasbi Chadli, F., Nazih, H., Krempf, M., Nguyen, P., Ouguerram, K., 2013. Omega 3 fatty acids promote macrophage reverse cholesterol transport in hamster fed high fat diet. PLoS One 8:e61109.

Labonte, E.D., Camarota, L.M., Rojas, J.C., Jandacek, R.J., Gilham, D.E., Davies, J.P., Ioannou, Y.A., Tso, P., Hui, D.Y., et al., 2008. Reduced absorption of saturated fatty acids and resistance to diet-induced obesity and diabetes by ezetimibe-treated and Npc1l1-/- mice. Am J Physiol Gastrointest Liver Physiol 295:G776-83.

Lin, X., Racette, S.B., Ma, L., Wallendorf, M., Ostlund, R.E., Jr., 2017. Ezetimibe Increases Endogenous Cholesterol Excretion in Humans. Arterioscler Thromb Vasc Biol 37:990-96.

MacArthur, J.M., Bishop, J.R., Stanford, K.I., Wang, L., Bensadoun, A., Witztum, J.L., Esko, J.D., 2007. Liver heparan sulfate proteoglycans mediate clearance of triglyceride-rich lipoproteins independently of LDL receptor family members. J Clin Invest 117:153-64.

Mazzone, T., Reardon, C., 1994. Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL3. J Lipid Res 35:1345-53.

Millar, J.S., Maugeais, C., Fuki, I.V., Rader, D.J., 2002. Normal production rate of apolipoprotein B in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 22:989-94.

Nagasaki, T., Akanuma, Y., 1977. A new colorimetric method for the determination of plasma lecithin-cholesterol acyltransferase activity. Clin Chim Acta 75:371-5.

Naples, M., Baker, C., Lino, M., Iqbal, J., Hussain, M.M., Adeli, K., 2012. Ezetimibe ameliorates intestinal chylomicron overproduction and improves glucose tolerance in a diet-induced hamster model of insulin resistance. Am J Physiol Gastrointest Liver Physiol 302:G1043-52.

Nomura, M., Ishii, H., Kawakami, A., Yoshida, M., 2009. Inhibition of hepatic Niemann-Pick C1-like 1 improves hepatic insulin resistance. Am J Physiol Endocrinol Metab 297:E1030-8.

Nozaki, Y., Fujita, K., Yoneda, M., Wada, K., Shinohara, Y., Takahashi, H., Kirikoshi, H., Inamori, M., Kubota, K., et al., 2009. Long-term combination therapy of ezetimibe and acarbose for non-alcoholic fatty liver disease. J Hepatol 51:548-56.

Ouchi, Y., Sasaki, J., Arai, H., Yokote, K., Harada, K., Katayama, Y., Urabe, T., Uchida, Y., Hayashi, M., et al., 2019. Ezetimibe Lipid-Lowering Trial on Prevention of Atherosclerotic Cardiovascular Disease in 75 or Older (EWTOPIA 75): A Randomized, Controlled Trial. Circulation 140:992-1003.

Park, H., Shima, T., Yamaguchi, K., Mitsuyoshi, H., Minami, M., Yasui, K., Itoh, Y., Yoshikawa, T., Fukui, M., et al., 2011. Efficacy of long-term ezetimibe therapy in patients with nonalcoholic fatty liver disease. J Gastroenterol 46:101-7.

Reeskamp, L.F., Meessen, E.C.E., Groen, A.K., 2018. Transintestinal cholesterol excretion in humans. Curr Opin Lipidol 29:10-17.

Repa, J.J., Buhman, K.K., Farese, R.V., Jr., Dietschy, J.M., Turley, S.D., 2004. ACAT2 deficiency limits cholesterol absorption in the cholesterol-fed mouse: impact on hepatic cholesterol homeostasis. Hepatology 40:1088-97.

Schotz, M.C., Scanu, A., Page, I.H., 1957. Effect of triton on lipoprotein lipase of rat plasma. Am J Physiol 188:399-402.

Sehayek, E., Hazen, S.L., 2008. Cholesterol absorption from the intestine is a major determinant of reverse cholesterol transport from peripheral tissue macrophages. Arterioscler Thromb Vasc Biol 28:1296-7.

Siri, P., Candela, N., Zhang, Y.L., Ko, C., Eusufzai, S., Ginsberg, H.N., Huang, L.S., 2001. Post-transcriptional stimulation of the assembly and secretion of triglyceride-rich apolipoprotein B lipoproteins in a mouse with selective deficiency of brown adipose tissue, obesity, and insulin resistance. J Biol Chem 276:46064-72.

Tchoua, U., D'Souza, W., Mukhamedova, N., Blum, D., Niesor, E., Mizrahi, J., Maugeais, C., Sviridov, D., 2008. The effect of cholesteryl ester transfer protein overexpression and inhibition on reverse cholesterol transport. Cardiovasc Res 77:732-9.

Treguier, M., Briand, F., Boubacar, A., Andre, A., Magot, T., Nguyen, P., Krempf, M., Sulpice, T., Ouguerram, K., 2011. Diet-induced dyslipidemia impairs reverse cholesterol transport in hamsters. Eur J Clin Invest 41:921-8.

Tremblay, A.J., Lamarche, B., Cohn, J.S., Hogue, J.C., Couture, P., 2006. Effect of ezetimibe on the in vivo kinetics of apoB-48 and apoB-100 in men with primary hypercholesterolemia. Arterioscler Thromb Vasc Biol 26:1101-6.

Uto-Kondo, H., Ayaori, M., Sotherden, G.M., Nakaya, K., Sasaki, M., Yogo, M., Komatsu, T., Takiguchi, S., Yakushiji, E., et al., 2014. Ezetimibe enhances macrophage reverse cholesterol transport in hamsters: contribution of hepato-biliary pathway. Biochim Biophys Acta 1841:1247-55.

van Heek, M., Austin, T.M., Farley, C., Cook, J.A., Tetzloff, G.G., Davis, H.R., 2001. Ezetimibe, a potent cholesterol absorption inhibitor, normalizes combined dyslipidemia in obese hyperinsulinemic hamsters. Diabetes 50:1330-5.

Wang, X., Ren, Q., Wu, T., Guo, Y., Liang, Y., Liu, S., 2014. Ezetimibe prevents the development of nonalcoholic fatty liver disease induced by highfat diet in C57BL/6J mice. Mol Med Rep 10:2917-23.

Yan, Y., He, F., Li, Z., Xu, R., Li, T., Su, J., Liu, X., Zhao, M., Wu, W., 2019. The important role of apolipoprotein A-II in ezetimibe driven reduction of high cholesterol diet-induced atherosclerosis. Atherosclerosis 280:99-108.

Zheng, S., Hoos, L., Cook, J., Tetzloff, G., Davis, H., Jr., van Heek, M., Hwa, J.J., 2008. Ezetimibe improves high fat and cholesterol diet-induced non-alcoholic fatty liver disease in mice. Eur J Pharmacol 584:118-24.

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Legends for tables and figures

Table 1. Body weight and plasma biochemical parameters after overnight fast in hamsters fed a control chow diet, a high cholesterol diet or a high cholesterol diet supplemented with 0.01% Ezetimibe over 4 weeks

Mean ± SEM, n=8 per group, *P<0.05, **P<0.01, ***P<0.001 vs Chow, +P<0.05 vs HF.

Figure 1

Distribution of cholesterol (A) and triglycerides (B) in the plasma of overnight fasted hamsters after a 4 week chow diet, high cholesterol diet or high cholesterol diet + 0.01% Ezetimibe analyzed by gel filtration chromatography (pooled plasma). Values are means ± SEM (n = 8 per group, *** P < 0.001 vs. chow fed hamsters).

Figure 2

Liver weight (in gram) and hepatic total cholesterol and triglycerides levels (in mg/g of liver) in hamsters fed a 4 week chow, high cholesterol diet or high cholesterol + 0.01% Ezet diet. Values are means ± SEM (n = 8 per group, *** P < 0.001 vs. chow fed hamsters).

Figure 3

After a 4-week chow, high fat diet or high cholesterol + 0.01% Ezet diet, Tyloxapol (500mg/kg BW) was administered intravenously to overnight fasted hamsters. Hamsters were bled at time 0, 30, 60, 90 and 120 min after injection. Plasma samples from each time point were used to determine plasma triglycerides levels over time (A). Triglycerides production (B) was calculated by subtracting the values at time 30 minutes from those at time 120 minutes in chow, HF or HF+0.01%Ezet fed hamsters.

Figure 4

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Diets effect on reverse cholesterol transport after labeled macrophages injection. **A**: 3H-tracer kinetic in plasma. **B**: Liver 3H-tracer at 72 h. **C**: 3H-tracer in fecal cholesterol and bile acids. Data are expressed as percent dpm injected and means±SEM (n = 6 per group; * $p<0.05$ different from Control; +$p<0.05$ different from HF)

Figure 5 :

Diets effect on gene expression in liver (A) and intestine (B). means±SEM (n = 6 per group; * $p<0.05$ different from Control; +$p<0.05$ different from HF)

	Chow	High fat diet (HF)	High fat diet + 0.01% Ezetimibe (HFe)
Body weight (g)	105.9 ± 2.48	104.7 ± 3.21	103.9 ± 1.81
Fasted blood glucose (mg/dl)	106.5 ± 4.64	137.88 ± 8.84**	139.75 ± 5.98***
Total cholesterol (g/l)	1.35 ± 0.05	2.65 ± 0.19***	2.04 ± 0.11*** †
HDL-cholesterol (g/l)	1.22 ± 0.1	1.57 ± 0.1*	1.48 ± 0.03**
HDL-cholesterol/total cholesterol	0.96 ± 0.05	0.61 ± 0.07***	0.75 ± 0.05**
Free cholesterol (mg/dl)	25.3 ± 0.94	48.17 ± 3.13**	41.87 ± 1.79***
Triglycerides (g/l)	1.22 ± 0.12	2.7 ± 0.40**	3.15 ± 0.29
Free fatty acids (mmol/l)	0.51 ± 0.06	0.47 ± 0.05	0.58 ± 0.03
CETP activity (pmol/h/µl)	26.08 ± 1.16	29.19 ± 2.14	26.78 ± 1.29
LCAT activity (nmol/h/ml)	350.65 ± 28.102	617.29 ± 47.54***	674.39 ± 51.57***

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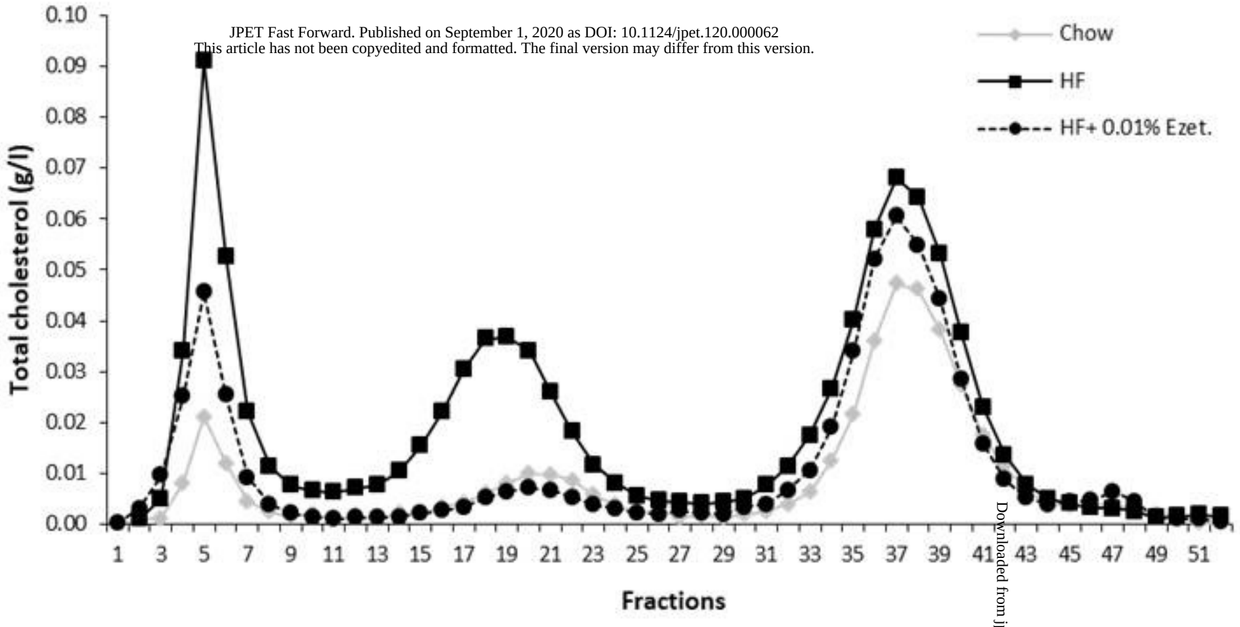
A



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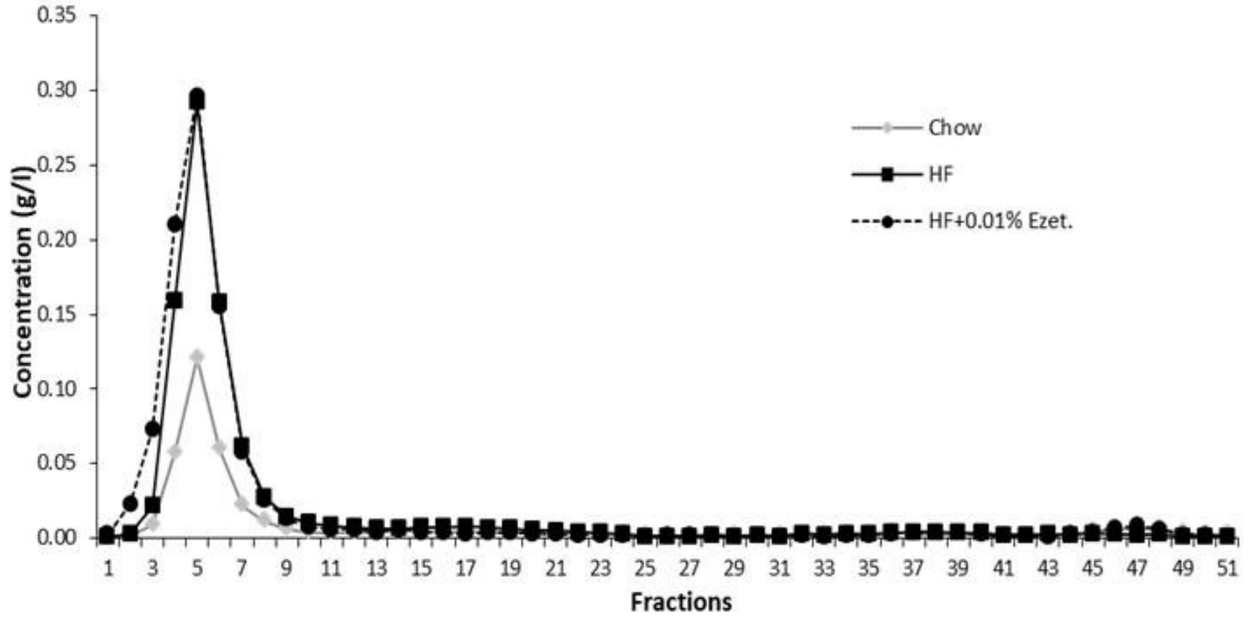

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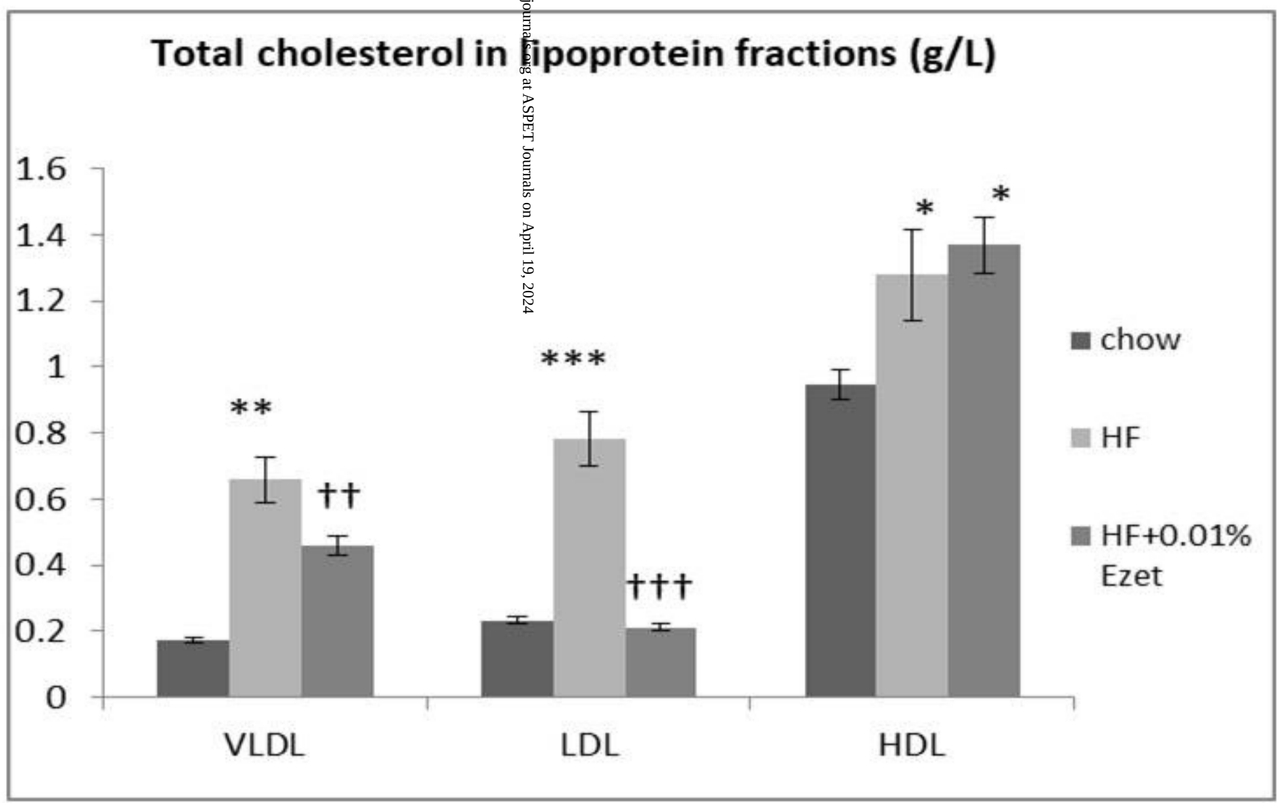


Figure 1

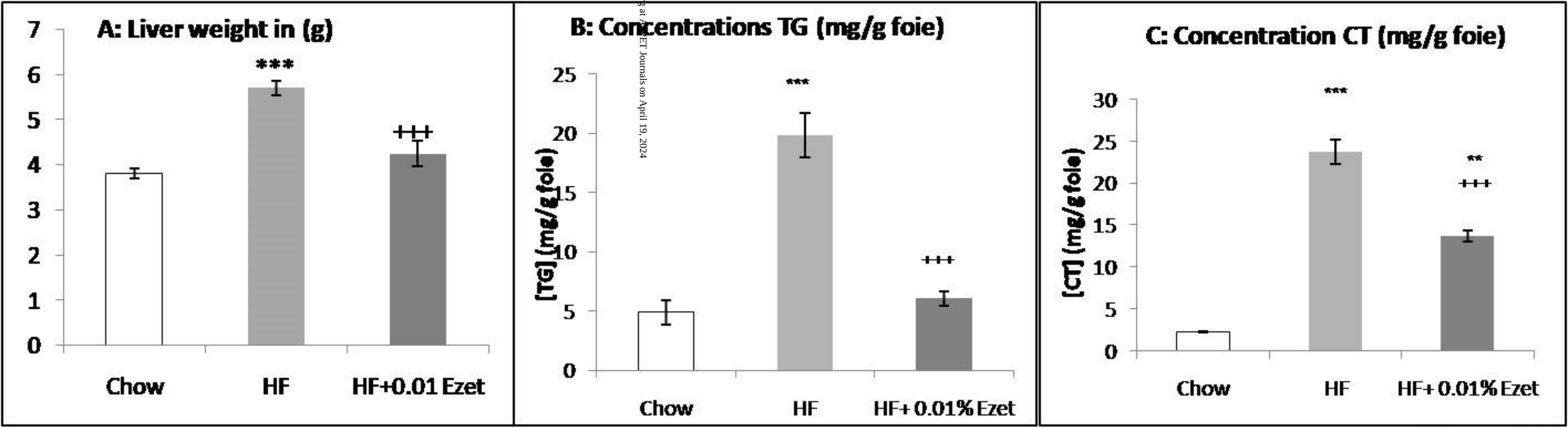
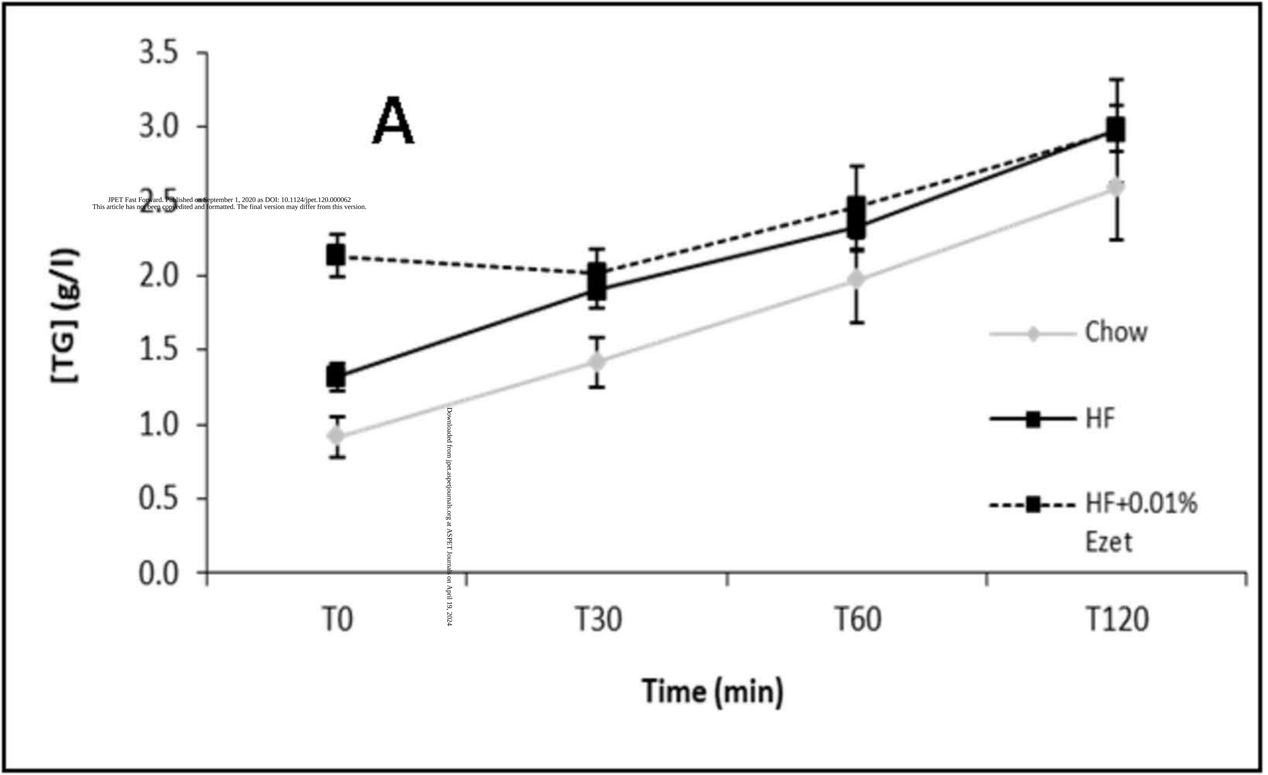


Figure 2



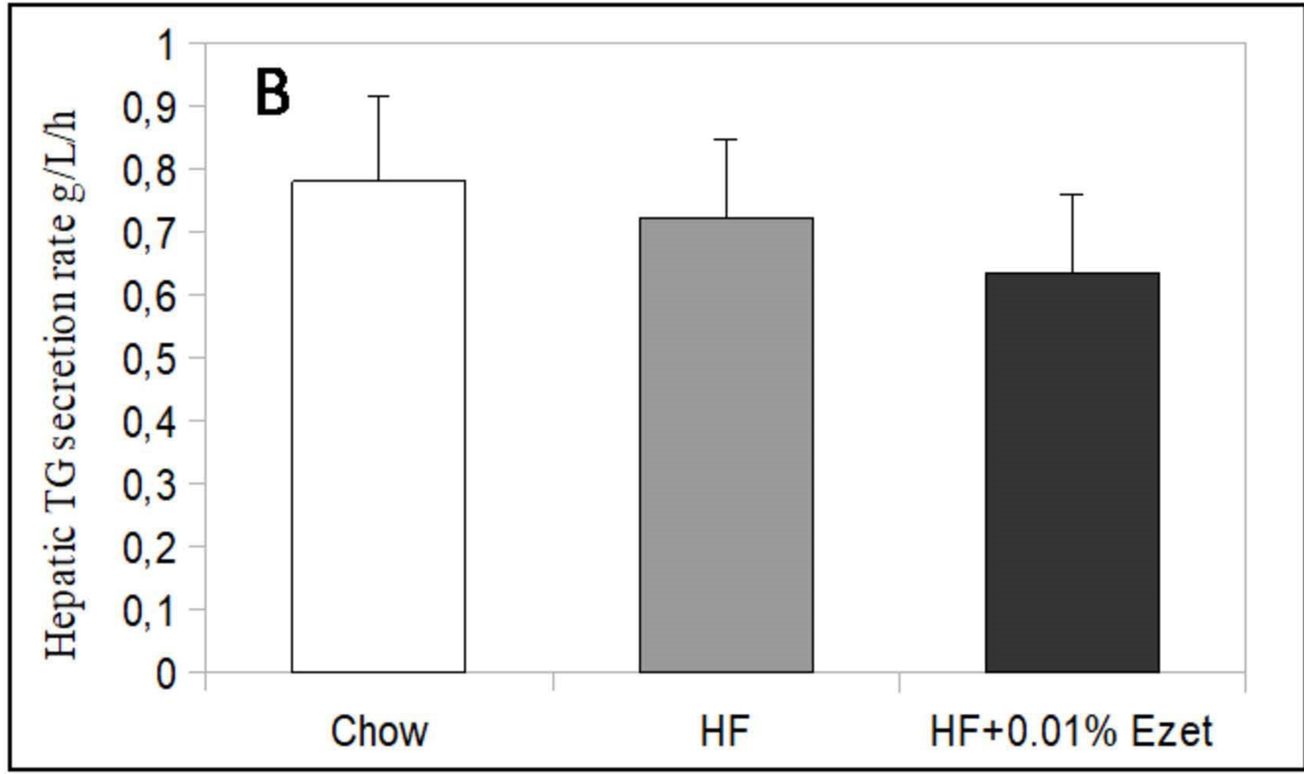


Figure 3

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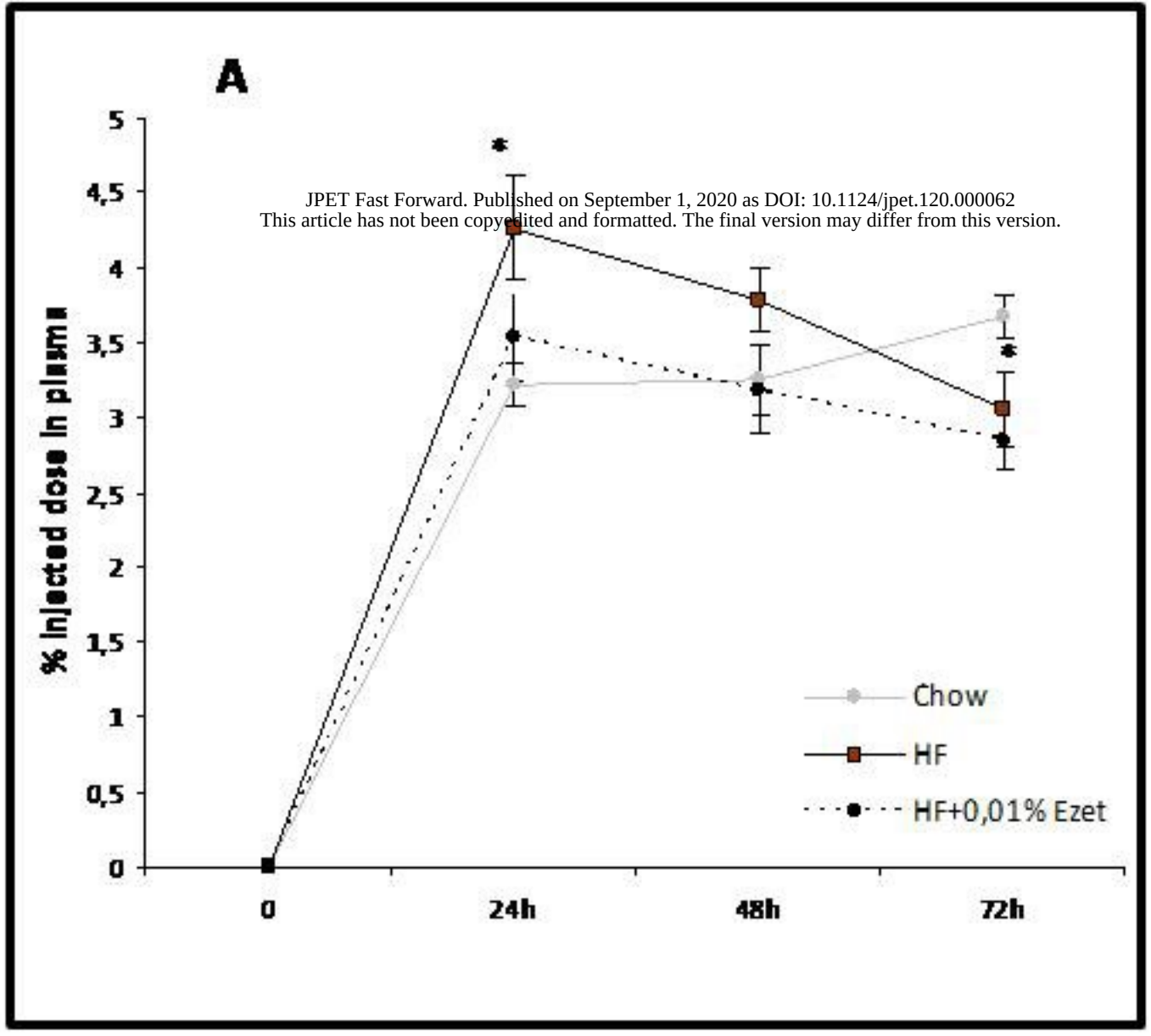
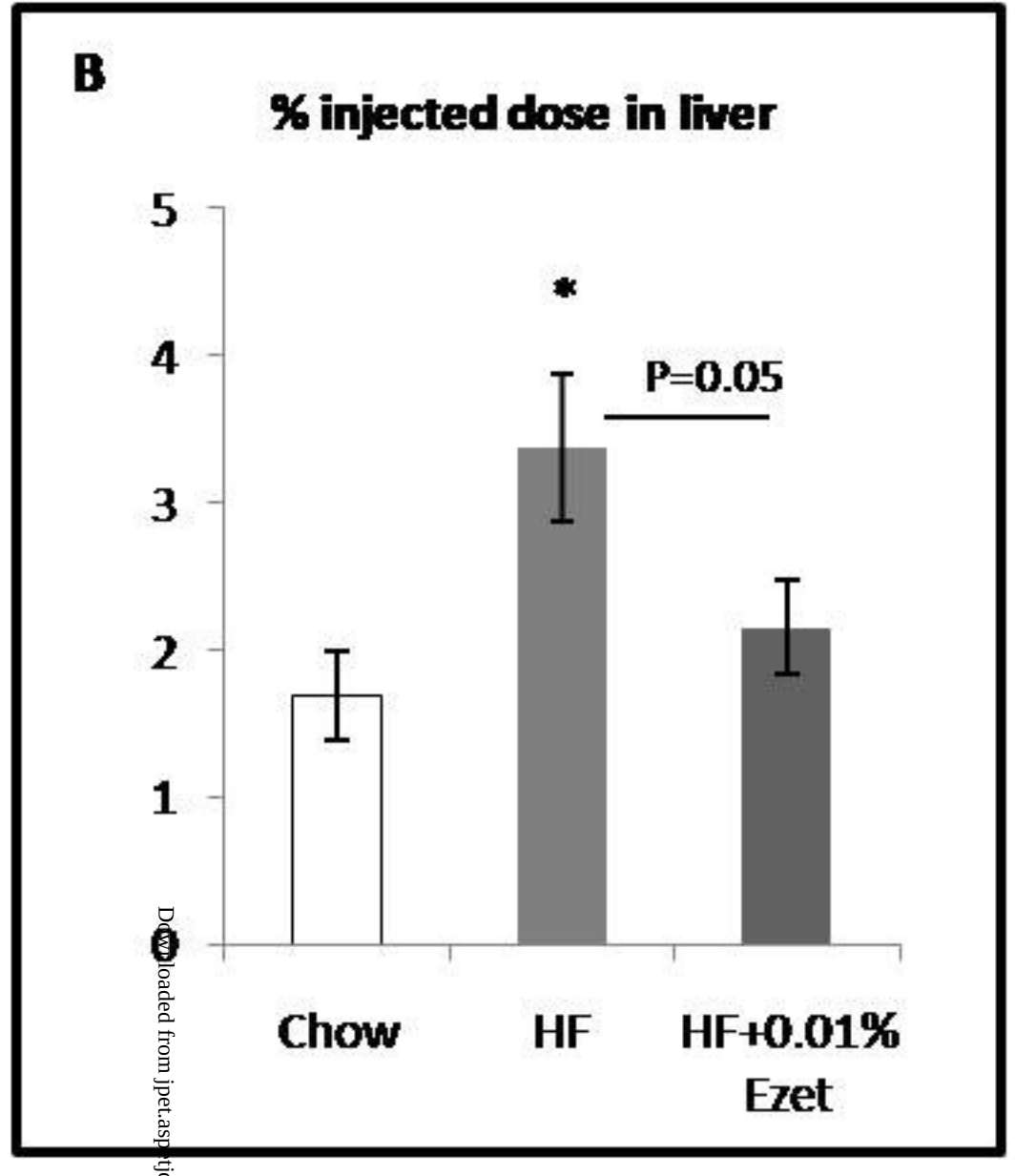




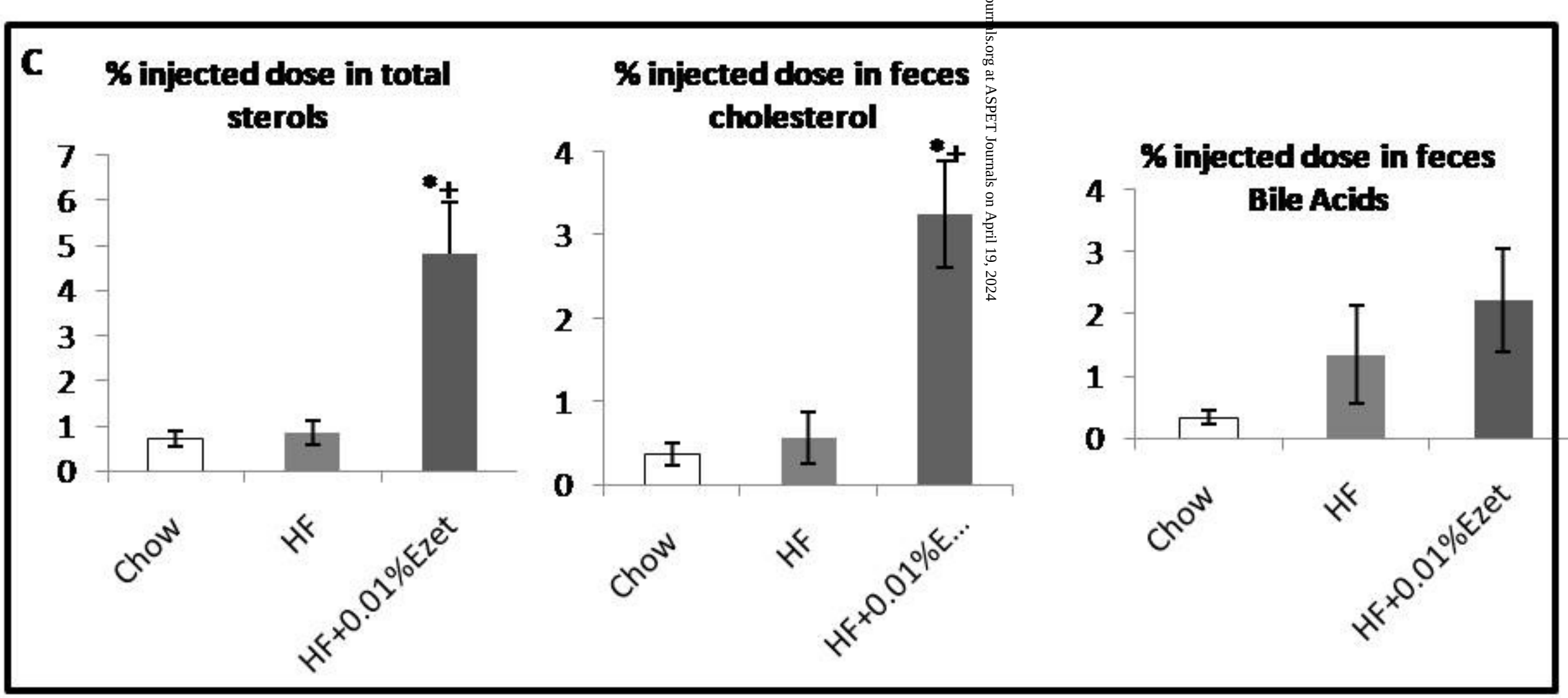
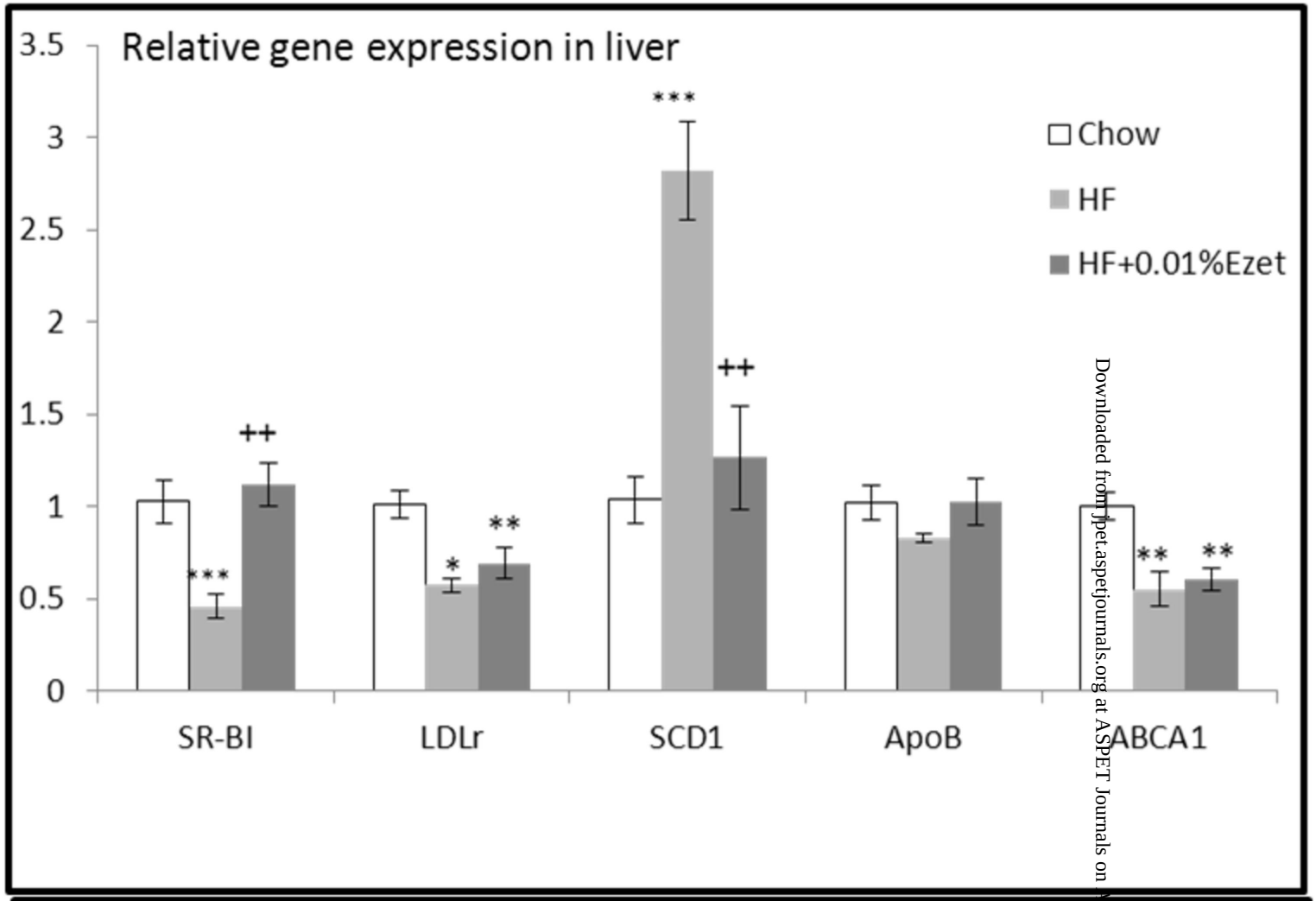
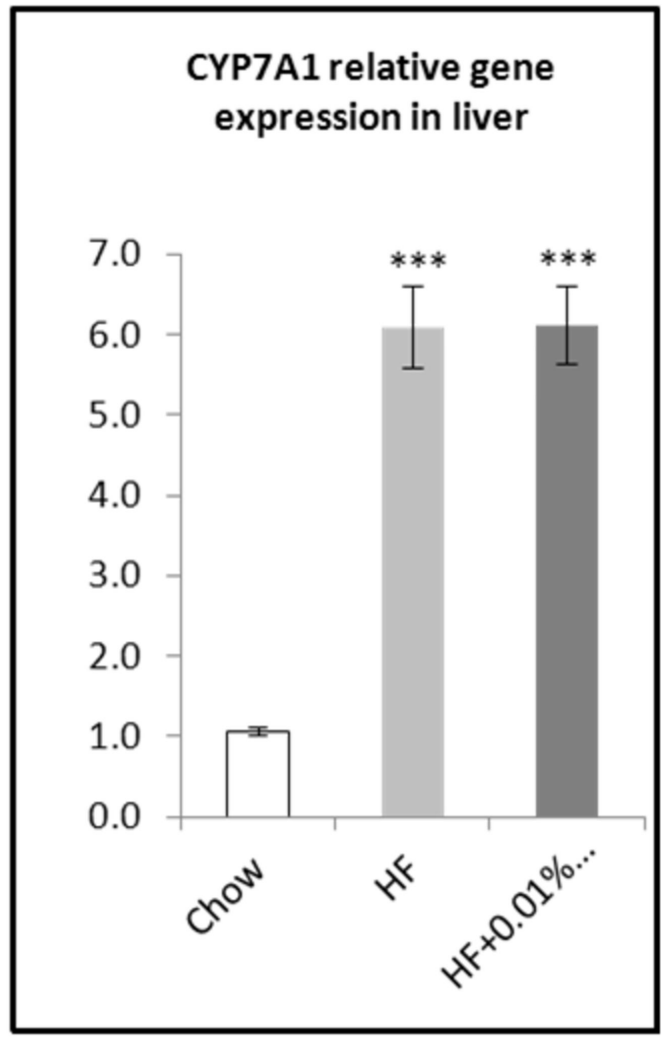


Figure 4

A





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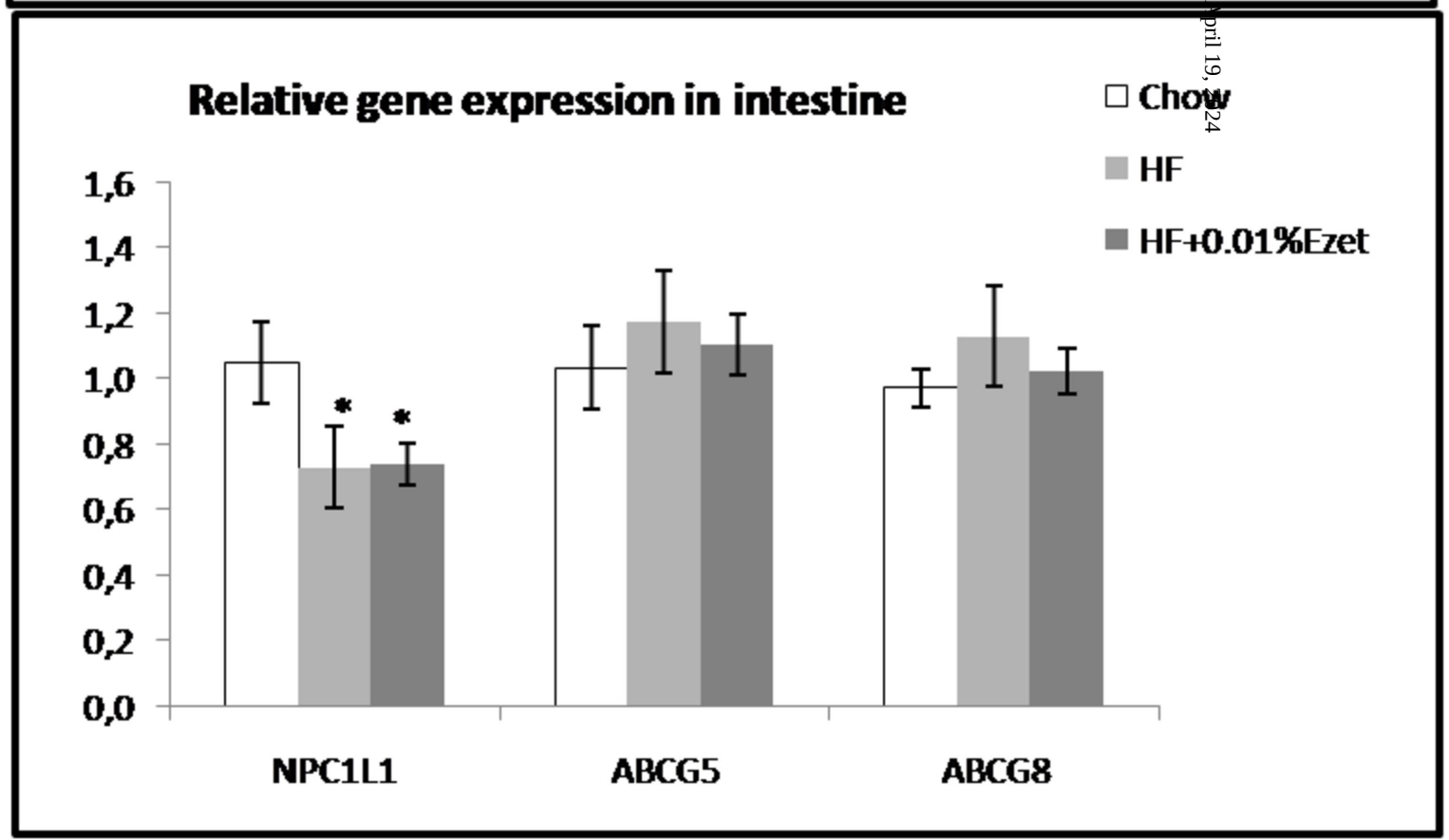

Figure 5