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Maternal hypercholesterolemia enhances oxysterol concentration in mothers and newly-weaned offspring but is attenuated by maternal phytosterol supplementation

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Abstract

In hypercholesterolemic pregnancies, the maternal environment is characterized by excessive levels of atherogenic lipids that may increase cardiovascular disease (CVD) risk in mothers and their offspring. We examined the influence of maternal hypercholesterolemia and phytosterol (PS) intervention on the concentration and metabolism of oxysterols, bioactive oxygenated cholesterol derivatives that regulate arterial health and lesion progression, in mothers and their newly-weaned offspring. Twenty-one female apoE^{-/-} mice were randomly assigned to three different diets throughout gestation and lactation: i) chow (chow); ii) high cholesterol (CH; 0.15%); and iii) CH with added PS (2%, CH/PS). At the end of the lactation period, mothers and pups were euthanized for serum and hepatic oxysterol analyses, hepatic transcriptional profiling of hepatic sterol regulatory targets, and atherosclerosis. Hypercholesterolemic dams and their pups demonstrated increased ($p < 0.05$) serum oxysterols [including 24 hydroxycholesterol (24HC), 25HC, 27HC, 7 α HC, 7 β HC, and 7 ketocholesterol (KC)] compared with the chow group that were normalized by maternal PS supplementation. Hepatic oxysterol concentrations followed a similar pattern of response in mothers but were not altered in newly-weaned pups. Hepatic mRNA expression suggested a pattern of enhanced *abca1/g1* HDL-mediated efflux but a reduction in biliary *abcg5/g8* export in both dams and their pups. Although arterial lesions were not apparent in newly-weaned pups, CH dams demonstrated enhanced atherosclerosis that was reduced upon PS intervention. These results demonstrate that offspring from hypercholesterolemic pregnancies have enhanced

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circulating oxysterol concentrations and highlights the potential utility of PS as a lipid-lowering option during hypercholesterolemic pregnancies for which there is currently limited options.

Keywords

cholesterol; oxysterols; phytosterols; maternal programming; offspring

1. Introduction

A number of pre-pregnancy factors (poor nutrition, obesity) and pathological pregnancy complications such as glucose intolerance [1], gestational diabetes [2], polycystic ovary syndrome [3], and preeclampsia [4] may result in a higher than normal peak in blood lipid concentrations throughout gestation. Although elevated blood cholesterol is recognized as an established cardiovascular disease (CVD) risk factor for the general population, hypercholesterolemia in women of childbearing years is particularly worrisome as excessive dyslipidemia during pregnancy increases maternal CVD risk and exposes the developing fetus to atherogenic lipoproteins and cholesterol oxidation products that has been shown to alter placental metabolism genes [5, 6], enhance early fatty streak development [7], and increase atherosclerosis in adult offspring [8].

As an underlying cause of CVD, atherosclerosis is recognized as a chronic inflammatory condition where an early fatty streak progresses to an advanced arterial plaque through a series of defined stages that include the uptake, retention, and oxidation of lipoproteins, particularly LDL, at the site of an arterial injury [9]. The cholesterol within LDL can be oxidized through enzyme-mediated or non-enzymatic processes to form a wide range of oxygenated derivatives of cholesterol, more commonly referred to as oxysterols. These oxysterols, including 7 α -hydroxycholesterol (7 α HC), 7-ketocholesterol (7KC), 4 β -hydroxycholesterol, 22-hydroxycholesterol, 24-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC), and 27-hydroxycholesterol (27HC), are bioactive signaling molecules that are believed to facilitate crosstalk between lipids and inflammatory mediators to influence arterial lesion development [10]. This broad class of oxygenated cholesterol derivatives have diverse and opposing metabolic functions with respect to atherosclerosis. Several oxysterols have the capacity to trigger inflammation and induce lesion growth and development (i.e. 27HC, 7KC) whereas other endogenous oxysterols repress inflammatory signaling and stimulate lesion regression (i.e. 24HC, 27HC) [11–14]. In addition to their role in arterial lesion development, hepatic oxysterol concentrations are recognized as biomarkers of hepatic oxidative damage that function as signaling molecules in the development and progression of non-alcoholic fatty liver disease [15]. By acting as ligands for liver-X-receptor (*Lxr*), oxysterols are thought to induce HDL-cholesterol-mediated efflux through ATP binding cassette A1 and G1 and biliary cholesterol efflux through *abcg5/g8* [16, 17].

Although oxysterols have been shown to be markers of atherogenic dyslipidemia in adolescence cohorts [18], it is unknown if offspring oxysterol status is affected by maternal dyslipidemia. However, previous work has shown that maternal oxysterol status is associated

with pregnancy induced hypertension [19, 20] and increased inflammation and toxicity of placental tissues [21, 22]. Using apoE deficient mouse and Syrian golden hamster models, we have previously reported that newly-weaned offspring born to hypercholesterolemic mothers have increased serum lipid/lipoprotein concentrations and hepatic cholesterol accumulation and that these lipid abnormalities are prevented through maternal supplementation of phytosterols (PS), plant-based cholesterol-lowering compounds [23, 24]. This study was undertaken to assess how maternal hypercholesterolemia and phytosterol intervention affects oxysterol profiles in serum and liver, hepatic sterol regulatory gene expression, and arterial atherosclerotic lesion development in dams and their newly-weaned offspring using an apoE-deficient mouse model.

2. Materials and Methods

2.1. Animals and Diets:

Twenty-one mature (2-month old) female mice homozygous for disruption of the apoE gene (apoE2/2, strain B6.129P2-Apoetm1Unc > /J, stock no. 002052) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed separately at the Animal Care Facility at the University at Buffalo in a temperature-controlled room (20°C, 12h light/dark cycle). Mice were randomly assigned (n = 7/group) to 1 of 3 commercial unpurified diets (Teklad 2019 Harlan Laboratories): i) a standard chow diet (**Chow**) ii) a cholesterol-enriched chow diet (**CH**) (0.15% wt:wt, TD.140285); and iii) the cholesterol-enriched chow diet supplemented with phytosterols (**CH/PS**) (2% wt:wt; TD.140286; phytosterols sourced from Forbes Medi-Tech Corporation). In-house analysis confirmed that, as a percentage of total sterols, the phytosterol supplement was composed largely of β -sitosterol (>75%), with minor amounts of sitosterol (<15%) and campesterol (<7%). Females were mated with male apoE -/- breeders for 1 wk (1 male per 2 females). After parturition, litters were randomly culled to 6 pups per dam to minimize variability in postnatal pup growth influenced by litter size (33). Throughout the suckling period the dams remained on their respective diets. To prevent the pups from consuming the diet as they matured, food pellets were provided on raised caged platforms that were only accessible by the mother and cages were changed daily. At weaning (postnatal day 21), the dams (food deprived for 15 hours) and pups were anesthetized with isoflurane for blood collection by cardiac puncture and then euthanized by exsanguination and heart removal. Livers were quickly excised, weighed, flash frozen in liquid nitrogen, and stored at -80°C for further processing and analyses. The mice used in this experiment were cared for in accordance with the guidelines established by the Institutional Animal Care and Use Committee. All procedures were reviewed and approved by the Animal Care Committee at the University at Buffalo (protocol no. PTE16082N).

2.2. Oxysterol analyses:

Oxysterols (7 α HC, 7 β HC, 24HC, 25HC, 27HC, and 7KC) were determined by liquid chromatography-selected ion monitoring-stable isotope dilution-atmospheric pressure chemical ionization-mass spectroscopy (LC-SIM-SID-APCI-MS) as previously described [25]. Snap frozen, pulverized liver homogenates (200 μ L of 100mg/mL wet weight) and serum (200 μ L) extracts were prepared. Briefly, total sterols and oxysterols in 200 μ L of sample were released from steryl esters by room temperature saponification (800 μ L of

0.35M KOH in ethanol containing 0.05% butylated hydroxytoluene and under argon atmosphere), neutralized with phosphoric acid (22uL, 70%), centrifuged and the clear supernatant extracted on HyperSep C-18 solid phase extraction (SPE) tubes. SPE eluent was evaporated to dryness under nitrogen and injected into a reversed phase, C-18 LC column (25 cm X 4.6 mm id, 5 micron) using a gradient mobile phase of 90% to 100 methanol containing 0.1% formic acid. The Shimadzu 10ADVP LC system was interfaced with a Shimadzu 2010A MS by APCI interface in positive ion mode. Oxysterols were detected by SIM of the $[M+H-H_2O]^+$ molecular ion of the hydroxyl groups present on each oxysterol. 7KC was monitored as the $[M+H]^+$ ion of the 7-keto group. Deuterated (d7) 24HC, (d7) 7 α HC and (d7) 7KC were used as stable, isotope-labeled, internal standards. We, and others [26], have found that oxysterol analytes are stable at -80°C storage. The assay has been validated in accordance with the FDA guidance for bioanalytical methods [27]. Separate matrix studies were conducted to validate the assay in liver tissue. The coefficient of variation of each oxysterol analyte was 8.8%.

2.3. mRNA extraction and real time RT-PCR:

RNA extraction and real-time RT-PCR were conducted according to previously published procedures system according to previously established protocols [28]. Total RNA was isolated from whole liver tissue using RNeasy mini Kit (Qiagen). RNA concentration and integrity was determined with spectrophotometry (260 nm) and agarose gel electrophoresis, respectively. RNA preparation and real-time RT-PCR were conducted using a one-step QuantiFast SYBR Green RT-PCR kit (Qiagen) with a Biorad CFX96 Touch real time PCR system. Gene expression was analyzed using the $2^{-(\Delta\Delta C_t)}$ method. Sequences of sense and antisense primers for target and housekeeping genes were based on previously published reports for β -actin [29], *Abca1*, *Abcg1*, *Abcg5*, *Abcg8*, *Srebp2* [30], *Lxr* [31], *Osbp3*, *Osbp7*, *Osbp8* [32], *Cyp7a1*, *Cyp27a1* [33], *Cyp46a1* [34], *11 β -hsd1* [35], *Ch25h* [36], *Il-1 β* , *Il-6*, and *Tnf- α* [37].

2.4. Hepatic cytokine extraction and quantification:

Cytokines were extracted from 100 mg of hepatic tissue suspended in a 0.1% Igepal PBS solution based on previously published methods [38, 39]. Hepatic Interleukin 6 (*Il-6*) and Tumor Necrosis Factor- α (*Tnf- α*) were measured by Enzyme-Linked Immunosorbent Assay (Invitrogen and R&D Systems).

2.5. Arterial atherosclerotic lesion histology and quantification:

Hearts were harvested at sacrifice and fixed in 10% buffered formalin for histological and morphometrical examination of the aortic roots as previously described [40, 41]. Briefly, after routine processing procedures, the specimens were blocked in paraffin and sections were cut at approximately 4 micron thickness until the aortic root was appeared. Then, several sections were kept to represents the whole length of aortic roots. These sections were stained with hematoxylin and eosin plus trichrome as previously described [40, 41]. Six of these stained sections taken at the beginning, middle and end of the aortic roots were examined for morphological structure of the aortic root; while 3 sections representing the whole aortic root length was used for morphometric analysis of atherosclerotic lesions using Image ProPlus system as previously described [40, 41].

2.6. Statistical analyses:

Litters from each dam were considered as a single observation. Data were analyzed with a general linear model ANOVA with a Bonferroni post-hoc test using SPSS 16 for Mac (SPSS Inc, Chicago, IL). Data are presented as mean $\pm$ SEM. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Oxysterol Profile:

Hypercholesterolemic dams demonstrated increased ($p < 0.05$) serum oxysterol concentrations (including 24HC, 25HC, 27HC, 7 α HC, 7 β HC, and 7KC) compared with Chow dams, however, maternal PS supplementation normalized serum oxysterol concentrations to Chow levels (Fig 1, a–f). With the exception of hepatic 27HC that did not differ ($p > 0.05$) between groups, hepatic oxysterol concentrations followed a similar pattern with increases ($p < 0.05$) in hypercholesterolemic dams and normalization to at or near Chow-fed levels in mothers supplemented with PS (Fig 1, g–l). Hypercholesterolemic dams demonstrated increased ($p < 0.05$) hepatic cholesterol deposition (48.5 ± 6.6 versus 15.6 ± 1.2 $\mu\text{mol/g}$ for chow) which were normalized ($p < 0.05$) to chow levels by PS supplementation (14.7 ± 3.2 $\mu\text{mol/g}$).

With the exception of 27HC, pups born to hypercholesterolemic mothers exhibited increases ($p < 0.05$) in all serum oxysterols compared with Chow pups, however, this increase was abated in pups from PS-supplemented mothers (Fig 2, a–f). Little change in hepatic oxysterol concentrations in pups were observed, however, 7 β HC concentration was increased ($p < 0.05$) in CH pups and normalized to Chow-levels in pups born to PS-supplemented mothers (Fig 2b). Similar to mothers, pups born to hypercholesterolemic mothers demonstrated increased ($p < 0.05$) hepatic cholesterol (18.3 ± 2.5 versus 13.1 ± 0.4 $\mu\text{mol/g}$) which was normalized ($p < 0.05$) by maternal PS intervention (11.3 ± 1.8 $\mu\text{mol/g}$).

3.2. Hepatic mRNA expression:

Hepatic mRNA expression in the dams is presented in Table 1. Although no change ($p > 0.05$) was observed in *Abca1* expression, *Abcg1* expression was enhanced (2.4 fold of chow, $p < 0.05$) in hypercholesterolemic dams but normalized to Chow level through maternal PS supplementation. Alternatively, CH dams demonstrated reductions in both *Abcg5* (0.49 fold of Chow) and *Abcg8* (0.52 fold of Chow) that was normalized to Chow levels in PS-supplemented dams. Although no change in *Lxr* expression was noted between dams, *Srebp2* mRNA expression was reduced ($p < 0.05$) in CH dams compared with Chow, but normalized to Chow levels in PS-supplemented dams. Oxysterol binding protein expression (*Ospb3*, 7, and 8) did not differ ($p > 0.05$) between the treatment groups. The expression of *Cyp7a1*, the rate-limiting enzyme in bile acid synthesis, was numerically increased ($p = 0.10$) in the CH versus the chow group and reduced ($p < 0.05$) in CH/PS dams in comparison with the Chow and CH groups. Compared with the chow group, *Cyp27a1* was reduced ($p < 0.05$) to a similar degree in CH and CH/PS dams.

Although *Cyp46a1* and *Ch25h* was not altered between the treatment groups, 11 β -Hsd1 was reduced ($p < 0.05$) in CH dams compared with chow but normalized to chow levels in the CH/PS group.

Although *Il-1 β* expression did not differ ($p > 0.05$) between the Chow and CH groups, it was reduced ($p < 0.05$) in mothers supplemented with PS compared with CH dams. CH mothers demonstrated a reduction in the expression of IL-6 (0.5 fold of Chow, $p < 0.05$) that was normalized by PS intervention. *Tnf- α* expression was increased in CH mothers (1.8 fold of Chow, $p < 0.05$) which was normalized in the PS group.

Hepatic mRNA expression in the offspring is presented in Table 2. mRNA expression of hepatic *Abca1* and *gl* were increased (1.8 and 1.2-fold of Chow, respectively, $p < 0.05$) in offspring from hypercholesterolemic dams and normalized to Chow levels through maternal PS supplementation. Although *Abcg5* expression did not differ ($p > 0.05$) between treatment groups, *Abcg8* mRNA was lowered (0.5 fold of Chow, $p < 0.05$) in CH pups and normalized in pups from PS-supplemented mothers. *Lxr* mRNA expression did not differ between Chow and CH pups but was increased (2.5 fold of chow, $p < 0.05$) in pups from PS-supplemented dams. Oxysterol binding protein expression (*Oshp3*, 7, and 8) did not differ ($p > 0.05$) between the pups from the different treatment groups. Pups from CH dams demonstrated an increase in *Cyp7a1* mRNA abundance (3.6 fold of Chow, $p < 0.05$) that was normalized in pups from CH/PS mothers, however, no change ($p > 0.05$) in *Cyp27a1* and *Cyp46a1* was noted between treatment groups.

Although *Il-1 β* expression did not differ ($p > 0.05$) between treatment groups, offspring from CH pups demonstrated increased ($p < 0.05$) expression of *Il-6* expression (1.8 fold of Chow) which was normalized in pups from PS-supplemented mothers. In contrast to what was observed with the dams, offspring from the CH and CH/PS mothers demonstrated a reduction ($p < 0.05$) in the mRNA expression of *Tnf- α* compared with offspring from Chow mothers (0.5 and 0.4 fold of Chow, respectively).

3.3 Hepatic Cytokine Protein Concentrations:

Despite altered mRNA expression, hepatic *Il-6* protein concentrations (ng/mL extract) did not differ ($p > 0.05$) between Chow (0.22 ± 0.01) CH (0.18 ± 0.02), and CH/PS (0.16 ± 0.02) dams. Similarly, hepatic *Tnf- α* protein levels were unchanged ($p > 0.05$) in dams fed Chow, CH, or CH/PS (1.73 ± 0.26 , 1.83 ± 0.32 , and 1.41 ± 0.20 μ g/mL extract, respectively).

Hepatic *Il-6* protein concentrations (ng/mL extract) were not altered ($p > 0.05$) in pups born to mothers fed Chow (0.16 ± 0.01), CH (0.17 ± 0.01) or CH/PS (0.17 ± 0.01). Similarly, no change ($p > 0.05$) in hepatic *Tnf- α* protein levels were observed in pups born to Chow (1.98 ± 0.32 μ g/mL extract) compared to those fed CH or CH/PS (3.01 ± 0.67 and 1.74 ± 0.38 μ g/mL extract, respectively).

3.4. Atherosclerosis:

Maternal hypercholesterolemia significantly increased ($p < 0.05$) atherosclerotic lesion size in the aortic root of mothers, however, lesions were less developed in the mothers supplemented with PS (Fig 3). Representative photomicrograph sections illustrated a higher

degree of atherosclerosis in hypercholesterolemic dams which were normalized with PS intervention (Fig.3 a–c). Atherosclerotic lesions were not apparent in newly-weaned pups at this stage of development from any treatment group (representative photomicrograph sections shown in Fig.3 d–f).

4. Discussion

In this study of the impact of maternal hypercholesterolemia on oxysterol status in both mother and offspring, several novel findings were noted. First, a hypercholesterolemic maternal environment during pregnancy and lactation is associated with an increase in maternal oxysterol status that is also evident in their newly-weaned offspring. Second, supplementation of the maternal diet with PS is effective at normalizing oxysterol concentrations in both the mother and her offspring. Third, hepatic mRNA expression suggested a pattern of enhanced *Abca1/g1* HDL-mediated efflux but a reduction in biliary *Abcg5/g8* export in both hypercholesterolemic dams and their pups. Finally, although arterial atherosclerotic lesions were not apparent in newly-weaned pups, hypercholesterolemic dams demonstrated an increase in the size and severity of atherosclerotic lesions that were reduced upon PS intervention, suggesting that PS may be a suitable CVD risk-reduction strategy for hypercholesterolemic pregnancies.

Although previous work has shown that excessive maternal dyslipidemia during pregnancy can alter fetal cholesterol metabolism and predispose adult offspring to metabolic abnormalities [42–44], surprisingly little work has assessed the relationship between maternal dyslipidemia and oxysterol status, despite their central role in regulating cellular sterol metabolism and atherosclerosis. Here, we have shown that a hypercholesterolemic environment during pregnancy is associated with increased maternal oxysterol concentrations that are correspondingly increased in their newly-weaned offspring. As oxysterols are bioactive mediators of oxidative stress, inflammation, apoptosis, and promote vascular smooth muscle cell calcification and atherosclerotic plaque instability [45–48], this observation may indicate enhanced CVD risk susceptibility for both mother and the offspring of hypercholesterolemic pregnancies [49–51]. On the other hand, the normalization of circulating oxysterols in PS-supplemented mothers and pups is noteworthy and supportive of our previous work reporting reduced lipid and lipoprotein particle numbers in this model [23]. Although PS have consistently been reported to lower blood cholesterol in both animals and humans, information on how they alter oxysterol metabolism are surprisingly scarce. In a recent crossover study in 13 healthy human subjects, consumption of a plant stanol enriched margarine for 3 weeks reduced serum 7 α HC but did not alter other oxysterols including 7 β HC, 7KC, 24HC, and 27HC [52]. It is unknown whether the observed lowering of oxysterols in offspring of PS-supplemented dams is a direct result of the improvement in maternal lipid status, altered lipid profile of maternal milk, or through some direct molecular action of PS ingested through breast milk. Regardless of the mechanism, reduction in oxysterols and other surrogate lipid risk markers previously observed may suggest CVD risk protection in pups exposed early to PS through the maternal diet. Although we did not see evidence of arterial atherosclerotic lesions in pups at this early stage of development, accelerated aortic lesions observed in CH versus chow dams were reduced through PS supplementation, suggesting that PS may be an effective therapy to

reduce CVD risk in hypercholesterolemic mothers. The effects of PS on arterial atherosclerotic lesion growth and regression are contested [53–56], however, previous work in apoE [40, 57] and LDLr [58, 59] deficient adult mice have demonstrated similar reductions in the size and severity of arterial atherosclerotic lesions in response to PS supplementation, albeit, not during pregnancy. Particular scrutiny to the safety of early PS exposure is of paramount importance in translating PS supplementation during pregnancy, particularly given the discussion around potential negative vascular and atherogenic effects of PS and their oxidized derivatives [60, 61]. However, the lack of any adverse atherogenic consequence of early PS exposure in this study may support the safety of maternal PS intervention, at least at this early developmental stage. However, any long-term CVD risk related to early cholesterol or PS exposure in this model remains to be determined and should be the focus of future work.

In addition to changes in serum oxysterol concentrations, hepatic cholesterol and oxysterol concentrations were elevated in CH compared to the Chow dams. Surprisingly, with the exception of an increase in 7 β HC, we observed little change in hepatic oxysterol concentrations in CH pups despite increased hepatic cholesterol status compared with offspring from Chow-fed mothers. Changes in hepatic oxysterol concentrations have been previously reported in response to diet-induced obesity [62] and nonalcoholic steatohepatitis (NASH) [63]. Oxysterols can be generated through non-enzymatic routes and redundant enzymatic pathways, making it difficult to track their origin [64]. Although we were unable to demonstrate consistent correlations between hepatic oxysterol status and the expression of their respective synthetic enzymes or oxysterol binding proteins, as has been reported previously [62], changes in the expression of several of these enzymes may provide some indication of their origin and metabolism. First, CH-fed dams had a markedly increased concentration of hepatic 7 α HC with a numerical increase ($p=0.10$) in *Cyp7a1* mRNA expression, most likely indicative of increased bile acid synthesis as a protective measure to eliminate excessive cholesterol [65]. Secondly, although 7 β HC and 7KC were both increased in CH dams, the suppression of *11 β -Hsd1* expression with cholesterol supplementation, which acts to interconvert these two oxysterol species [66], suggests a non-enzymatic route of origin, particularly for 7 β HC as 7KC can also be produced through oxidation of 7-dehydrocholesterol by *Cyp7a1* [67]. Finally, pups from CH mothers demonstrated a dramatic increase (3.6 fold) in *Cyp7a1* expression, again, likely a hepatoprotective response against increased cholesterol influx that was reflected in serum 7 α HC concentrations, but perhaps surprisingly, not in the liver.

A noteworthy finding from this study was the contradictory pattern of an increased hepatic *Abca1/g1* but reduced *Abcg5/g8* transcription in both CH-fed dams and offspring, perhaps reflective of an increased HDL-mediated cholesterol efflux but reduced hepatic biliary sterol excretion [68, 69]. In support of the former, we previously observed increased HDL-particle number in newly-weaned offspring from hypercholesterolemic versus normalcholesterolemic mothers (unpublished observation). Although both the *Abca1/g1* and *Abcg5/g8* transport systems are primarily regulated at the transcriptional level through *Lxr* [17], the mRNA expression of *Lxr* itself was not altered between chow and CH pups. Interestingly, pups from CH/PS mothers demonstrated 2.5-fold increase in *Lxr* expression

compared with chow pups, however, the significance of this to hepatic sterol metabolism is not immediately evident.

Similar to our observation, previous work has reported a repression of hepatic *Abcg5/g8* expression despite an increased accumulation of sterols in several animal models of nonalcoholic fatty liver disease (NAFLD) and NASH [16, 70, 71]. Furthermore, experimentally induced liver inflammation has been shown to down regulate the expression of cholesterol efflux transporters and increase *Ldlr* and *Srebp2*, contributing to an accumulation of hepatic lipids [72–74]. Although we did not observe a consistent pattern in inflammatory gene expression between mothers and their offspring, hepatic *Tnf-α* and *Il-6* mRNA were elevated in CH mothers and their pups, respectively, compared with their Chow counterparts, possibly indicative of a pro-inflammatory response that often accompanies excessive hepatic cholesterol deposition [75, 76]. However, we did not observe any change in hepatic cytokine protein concentrations between the treatment groups. Although the conflicting pattern of hepatic *Tnf-α* and *Il-6* mRNA expression between CH mothers and their pups was unexpected, the effect of age and metabolic status on immune ontogeny [77] and possible transfer of immunomodulating factors to offspring through maternal milk [78, 79] may be contributed to this response.

In summary, results from this model suggest that mothers with hypercholesterolemia during pregnancy and lactation demonstrate enhanced atherosclerosis and increased oxysterol concentrations and give rise to newly-weaned offspring with excessive hepatic cholesterol deposition and correspondingly elevated serum oxysterol concentrations that are normalized through maternal PS supplementation. Enhanced circulating oxysterols from offspring of hypercholesterolemic pregnancies is concerning at this early developmental stage as it may indicate an increased life-long CVD risk susceptibility, although this remains to be established. Additionally, this study highlights the potential utility of PS as a lipid-lowering alternative during hypercholesterolemic pregnancies for which there are currently limited options.

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Serum

Figure 1:

Maternal oxysterol concentrations in serum (a-f, ng/mL) and liver (g-l, ng/mg) including 24-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC), 7 α -hydroxycholesterol (7 α HC), 7 β -hydroxycholesterol (7 β HC), and 7ketocholesterol (7KC). ^{abc}Indicates significant difference ($p < 0.05$); mean $\pm$ SE; $n = 7$ /group.

Serum

Liver

Figure 2:

Oxysterol concentrations in serum (a-f, ng/mL) and liver (g-l, ng/mg) in newly-weaned offspring including 24-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC), 7 α -hydroxycholesterol (7 α HC), 7 β -hydroxycholesterol (7 β HC), and 7ketocholesterol (7KC). ^{abc}Indicates significant difference ($p < 0.05$); mean $\pm$ SE; $n = 7$ /group.

Figure 3:

Top panel: Total atherosclerotic lesion area (mm²) of aortic root following lactation in dams fed a chow, cholesterol (CH) or cholesterol plus phytosterol (CH/PS) supplemented diet throughout gestation and lactation. Areas are means values taken from three sections representing the whole aortic root length. Bottom panel: Images are representative photomicrographs of trichrome-stained transverse sections of the aortic root in dams (a-c, arrows point to the atherosclerotic lesions) and their newly-weaned pups (d-f) from the three treatment groups. ^{abc}Indicates significant difference (p<0.05); mean ± SE; n=7/group.

Table 1:

mRNA expression of hepatic regulators following lactation in dams fed a chow, cholesterol (CH) or cholesterol plus phytosterol (CH/PS) supplemented diet throughout gestation and lactation^{*}.

Gene Target	Chow	CH	CH/PS
Cholesterol Efflux Targets			
<i>Abca1</i>	1.0 ± 0.28 ^a	1.29 ± 0.24 ^a	1.43 ± 0.10 ^a
<i>Abcg1</i>	1.0 ± 0.13 ^a	2.37 ± 0.41 ^b	0.97 ± 0.20 ^a
<i>Abcg5</i>	1.0 ± 0.07 ^a	0.49 ± 0.04 ^b	0.98 ± 0.17 ^a
<i>Abcg8</i>	1.0 ± 0.14 ^a	0.52 ± 0.07 ^b	0.92 ± 0.07 ^a
Molecular Regulators			
<i>Lxr</i>	1.0 ± 0.14 ^a	0.73 ± 0.09 ^a	0.88 ± 0.12 ^a
<i>Srebp2</i>	1.0 ± 0.20 ^a	0.50 ± 0.10 ^b	1.30 ± 0.12 ^a
Oxysterol Regulatory Targets			
<i>Osbp3</i>	1.0 ± 0.25 ^a	0.92 ± 0.15 ^a	0.69 ± 0.21 ^a
<i>Osbp7</i>	1.0 ± 0.12 ^a	1.01 ± 0.13 ^a	0.81 ± 0.18 ^a
<i>Osbp8</i>	1.0 ± 0.05 ^a	0.79 ± 0.09 ^a	0.94 ± 0.13 ^a
<i>Cyp7a1</i>	1.0 ± 0.11 ^{a,b}	1.4 ± 0.17 ^a	0.64 ± 0.19 ^b
<i>Cyp27a1</i>	1.0 ± 0.08 ^a	0.56 ± 0.10 ^b	0.55 ± 0.05 ^b
<i>Cyp46a1</i>	1.0 ± 0.26 ^a	1.30 ± 0.22 ^a	1.40 ± 0.26 ^a
<i>11β-hsd1</i>	1.0 ± 0.07 ^a	0.60 ± 0.09 ^b	0.90 ± 0.07 ^a
<i>Ch25h</i>	1.0 ± 0.11 ^a	1.05 ± 0.10 ^a	0.74 ± 0.08 ^a
Inflammatory Targets			
<i>Il-1β</i>	1.0 ± 0.8 ^{a,b}	1.22 ± 0.13 ^a	0.77 ± 0.13 ^b
<i>Il-6</i>	1.0 ± 0.11 ^a	0.53 ± 0.04 ^b	0.79 ± 0.21 ^{a,b}
<i>Tnf-α</i>	1.0 ± 0.36 ^a	1.86 ± 0.19 ^b	1.11 ± 0.34 ^{a,b}

^{*} mean ± SE; n=7/group

Table 2:

mRNA expression of hepatic regulators in newly-weaned pups from mothers fed a chow, cholesterol (CH) or cholesterol plus phytosterol (CH/PS) supplemented diet throughout gestation and lactation^{*}.

Gene Target	Chow	CH	CH/PS
Cholesterol Efflux Targets			
<i>Abca1</i>	1.0 ± 0.19 ^a	1.81 ± 0.12 ^b	1.30 ± 0.18 ^a
<i>Abcg1</i>	1.0 ± 0.11 ^a	1.21 ± 0.11 ^b	0.92 ± 0.07 ^a
<i>Abcg5</i>	1.0 ± 0.25 ^a	1.19 ± 0.42 ^a	1.13 ± 0.41 ^a
<i>Abcg8</i>	1.0 ± 0.14 ^{a,b}	0.50 ± 0.22 ^b	1.14 ± 0.07 ^a
Molecular Regulators			
<i>Lxr</i>	1.0 ± 0.16 ^a	1.29 ± 0.42 ^a	2.50 ± 0.36 ^b
<i>Srebp2</i>	1.0 ± 0.10 ^a	0.99 ± 0.25 ^a	1.31 ± 0.23 ^a
Oxysterol Regulatory Targets			
<i>Osbp3</i>	1.0 ± 0.13 ^a	0.95 ± 0.29 ^a	1.24 ± 0.11 ^a
<i>Osbp7</i>	1.0 ± 0.05 ^a	1.09 ± 0.33 ^a	1.57 ± 0.15 ^a
<i>Osbp8</i>	1.0 ± 0.12 ^a	1.19 ± 0.21 ^a	1.10 ± 0.11 ^a
<i>Cyp7a1</i>	1.0 ± 0.34 ^a	3.66 ± 0.42 ^b	1.50 ± 1.12 ^a
<i>Cyp27a1</i>	1.0 ± 0.19 ^a	0.82 ± 0.25 ^a	0.74 ± 0.19 ^a
<i>Cyp46a1</i>	1.0 ± 0.15 ^a	0.81 ± 0.10 ^a	0.84 ± 0.13 ^a
<i>11β-hsd1</i>	1.0 ± 0.11 ^a	1.44 ± 0.20 ^a	1.24 ± 0.11 ^a
<i>Ch25h</i>	1.0 ± 0.78 ^a	0.54 ± 0.05 ^b	0.42 ± 0.21 ^b
Inflammatory Targets			
<i>Il-1β</i>	1.0 ± 0.32 ^a	1.14 ± 0.29 ^a	1.23 ± 0.24 ^a
<i>Il-6</i>	1.0 ± 0.11 ^a	1.86 ± 0.15 ^b	1.03 ± 0.13 ^a
<i>Tnf-α</i>	1.0 ± 0.19 ^a	0.36 ± 0.11 ^b	0.40 ± 0.18 ^b

^{*} mean ± SE; n=7/group