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Examining the potential benefits of (-)-epicatechin, (+)-catechin, and rutin on maternal and offspring cardiovascular outcomes in LDLr-/- mice exposed to an atherogenic environment during early development

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ABSTRACT

Background: Maternal nutritional status can impact numerous early developmental processes. In certain cases, these effects can influence the risk their offspring can have for select chronic diseases later in life. Consequently, in this article we report on the effects of maternal consumption of high levels of certain flavonoids on the development of coronary artery disease (CAD) in an atherosclerosis-prone mutant mouse model.

Methods: LDLr -/- mutant mice were fed a control fat (CF), high fat (HF), or the HF diet supplemented with epicatechin and catechin (HFEC) or rutin (HFRU), prior to pregnancy and during lactation, in order to explore whether the flavonoids influenced markers of vascular health in the lactating dams (lactation day (LD) 21). Post-weaning (postnatal day (PND) 22), offspring were challenged with an atherogenic environment (HF diet in the absence of flavonoids) and vascular health markers were assessed in the adult offspring (PND 60).

Results: Dams fed the HF diet had elevated markers of atherosclerosis on LD 21 when compared to the dams fed with the control diet. Flavonoid consumption prior to pregnancy and during lactation had inconsistent effects on maternal markers of atherosclerosis (plasma cholesterol, aortic lipid accumulation, and oxidative stress biomarkers) at LD 21 compared to dams fed the HF diet without flavonoids. At PND 60, there were no differences in vascular health markers among the groups of LDLr -/- offspring whose mothers consumed the CF or the HF diet with or without flavonoids during lactation.

Conclusions: Maternal consumption of the flavonoid-supplemented HF diets had modest effects on maternal markers of atherosclerosis. The exposure of offspring to the flavonoid-supplemented HF diets during early lactation had little effect on the cardiovascular parameters assessed in the adult offspring.

Key Words: (-)-epicatechin, (+)-catechin, rutin, flavonoids, flavanols, flavonols, coronary artery disease, lactation, development

BACKGROUND

Developmental programming refers to events that occur during critical periods of development which can influence long-term health outcomes. These outcomes are commonly attributed to direct damage, impaired development, or long-term functional rewiring of developing organisms [1]. With respect to coronary artery disease (CAD), it has been shown in humans, as well as in rabbit and rodent models, that fatty streaks—an early sign of atherosclerosis—can develop in the fetus and newborn of hypercholesterolemic mothers [2, 3, 4, 5]. Additionally, *in utero* atherogenic conditions, promoted by maternal hypercholesterolemia or other risk factors can prime the offspring to develop CAD later in life, in the absence of atherosclerotic lesions at birth [6, 7]. Given the examples above, it is reasonable to argue that interventions aimed at curbing the onset and/or progression of CAD can include interventions during pregnancy and lactation, as these are periods when an individual demonstrates great susceptibility and plasticity to environmental influences.

There has been an increasing interest in the concept that the regular consumption of a plant-based diet, particularly one that includes food items rich in select types of flavonoids, including flavanols and flavonols, can improve cardiovascular health [8, 9, 10, 11, 12]. Findings from recent meta-analyses of data from prospective cohort studies, demonstrate a significant association between high intakes of flavonoid-rich foods and beverages and reduced CAD risk, which consistently support this concept [13, 14]. It should be noted that the participants of these studies were older men and women with a range of diseases, including atherosclerosis. The potential anti-atherogenic effects of high intakes of flavonoids in the context of pregnancy and development has received limited attention.

Flavanols and flavonols have been reported to modulate a range of cellular properties and functions, including the promotion of endothelial cell repair and vasomotility, the inhibition of platelet aggregation and activation, and the modulation of inflammatory and oxidative reactions [15, 16, 17, 18, 19, 20, 21, 22, 23], all factors which can reduce the risks of CAD. Importantly, supplementing atherogenic diets with flavanols or flavonols, either as plant extracts or in their purified form, has been reported to mitigate the development of atherosclerotic lesions in adult mutant mice engineered to develop atherosclerosis in response to high fat consumption [24, 25, 22, 26, 27, 28, 29].

While interest in the potential health benefits of flavonoid intake is considerable, the potential adverse effects of consuming very large amounts of these phytochemicals have not been extensively studied. Many flavonoids are available for purchase as dietary supplements, and it is feasible to achieve levels of intake which markedly exceed what is provided in typical diets. There is limited literature on the subject. Accordingly, we examined the effects of maternal

consumption of high levels of certain flavonoid on the mother and progeny in a mouse model [30]. Our data revealed that the chronic consumption of maternal diets containing high amounts of flavanols (epicatechin and catechin) or flavonols (rutin) throughout gestation and lactation did not affect litter size, fetal development, postnatal survival, skeletal development or postnatal body weight of the offspring and were not associated with developmental toxic effects. A cautious interpretation of the data is that the consumption of flavonols and flavanols by pregnant women does not represent a risk. However, we also recognize that additional studies are required to examine the potential effects of the various different subclasses of flavonoids.

In the current study we tested the hypothesis that the consumption of high amounts of certain flavonoids by the mother during pregnancy would reduce the development of atherosclerosis in offspring, stimulated by the consumption of a high fat diet. We used LDLr knockout mice, a commonly used murine model to study diet-induced atherogenesis [31]. The advantage of using these mice is that the progression of atherosclerotic lesions and plaque formation, and the lipid profiles in response to pro-atherogenic western diets share the same pathology observed in humans [32, 33]. However, the lack of LDL receptors significantly compromises ovarian function and composition in LDLr mice [34]. Additionally, the severe dyslipidemia which results from feeding a high fat diet before and during pregnancy in the absence of these LDLr females impairs reproductive performance in these mice, which results in poor or no fertility [34], [Palinski personal communication], or in severe in utero growth retardation, with fetuses weighing below the 10^{th} percentile of wild type weights [35]. For these reasons, in the current study dams were fed a control fat diet. We also examined whether select flavonoids, introduced during early postnatal development, influenced select markers of vascular health in lactating dams and adult offspring challenged with an atherogenic environment post-weaning.

We chose to focus on catechin and epicatechin (flavanols) and rutin hydrate (a flavonol) for the following reasons. First, the respective flavonoids are present in common foods in the diet and are consumed in relatively high amounts by European and American adults [36, 37, 38]. Second, we observed that high intakes of these compounds during gestation and lactation are not associated with any marked negative developmental effects [30]. Third, the consumption of these classes of flavonoids has been associated with improvements in vascular health in diverse populations [9, 39, 40].

METHODS

Animals and Diets

Eight to ten-week old female, $LDLr^{-/-}$ mice with a C57/BL6 genetic background were produced in house from breeding pairs (B6.129S7-$LDLR^{tm1Her}$) purchased from Jackson Labs (Bar Harbor, ME). Mice were fed one of four diets (n=6-8 per group), a semi-purified, control fat (CF) diet containing 7% corn oil; a high fat (HF) diet containing 2.5% corn oil, 12.5% cocoa butter and 2.5% cholesterol (Dyets, Bethlehem, PA); the HF diet supplemented with a flavanol mixture of epicatechin: catechin (Sigma, St. Louis, MO), in a 4:1 ratio at 1% of the diet (8000 mg epicatechin + 2000 mg catechin/kg diet; HFEC); or the HF diet supplemented with rutin hydrate (Sigma, St. Louis, MO) at 1% of the diet (10000 mg rutin/kg diet; HFRU). The flavanol ratio of 4:1 (-)-epicatechin / (+)-catechin reflects the ratio reported in cocoa-containing products [41],

[42]. Diets were isocaloric and prepared in small batches containing *tert*- butylhydroquinone (Dyets), in order to minimize oxidation of the flavonoids.

Females were fed their respective diets for six weeks prior to breeding with LDLr -/- males fed a commercial stock diet (Purina, Laboratory Rodent Diet 5001). Females were bred overnight for a maximum of two weeks or until successful mating, as determined by the presence of a vaginal plug in the morning (designated gestation day (GD) 0.5). Pregnant dams (from all groups) were transferred to maternity cages immediately after plug identification and fed the CF diet through pregnancy until lactation day (LD) 4. On LD 4, dams were switched back to their respective diets (CF, HF, HFEC or HFRU) until LD 21. Maternal body weight was monitored weekly until LD 21 and food intake was recorded weekly until GD 0.5. Litter size was recorded and litters were reduced to six pups per dam on LD 4. On LD 21, dams were killed and pups were weaned and housed in maternity cages by sex. All LD 21 offspring were then fed the HF diet without additional flavonoids. Consequently, for the progeny, the pregnancy diet was CF and the lactation/post-weaning dietary groups were CF/HF; HF/HF; HFEC/HF; or HFRU/HF. Pup growth and mortality were recorded weekly until postnatal day (PND) 60. On PND 42, pups were individually housed in suspended stainless steel cages and food intake was recorded weekly until PND 60. This study was approved by the Animal Care and Use Administrative Advisory Committee of the University of California at Davis.

Tissue and Plasma Collection

Dams were killed by CO_2 inhalation and exsanguination. Blood was collected from the femoral artery in heparinized syringes and the plasma was recovered by centrifugation (2500 rpm for 15 min at 4^0C), and stored at -80^0C. The liver and kidneys were excised and rinsed in phosphate buffer saline (PBS). Tissues were weighed, snap-frozen in liquid nitrogen and stored at -80^0C. The heart was flushed with 0.5M EDTA/PBS and excised with the aorta. Advential fat was removed from heart and aorta under microscope. The descending aorta, including the aortic arch, was isolated and stored in 0.5M EDTA/PBS at 4^0C for lipid accumulation quantification. The heart was fixed overnight in 4% paraformaldehyde at 4°C for immunohistochemistry. At PND 60, the offspring were killed and blood, liver, kidneys, heart and aorta were collected using the methods described above.

Plasma Cholesterol

Maternal and offspring plasma cholesterol levels were determined by fluorometry using a commercial kit (Cayman Chemical, Ann Arbor, MI).

Lipid Accumulation

Maternal and offspring aortas were opened longitudinally (including aortic arch) and pinned down to expose the lumen. Aortas were rinsed with 2-isopropanol, incubated with Oil-Red-O stain for thirty minutes at room temperature, and rinsed with 2-isopropanol and 0.5M EDTA/PBS. Tissues were transferred to depressed slides and cover slipped. Slides were examined by light microscopy using a stereo zoom microscope (Nikon AZ100). The surface area coverage of stain indicator of lipid accumulation was determined using FIJI [43]. The surface

area coverage was determined by calculating the number of pixels in the stain threshold area(s) / total number of pixels in the selected areas.

Tissue Tumor Necrosis Factor alpha (TNF-α)

The concentration of TNF-α was determined on offspring liver lysates, using commercially available ELISA for mice (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Data are reported as pg cytokine per mg of liver protein determined by the Bradford assay (BioRad, Hercules, CA). Liver tissues were homogenized with ice-cold 20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210 M mannitol, 70mM sucrose, and a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Samples were sonicated and the supernates were recovered by high-speed centrifugation (15,00 x g for 7 minutes at 4^0C).

Immunohistochemistry

After fixation, maternal and offspring hearts were embedded in paraffin and cut in serial transverse sections (7 μM) using a microtome, and then collected on slides. Slides were deparaffinized and rehydrated in graded ethanol (100–70%). To increase epitope exposure, the sections were permeabilized with trypsin at 37°C. The sections were incubated overnight at 4°C with the following primary antibodies: MCP-1, eNOS, VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA); 4-HNE (Alpha Diagnostic, San Antonio, TX), SOD-1 (CuZnSOD) (Millipore, Temecula, CA), FBG (Sigma, St. Louis, MO) or with a negative control of corresponding serum solution. The following day, the sections were incubated at room temperature with the appropriate biotinylated secondary antibodies. The antigen-antibody complex was visualized with an avidin-horseradish peroxidase staining kit (ABC, Vector Laboratories, Burlingame, CA), in the presence of 3-3′diaminobenzidine and H_2O_2. The slides were counterstained toluidine blue and examined by light microscopy using a phase microscope (Olympus BX51). Personnel blinded to the treatment groups selected one stained aortic section from four different litters per treatment, per antibody to image. Antibodies' staining intensity and distribution were quantified using FIJI [43] and expressed as the average intensity of the pixels in the selected area / the total number of pixels in the selected area.

Statistical Analysis

Statistical analyses were performed using Small Stata version 12 (StataCorp, College Station, TX). Significant effects of maternal dietary treatments on lipid accumulation, immunohistochemistry, plasma cholesterol, and tissue TNF-α were determined through analyses of variance (ANOVA) and least square means. Generalized Linear Modeling accounting for clustering within litters determined significant effects of maternal dietary treatment on reproductive and postnatal outcomes. Significant effects of maternal and offspring dietary treatments on body weight were determined by repeated measures ANOVA. Data are expressed as mean ± standard error of the means (SEM); statistical significance was set at $P < 0.05$. Post hoc analysis was performed to evaluate the significance of observed differences among the groups using the Tukey method.

RESULTS:

Maternal Food Intake, Body Weight, and Tissue Weight

Average daily food intake prior to conception was similar among the treatment groups (data not shown). Maternal weight gain was similar among the groups, with the largest increases occurring during gestation (data not shown). Maternal kidney weights either on a relative (tissue/body weight ratio), or absolute, weight was similar among groups (data not shown). Maternal liver weights were significantly affected by the dietary treatments. Compared to CF fed mice, mice fed a HF diet had larger relative and absolute liver weights. The addition of the flavonoids to the HF diets did not prevent liver enlargement (data not shown).

Offspring Food Intake, Body Weight, and Tissue Weight

Weekly food intake was similar among groups during each week of food intake data collection (PND 42-48, PND 49-55, and PND 56-60), per litter as well as per sex (data not shown). Body weights were similar among treatment groups over time from PND 21 through PND 60 (data not shown). There were no differences among PND 60 offspring, per litter, liver or kidney weight on an absolute or relative weight basis (data not shown).

Reproductive and Postnatal Outcomes

Feeding the LDLr -/- dams a CF diet during pregnancy resulted in successful breeding and dams delivered normal sized litters (Table 1).

Table 1. Reproductive and Postnatal Outcomes assessed at PND 60.

Group*	Litter Size	Males per Litter	Females per Litter	Eye Anomalies per Litter	Malocclusion Anomalies per Litter	Percent Anomalies per Litter
CF/HF	4.0 ± 0.7	2.1 ± 0.5	1.9 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
HF/HF	4.2 ± 0.5	2.0 ± 0.6	2.2 ± 0.5	16.7 ± 0.2	33.3 ± 0.3	13.9 ± 6.3
HFEC/HF	4.5 ± 0.4	2.0 ± 0.5	2.5 ± 0.3	12.5 ± 0.1	25.0 ± 0.16	9.8 ± 5.0
HFRU/HF	4.3 ± 0.7	2.3 ± 0.3	2.0 ± 0.5	0.0 ± 0.0	16.7 ± 0.2	6.1 ± 3.9

Values are mean ± SEM

*Diets represent dietary interventions fed to dams during lactation and to offspring post weaning.

However, the 6-8 weeks of HF feeding prior to breeding resulted in a non-significant increase in the incidence of anomalies in the progeny when assessed at PND 60. The addition of high amounts of flavonoids to the HF diet did not prevent the anomalies. The observed anomalies included micropthalmia of the left and/or right eye or the presence of malocclusion. Malocclusion was determined by identifying overgrown incisors, thus indicating misalignment of the mandibular and maxillary teeth. If a mouse had more than one gross anomaly, then the anomaly was only counted as one. Approximately 14% per litter of the HF litters had at least one offspring with one or more anomalies, compared to the CF group that had no anomalies (0%). The HFEC and HFRU groups had anomaly frequencies that were intermediate between the two (10%, and 6% per litter, respectively) (Table 1). There were no differences in postnatal pup

survival among any of the groups until PND 60. From LD 0 until LD 4, when they were fed the CF diets, offspring average survival rate per litter was 87%, 88%, 89%, and 100% for pups of dams fed the CF, HF, HFEC, and HFRU diets respectively. From LD 4 to LD 21, survival rates were 90-92%, and 87-100% for PND 22 to PND 60.

Maternal and Offspring Plasma Cholesterol

Plasma cholesterol levels were measured at the end of the lactation period in dams (LD 21) and at PND 60 in the offspring. Feeding dams with the HF diet for 6-8 weeks prior to breeding and during the lactation period was associated with moderate increases in plasma cholesterol; the HFRU-fed dams had significantly higher plasma cholesterol levels compared to dams fed the CF and HF diets (Table 2). For the offspring, there were no differences in plasma cholesterol levels among the different groups (Table 2).

Table 2. Plasma cholesterol levels and aortic lipid accumulation measured in LD 21 dams and PND 60 offspring.

Plasma cholesterol (mg/dL)				
LD 21 Dams*			**PND 60 Offspring****	
CF (n = 8)	482.2 ±		CF/HF (n =	533.7 ±
HF (n= 5)	578.6 ±		HF/HF (n =	603.2 ±
HFEC (n =	631.6 ±		HFEC/HF (n	531.8 ±
HFRU (n =	843.5 ±		HFRU/HF	616.9 ±
Lipid Accumulation (% coverage)				
LD 21 Dams*			**PND 60 Offspring****	
CF (n = 6)	8.1 ± 1.8[a]		CF/HF (n =	15.1 ± 1.2
HF (n= 6)	26.5 ± 4.2[b]		HF/HF (n =	17.9 ± 2.4
HFEC (n =	19.5 ± 1.7[b]		HFEC/HF (n	15.2 ± 2.3
HFRU (n =	29.9 ± 2.3[b]		HFRU/HF	16.4 ± 2.0

Values are mean ± SEM; values not sharing the same superscripts are significantly different at $P<0.05$

*Diets represent dietary intervention fed to females pre-pregnancy and during lactation.

**Diets represent dietary interventions fed to dams during lactation and to offspring post weaning.

Offspring Liver Tumor Necrosis alpha (TNF-α)

Feeding the dams the high fat diet during lactation influenced the offspring liver TNF-α levels as measured by ELISA (Figure 1).

Specifically, liver TNF-α levels were significantly higher in the HF/HF treatment group compared to the HFEC/HF group. In female offspring, TNF-α levels of the HF/HF group were significantly higher than the other three groups (data not shown). When comparing the two

flavonoid exposed offspring groups (EC/HF and RU/HF) to the HF/HF only, there was a significant decrease in TNF-α level (p=0.0027) in both flavonoid exposed groups.

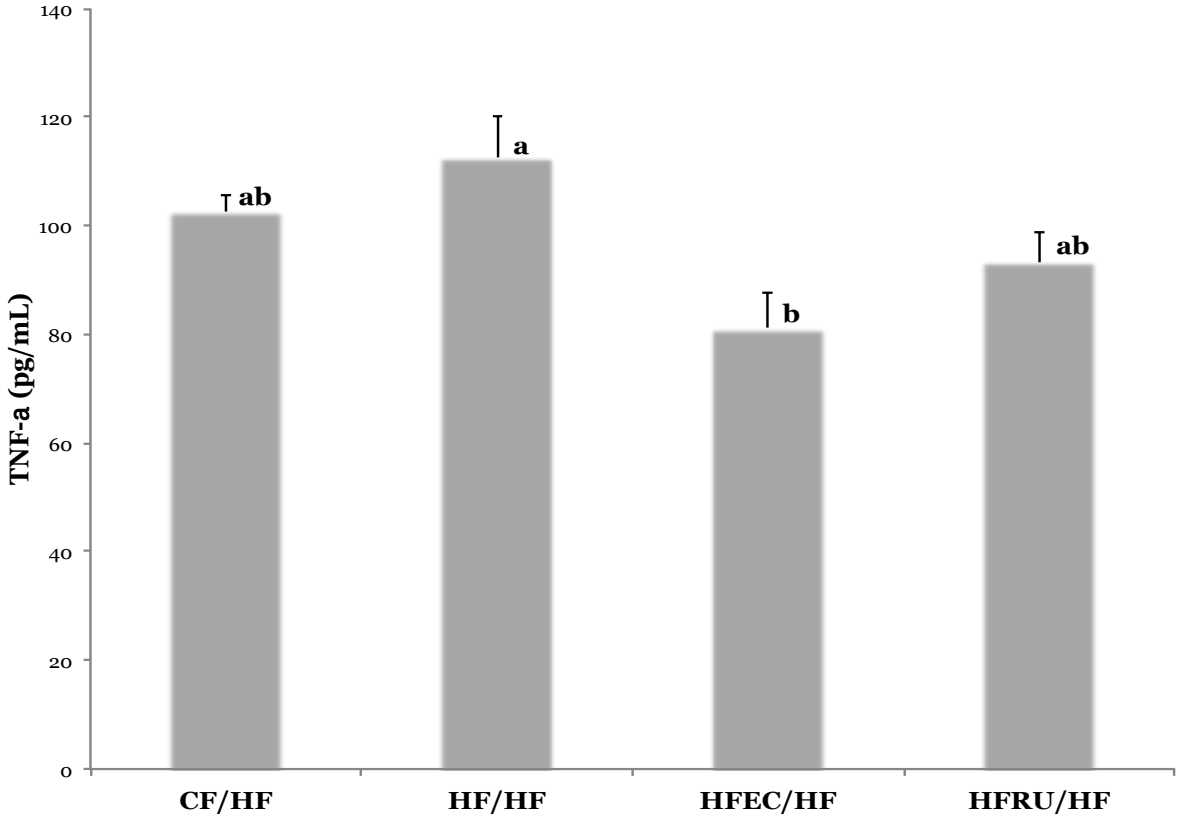


Figure 1. Liver TNF-α level in PND 60 offspring.
Values are mean ± SEM; values not sharing the same superscripts are significantly different at P<0.05

Maternal and Offspring Aortic Lipid Accumulation

There was a marked increase in lipid accumulation and lesion size in maternal aortas of dams fed a HF diet compared to dams fed a CF diet, as measured by Oil-Red-O staining (Table 2). In the offspring, there were no significant effects of treatment (Table 2).

Maternal and Offspring Aortic Sinus Characteristics

The aortic sinus of dams (LD 21) and offspring (PND 60) were processed for immuno-detection of a series of cardiovascular-related biomarkers. Following exposure to an atherogenic diet throughout lactation, dams fed the HF diet showed increased aortic staining of biomarkers indicative of macrophage infiltration (MCP-1), cytokine mobilization (VCAM) and ROS and RNS presence (eNOS, SOD-1 and 4-HNE). All parameters were markedly increased in HF dams, with the addition of flavonoids to the HF diet being associated with a decreased expression of most all the biomarkers. However, while these results suggest that there is some attenuation effect by both classes of flavonoids, the quantitative analysis of the staining intensity was not statistically different between groups (Figure 2; Table 3).

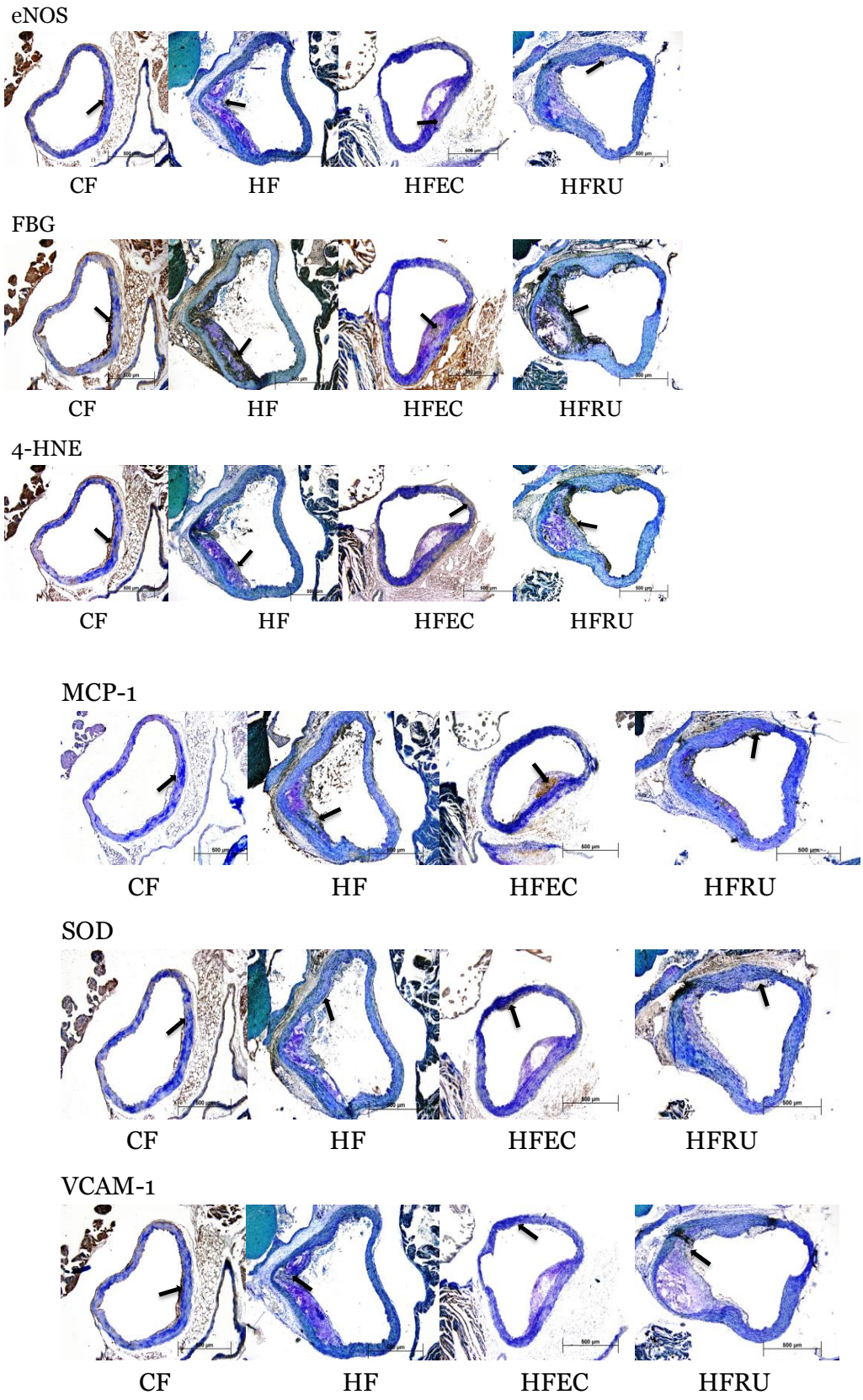


Figure 2. LD 21 maternal aorta expression of cardiovascular biomarker

Table 3. Expression of select biomarkers measured in aortic sinus of LD 21 dams.

	CF* **(n = 4)**	**HF (n = 4)**	**HFEC (n = 4)**	**HFRU (n = 4)**
eNOS	$1.1 \times 10^8 \pm 9.3 \times 10^6$	$1.6 \times 10^8 \pm 2.4 \times 10^7$	$1.4 \times 10^8 \pm 10.0 \times 10^6$	$1.5 \times 10^8 \pm 2.9 \times 10^7$
FBG	$1.1 \times 10^8 \pm 1.0 \times 10^7$	$1.8 \times 10^8 \pm 1.5 \times 10^7$	$1.7 \times 10^8 \pm 2.1 \times 10^7$	$1.8 \times 10^8 \pm 2.7 \times 10^7$
4-HNE	$1.1 \times 10^8 \pm 1.1 \times 10^7$	$1.9 \times 10^8 \pm 1.7 \times 10^7$	$1.4 \times 10^8 \pm 1.0 \times 10^7$	$1.7 \times 10^8 \pm 2.8 \times 10^7$
MCP-1	$1.1 \times 10^8 \pm 1.7 \times 10^6$	$1.5 \times 10^8 \pm 3.3 \times 10^7$	$1.6 \times 10^8 \pm 2.3 \times 10^7$	$1.4 \times 10^8 \pm 3.3 \times 10^7$
SOD-1	$1.2 \times 10^8 \pm 1.1 \times 10^7$	$1.6 \times 10^8 \pm 2.6 \times 10^7$	$1.5 \times 10^8 \pm 2.2 \times 10^7$	$1.4 \times 10^8 \pm 2.4 \times 10^7$
VCAM-1	$1.2 \times 10^8 \pm 1.2 \times 10^7$	$1.8 \times 10^8 \pm 1.8 \times 10^7$	$1.5 \times 10^8 \pm 1.7 \times 10^7$	$1.6 \times 10^8 \pm 2.2 \times 10^7$

Values are mean ± SEM of stain intensity (pixel intensity/pixels)
*Diets represent dietary intervention fed to females pre-pregnancy and during lactation.

In the offspring, the aorta immuno-staining profiles of the biomarkers in response to the various diets was opposite to that measured in the dams, although none of these group differences reached statistical significance (Figure 3; Table 4).

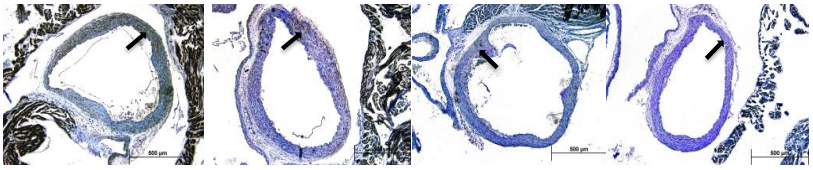
Table 4. Expression of select biomarkers measured in aortic sinus of PND 60 offspring.

	CF/HF* **(n = 4)**	**HF/HF (n = 4)**	**HFEC/HF (n = 4)**	**HFRU/HF (n = 4)**
eNOS	$1.7 \times 10^8 \pm 2.3 \times 10^7$	$1.2 \times 10^8 \pm 1.4 \times 10^7$	$1.6 \times 10^8 \pm 3.3 \times 10^7$	$1.2 \times 10^8 \pm 1.7 \times 10^7$
FBG	$1.9 \times 10^8 \pm 3.3 \times 10^7$	$1.2 \times 10^8 \pm 1.3 \times 10^7$	$1.5 \times 10^8 \pm 3.6 \times 10^7$	$1.2 \times 10^8 \pm 1.9 \times 10^7$
4-HNE	$1.8 \times 10^8 \pm 2.3 \times 10^7$	$1.2 \times 10^8 \pm 1.7 \times 10^7$	$1.6 \times 10^8 \pm 3.5 \times 10^7$	$1.4 \times 10^8 \pm 1.4 \times 10^7$
MCP-1	$1.6 \times 10^8 \pm 2.2 \times 10^7$	$1.3 \times 10^8 \pm 1.5 \times 10^7$	$1.3 \times 10^8 \pm 1.7 \times 10^7$	$1.1 \times 10^8 \pm 1.2 \times 10^7$
SOD-1	$1.8 \times 10^8 \pm 2.1 \times 10^7$	$1.4 \times 10^8 \pm 1.5 \times 10^7$	$1.4 \times 10^8 \pm 3.0 \times 10^7$	$1.2 \times 10^8 \pm 1.4 \times 10^7$
VCAM-1	$1.8 \times 10^8 \pm 2.7 \times 10^7$	$1.3 \times 10^8 \pm 2.0 \times 10^7$	$1.6 \times 10^8 \pm 3.7 \times 10^7$	$1.2 \times 10^8 \pm 1.0 \times 10^7$

Values are mean ± SEM of stain intensity (pixel intensity/pixels)
*Diets represent dietary interventions fed to dams during lactation and to offspring post weaning.

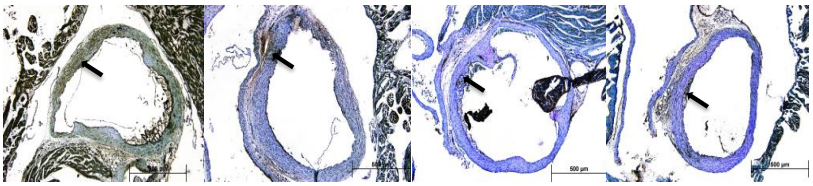
Figure 3. PND 60 offspring aorta expression of cardiovascular biomarkers

eNOS



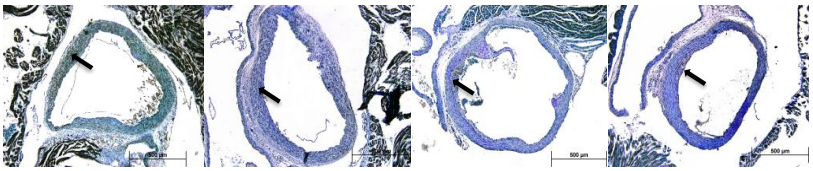
CF HF HFEC HFRU

FBG



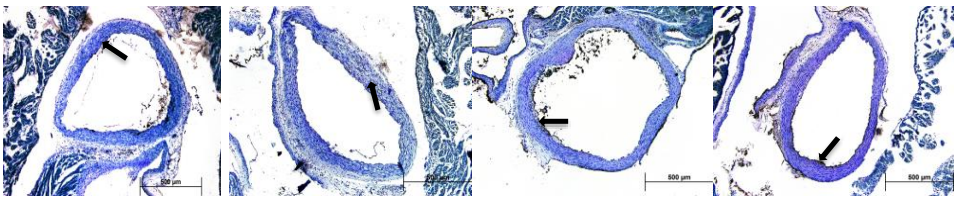
CF HF HFEC HFRU

4-HNE



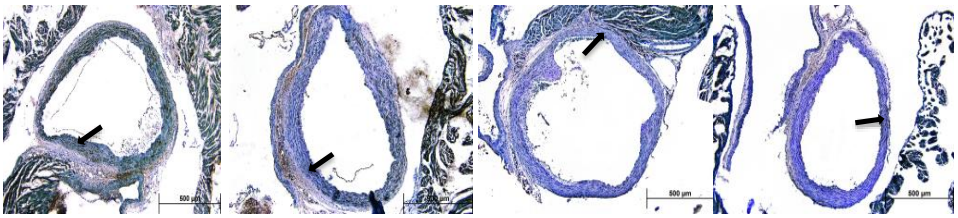
CF HF HFEC HFRU

MCP-1



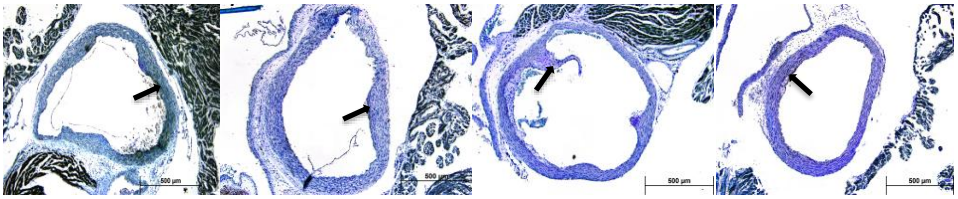
CF HF HFEC HFRU

SOD



CF HF HFEC HFRU

VCAM-1



CF HF HFEC HFRU

Whereas the HF diet resulted in increased expression of aortic markers, thereby suggesting the development of atherosclerotic plaque in dams (these plaques were visible by histology, (see arrows Figure 3), the aortic sinus of offspring fed a HF diet post weaning showed reduced expression of the inflammatory biomarkers and the absence of plaque formation (Figure 3; Table 4). Supplementation of the HF diet with flavonoids did not influence the expression of these indices.

DISCUSSION

The early postnatal environment can be a critical period with respect to influencing an individual's subsequent risk for the development of certain chronic diseases, such as atherosclerosis. In this study, we used LDLr -/- mice to explore the extent to which the addition of epicatechin and catechin or rutin, at 1% of the diet beginning prior to pregnancy and during lactation, affected markers of maternal vascular health in response to a high fat modified western type diet. Additionally, we assessed the effect of flavonoid exposure during lactation on markers of postnatal vascular health when offspring were challenged with a pro-atherogenic environment in the post-weaning period.

Using this model, our results demonstrate that LDLr -/- dams were hypercholesterolemic and particularly susceptible to the pro-atherogenic effects of a high fat diet, as evidenced by the increased lipid accumulation in the aortic region. Our data also revealed that the addition of flavonoids to this fat-rich diet failed to mitigate these adverse effects. We also observed that the offspring were hypercholesterolemic and developed atherosclerotic lesions in response to a HF diet challenge of 40 days post weaning. The atherogenic phenotype in the progeny was similar among all offspring, independent of the maternal diet pre-gestation and during lactation. Supplementation of the maternal HF diets with different flavonoids during lactation did not help the offspring to develop protection against a subsequent lipidemic challenge, as there was no difference in markers of vascular health between the treatment groups. Therefore, our results reveal that manipulating the maternal diet during the pre-pregnancy and lactation periods did not result in positive or detrimental developmental programming effects in LDLr -/- offspring when challenged with a HF diet post weaning. In contrast, Napoli et al demonstrated that feeding LDLr-/- mice a HF diet supplemented with varying levels of cholesterol during gestation and chow post birth resulted in a two-fold increase in aortic lesion size in the offspring, compared to offspring of LDLr-/- knockout dams fed a CF diet during pregnancy [3].

LDLr -/- mice are extremely receptive to developing atherosclerotic lesions when fed a diet high in saturated fat [31], [44], and LDLr -/- mice fed a diet consisting of 15% fat and 1.25% cholesterol for approximately 6 weeks readily form atherosclerotic lesions and become hypercholesterolemic with serum cholesterol levels greater than 400 mg/dL [45]. In our study, LDLr -/- dams and offspring were fed the HF diet for a period of 6 to 8 weeks and displayed biochemical and clinical signs of atherosclerosis consistent with the model. Our results show that the inclusion in the diet of two types of flavonoids at relatively high levels failed to significantly modulate the pro-atherogenic effects of the HF diet in this genetically susceptible hypercholesterolemic mutant mouse model. For dams, the potential anti-atherogenic agents were ingested with the HF diet and for the offspring, the exposure to the flavonoids was early in life (through lactation), prior to the HF challenge. These results conflict with those of numerous

reports showing that various flavonoids given as supplements or as plant-derived extracts can limit atherosclerotic lesion progression in response to a high fat diet in both the LDLr and the ApoE mutant mouse models [46, 24, 25, 47, 22, 29, 48]. A number of factors may have contributed to these different outcomes. To the best of our knowledge, the putative anti-atherogenic effects of flavonoids have not been tested in the context of developmental programming. It is possible that the state of pregnancy and lactation had an effect on the metabolism or distribution of the flavonoids, the hemodynamic conditions, or the vascular endothelium of dams and offspring that interfered with the mechanisms of actions of these compounds. Alternatively, differences in experimental conditions including the types and levels of flavonoids that were chosen and the duration of exposure may have contributed to the lack of effects observed in the study.

The pathogenesis that underscores the development and progression of atherosclerosis is complex and comprises a series of interacting factors that ultimately disrupt the vascular endothelium and promote lesions formation [49, 50]. Initially, lesions are instigated by the accumulation and oxidation of low density lipoprotein-cholesterol (LDL-C) at the endothelium surface, in the intima, in macrophages and lowering LDL-C levels is the recommended approach to reduce atherosclerotic lesion formation even in cases of familial hypercholesterolemia (FH), characterized by elevated serum cholesterol and LDL-C levels because of mutations in the LDLr gene [51, 51, 52, 3, 53, 54]. Flavonoids, specifically catechins added directly to the diet or as green tea extract can modulate the expression of several genes that regulate LDL-C metabolism, including the LDLr transcription factor SREBP-1 [55, 46, 43, 56]. Such an effect was not observed in our study, as plasma cholesterol levels remained elevated in the LDLr -/- mice, a model characterized by impaired lipoprotein clearance and LDL-C accumulation. Likewise, a majority of studies showed no reduction of plasma cholesterol levels in LDLr -/-mice fed diets supplemented with different forms of flavonoids [57, 29, 26, 28, 48]. Thus with respect to lipoprotein metabolism and reduction of atherosclerosis, it appears that more aggressive interventions are required in these mice (as in individuals with FH), to overcome the strong genetic disposition to develop hypercholesterolemia.

We examined whether high intakes of flavanols or flavonols in the context of maternal hypercholesterolemia attenuated the vascular oxidative and/or inflammatory reactions that characterize the pathogenesis of atherosclerosis. The presence of epicatechin and catechin in the diet during lactation did lower TNF-□ level compared to the HF/HF group. Consistent with other studies, decreased TNF-□ expression by flavonoids has been reported by inhibiting the activation of nuclear factor-kappa beta (NF-κB) through suppression of NF-κB mobilization [58, 59, 60, 61]. Lipid accumulation in the aorta, as well as the expression of markers of endothelial reactivity in the aortic sinus of dams and offspring exposed to high intakes of flavonoids and fat, was found to be similar to that of dams and offspring fed diets rich in fat. The lack of impact by flavonoids in the vascular endothelium of LDLr -/- dams and offspring was also observed by Natsume and Baba [28], who discovered no effects of flavonoids in the vascular endothelium, reported no changes in fatty plaque lesion size, atheroma formation and expression of various oxidative and inflammatory stress markers in aortas of ApoE -/- mice fed a HF diet supplemented with low dose cocoa polyphenols (0.4%) for 16 weeks. Studies conducted by Kleeman et al [21] also demonstrated no changes in plasma e-selectin and VCAM-1 levels in

Apo E -/- mice fed a HF diet supplemented with low dose quercetin (0.1%) for 15 weeks. In contrast, other studies have reported decreased expression of cell adhesion and chemokine markers in the aortic sinus of HF fed ApoE -/- mice treated with higher doses of quercetin [22], [29]. As discussed above, differences in research designs are likely to have contributed to the variability in results with respect to flavonoids' anti-atherogenic effectiveness. To our knowledge, there are no published articles or data on the effects of flavonoids on inflammatory and oxidative markers of atherosclerosis in hypercholesterolemic dams and their offspring. Although there is evidence that flavonoids are present and can be quantified in breast milk [62], in our study it is possible the flavonoids included in the milk were not large enough amounts, or what was transferred to the offspring were not in large enough amounts to observe any physiologic changes in the parameters we assessed. Therefore, we speculate that feeding LDLr -/- mice a HF diet post weaning was metabolically damaging and superseded any protective effects that could have been afforded by the addition of flavonoids in the diet during lactation.

Evidence that the HF diet resulted in severe reproductive stress in our model was provided by the observation that LDLr -/- dams fed this HF diet during gestation (with or without added flavonoids) could not sustain full term births although the dams were fertile (presence of vaginal plugs and fetuses *in situ* at necropsy). Full term viable litters could be generated only when we fed the LDLr -/- dams the atherogenic diets for 6 weeks prior to breeding but not during gestation. Moreover, when HF-fed dams were switched to a control diet for the duration of gestation, the dams fed the HF diets pre-breeding exhibited increased rates of congenital anomalies in the progeny. The most commonly observed anomalies were microphthalmia, and malocclusion. Asymmetric eye defects are common in C57BL/6J mice, particularly in female mice [63], and specific environmental factors like ethanol exposure or folic acid deficiency can increase their incidence [64, 65]. Here we show high fat to be an additional factor that can influence the baseline expression of this genetic susceptibility in C57BL mice. Interestingly, supplementation of the HF diet with high amounts of the flavonol rutin reduced the frequency of eye anomalies in the progeny to control levels. Others have reported the preventive effects of quercetin (a rutin aglycone) on the developmental toxicity of various reproductive toxicants [66, 67]. Supplementation of the HF with epicatechin and catechin group had a modest effect of reducing anomalies. Here our results and data demonstrate that high intakes of flavanols may also offer some benefits in pregnancies complicated by teratogens exposure. In any case, the high intakes of these flavonoids were well tolerated by the pregnant mice and their offspring. Additionally, we did not observe any adverse effects of epicatechin, catechin or rutin on postnatal survival and growth of offspring, consistent with results from our previous study showing no developmental toxicity of large maternal intakes of these flavonoids during pregnancy [30]. In the present study, the HF fat diet was teratogenic to LDLr -/- dams and their progeny, and inclusion of flavonoids to this diet moderated the adverse reproductive performance associated with the HF diet.

The concept of developmental programming proposes that environmental signals received by the fetus and neonates can shape the inherited developmental trajectory into postnatal phenotypes that closely match the *in utero* environment. This developmental plasticity driven by epigenetic mechanisms has been termed predictive adaptive responses [68, 69]. According to this model, prediction errors or mismatches contribute to reduced lifespan and increased

susceptibility to diseases in adulthood [70, 71, 72]. In this context, maternal nutrition and health provide the *in utero* environmental conditions that determine the plane of development. Consequently, malnutrition and diseases can result in congenital anomalies or death if these conditions are severe, or induce metabolic and functional adaptations in the fetus, for which there may be adverse consequences later in life contingent to the nutrition and health circumstances of postnatal life. Although the idea of developmental programming has been validated using different paradigms, only a few studies have tested the relative importance of lactation as a period of plasticity, independent of pregnancy. In general, these models showed that maternal overfeeding during the lactation period do not provide any predictive-adaptive advantages to offspring weaned to a high fat palatable diet as they develop obesity, hypertension or metabolic syndrome at greater rates than offspring that suckled dams fed a control diet [73, 74, 75, 76]. Therefore, although maternal undernutrition is known to program the fetus to future thriftiness, maternal overnutrition during lactation does not prime the neonate with the ability to squander when faced with a surfeit of calories or fat. Consistent with the above, our data revealed that maternal hypercholesterolemia and a HF diet during lactation did not elicit significant protective programming on the development, onset and severity of atherosclerosis later in life. As a result, for heart disease risks, the effects of maternal hypercholesterolemia and high fat feeding during lactation are similar to the outcomes reported in response to maternal hypercholesterolemia during pregnancy, which can increase atherosclerosis susceptibility later in life [77, 78, 79, 80].

CONCLUSIONS

In summary, the results of this study demonstrate that the chronic consumption of maternal diets containing high amounts of saturated fat with the addition of the flavanols (epicatechin and catechin) or flavonols (rutin) prior to gestation and throughout lactation had modest effects on maternal markers of atherosclerosis. In the offspring, when the HF diet was introduced at weaning, the inclusion of the flavonoids in the maternal diet during lactation had little effect on the parameters assessed. Consequently, in the context of early postnatal adaptation, using the LDLr -/- mouse model of atherosclerosis, it appears that the presence of saturated fat in the diet resulted in metabolic effects that persisted even with the exposure of a diet that included the addition of flavonoids at 1% during lactation.

Abbreviations: CAD: coronary artery disease; LDLr-/-: low density lipoprotein receptor knock out; CF: control fat diet; HF: high fat diet; HFEC: high fat diet supplemented with 1% mix of (-)-epicatechin and (+)-catechin; HFRU: high fat diet supplemented with 1% rutin GD: gestation day; LD: lactation day; PND: postnatal day; TNF-α: tumor necrosis factor-alpha; FBG: fibrinogen; 4-HNE: 4-hydroxynonenal; eNOS: endothelial nitric oxide synthase; MCP-1: monocyte chemoattractant protein-1; SOD-1: copper-zinc superoxide dismutase VCAM-1: vascular cell adhesion molecule; ANOVA: analysis of variance; SEM: standard error of the means; LDL-C: low density lipoprotein cholesterol; FH: familial hypercholesterolemia; NF-κB: nuclear factor-kappa beta

Competing Interests: The authors declare that they have no competing interests.

Author's Contributions: The author's contributions are as follows – MNRL, LL, and CLK conceived and designed the experiments; MNRL, MDG, and JP handled and cared for the animals; MNRL, LL, MDG, and JP collected all tissues, developed experiment protocols and performed the experiments; MNRL performed statistical analysis; MNRL, LL, JYU-A, and CLK were involved in data interpretation; MNRL and LL wrote the paper with editing contributions from all other authors

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