Cardiovascular Topics

Natural cocoa inhibits maternal hypercholesterolaemia-induced atherogenesis in rabbit pups

Richard Michael Blay, Saviour Kweku Adjenti, Kevin Kofi Adutwum-Ofosu, Bismarck Afedo Hottor, John Ahenkorah, Benjamin Arko-Boham, Frederick Kwaku Addai

Abstract

Atherosclerosis begins during foetal development and is enhanced by maternal hypercholesterolaemia during pregnancy. This study assessed the effect of natural cocoa on atherosclerosis in offspring conceived in maternal hypercholesterolaemia. Female rabbits were fed a cholesterol-enriched diet for two weeks and hypercholesterolaemia was confirmed, after which they were crossed with normocholesterolaemic males. One group of hypercholesterolaemic mothers (HCC) received natural cocoa powder (NCP) in their drinking water, whereas the other group (HC) received only water. Histological analysis of three segments of the aorta (arch, thoracic and abdominal) from offspring of both groups was compared with a control group (NC). Intima–media thickness of the aortic arch in offspring born to hypercholesterolaemic rabbits (HC: 146 µm) was higher compared to HCC (99 µm) and control rabbits (58.5 µm). All the sections from the aortic arch of the HC group had atherosclerotic lesions while none of the sections of the aortic arch from the NC and HCC groups had lesions present. Inferentially, regular and voluntary consumption of NCP during pregnancy may inhibit aortic atherogenesis in offspring of hypercholesterolaeic mothers.

Keywords: atherosclerosis, maternal hypercholesterolaemia, intima–media thickness, cocoa, antioxidants, foetal

Atherosclerosis is a progressive disease that is initiated by turbulent blood flow and the accumulation of lipids in the walls of large arteries, leading to dysfunction of the endothelium, and subsequently, the formation of lesions. The disease leads to complications such as myocardial infarction and stroke, which are known to cause the death of about 17 million people globally each year.

In humans, the process of atherogenesis begins during foetal development, and early lesions known as fatty streaks, containing cholesterol-rich macrophages or foam cells, occur in the first decade of life. There is therefore a long time lag between the onset of atherogenesis and clinical manifestation, and fatty streaks become precursors to advanced lesions later on in life.

The relationship between serum cholesterol levels and atherosclerosis has long been established, and cholesterol-lowering therapy is known to reduce atherosclerosis. The specific process that initiates atherosclerosis, however, needs to be further understood in order to develop effective therapeutic measures.

Three hypotheses have been proposed concerning the initiation of atherosclerosis, namely, the response-to-injury, the response-to-retention and the oxidative-modification hypotheses. According to the response-to-injury theory, atherosclerosis is initiated when endothelial cells are denuded due to damage to the cells. It is now known that endothelial injury alone does not initiate atherosclerosis, but injury results in the initiation of oxidation of low-density lipoprotein (LDL) and the activation of monocytes, which differentiate into macrophages and foam cells.

In the response-to-retention hypothesis, sub-endothelial retention of apolipoprotein B-containing lipoproteins in the walls of arteries is the key pathological event during atherosclerosis. The oxidative-modification hypothesis suggests that native LDL is oxidised in the vessel wall and the uptake of modified or oxidised LDL by macrophages leads to the formation of foam cells, which become the pivot for the development of advanced atherosclerotic lesions.

Putting together the evidence supporting the various hypotheses, accumulation of cholesterol and its oxidation are key in the initiation of atherosclerosis. Hypercholesterolaemia, a condition characterised by elevated cholesterol levels in blood, is therefore the principal risk factor for cardiovascular diseases, and understanding the role it plays during atherosclerosis is therefore likely to provide several novel treatment solutions.

Maternal hypercholesterolaemia during pregnancy results in the formation of significantly larger atherosclerotic lesions in foetuses, and the formation of advanced lesions in adult life progresses faster in offspring of hypercholesterolaeic mothers.
Maternal hypercholesterolaemia may enhance atherosclerosis by differentially dysregulating the expression of aortic genes in the offspring, resulting in a cascade of processes later in life that will increase lipid deposition and inflammation. Moreover, hypercholesterolaemia in pregnancy enhances endothelial dysfunction in foetal arteries and placental vasculature, thereby decreasing nitric oxide-dependent vasodilation while increasing oxidative stress. On the other hand, dietary intervention in humans using antioxidant-rich foods has an inverse relationship that will increase lipid deposition and inflammation. Moreover, consumption of natural cocoa powder in the prevention of foetal onset of atherosclerosis.

Cocoa powder is rich in antioxidants and several studies have shown that treating animals with cocoa powder inhibits the oxidation of LDL, reduces oxidative stress, inflammation and insulin resistance. Moreover, consumption of natural cocoa powder reduced hypercholesterolaemia and atherosclerosis in apolipoprotein E knock-out mice by reducing the expression of genes related to metabolism, apoptosis and inflammation. Our study therefore investigated the effect of maternal hypercholesterolaemia on the vascular morphology and atherosclerosis in the offspring of hypercholesterolaiamic rabbits. It also sought to validate potential beneficial effects of consumption of natural cocoa powder (NCP) in the prevention of foetal onset of atherosclerosis.

Methods

This study was approved by the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana. It was carried out in accordance with appropriate institutional regulations on the care and use of laboratory animals.

Ten New Zealand white female rabbits (age: 6 months, body weight: 1.5–2.8 kg) were obtained from the Animal Experimentation Unit of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Accra. The rabbits were transported to the animal house of the University of Ghana Medical School, Korle-Bu and kept for two weeks to acclimatise. The animals were housed under standard conditions of local temperature (30°C) and relative humidity (80%) and exposed to a 12-hour light/dark cycle.

Rabbits were then randomly assigned by lottery to three groups and housed individually in cages. The rabbits were arbitrarily numbered from one to 10 and the numbers were written on pieces of paper. After mixing up the pieces of paper, rabbits were placed in groups when the numbers were drawn.

The first two groups of four rabbits each were fed cholesterol-enriched feed (CEF). The CEF was prepared (adopted from Sun et al.27) by mixing standard feed (Kosher Feedmill Ltd, Accra) with 0.5% (w/w) cholesterol (Hopkin and William Ltd, London) and 10% (v/w) coconut oil (open market, Accra). The rabbits were fed CEF for two weeks, and when a routine lipid profile test confirmed hypercholesterolaemia, they were crossed with normocholesterolaeimc males.

The normal cholesterol range for rabbits is 0.14–1.86 mmol/l (Offert et al.28). Hypercholesterolaemia was defined as a total plasma cholesterol level higher than twice the upper level of the normal range.

One group of rabbits on CEF was given 2% (w/v) of NCP (GoodFood brand, Kakawa Enterprise Ltd, Accra) as an aqueous suspension instead of drinking water. This group (HCC) had 24-hour access to the NCP suspension, which they drank voluntarily, after being mated until they littered (28–30 days). The second group of rabbits on CEF (HC) were given 24-hour access to filtered tap water.

The third group of rabbits (n = 2), designated as normal control (NC), were given standard chow without cholesterol enrichment and filtered tap water throughout the duration of the experiment. Animals that were fed CEF were fed with standard chow after delivery and the cocoa drink was replaced with drinking water. Twelve pups were delivered by the HCC rabbits, six by the HC rabbits, and the NC rabbits delivered 12 pups.

Total levels of cholesterol were determined after an overnight fast by an enzymatic colorimetric test in a laboratory at the Medical Biochemistry Department (University of Ghana Medical School), using a semi-automated clinical analyser, Microlab 300 (Vital Scientific, the Netherlands).

Blood samples were obtained by the bleeding of the marginal ear vein. The skin over the ear of the rabbits was anaesthetised using a local anesthetic cream containing lidocain (Lignocaine 2% Jelly, Purna Pharmaceuticals, Belgium) after the fur over the ear was shaved and the skin sanitised with alcohol. Blood samples were then drawn from the marginal ear vein and stored in sterilised test tubes containing heparin.

Maternal blood samples were collected before treatment with the cholesterol-enriched diet and after two weeks of feeding with the cholesterol-enriched diet. Blood samples from the offspring at the end of the experiment were obtained by cardiac puncture after chloroform inhalation had anaesthetised them.

The rabbit pups were euthanised by chloroform inhalation one week after birth and perfusion-fixed using 10% normal saline, followed by 10% phosphate-buffered formalin at pH 7.3. The aorta was dissected to remove the arch, thoracic and abdominal segments, which were post-fixed in 10% phosphate-buffered formalin for two to seven days. The aortae were taken through a routine histological processing.

Every 10th section of the arch, thoracic and abdominal segments of the aorta with a thickness of 10 µm was stained and analysed. Sections were stained with haematoxylin and eosin (H & E) for assessing intima–media thickness. In order to assess collagen and elastic fibre deposition in the vascular walls, Verhoeff–Van Gieson (VVG) staining of the sections was performed.

To determine the presence or absence of atherosclerotic lesions, frozen sections of the aortic segments were stained with Oil red O. Sections were counter-stained in alum haematoxylin. Five sections each of the aortic arch, thoracic and abdominal aorta were selected 100 µm apart and examined qualitatively for the presence or absence of atherosclerotic lesions.

Micrographs of stained sections were obtained using a digital microscope eyepiece (Premiere MA 88) fitted to a Leica Galen III light microscope. The digital eyepiece was connected to a computer and images from the microscope were captured using Microsoft Publisher software 2003 version. Images were analysed with Photoshop CS 4 (Adobe Systems, San Jose, CA, 2008).

A stage graticule (Nikon, Japan) was used to calibrate the ruler tool in Photoshop by mounting it onto the stage of a microscope and a micrograph, taken using the digital eyepiece, was connected to the ×10 objective lens. Intima–media thickness was measured using the ruler tool in Photoshop. Two lines
(DD and EE) perpendicular to each other were drawn across the image of the artery through the centre, as shown in Fig. 1. Intima–media thickness readings were taken between points 1/2 and 3/4 on line EE and the average was calculated and recorded.

**Statistical analysis**

The results of the study were analysed using Graphpad Prism software (3.0). The t-test was used to compare the means of two groups, while one-way ANOVA was used to compare the means of three groups. Inherent in the Graphpad Prism is the F-test for variances to justify the t-test and the Bartlett’s test to justify ANOVA. A p-value less than 0.05 was considered to be statistically significant. Bonferroni’s multiple comparison test was done to show actual differences between the three groups.

**Results**

After feeding the female rabbits with a 0.5% cholesterol diet, the mean total plasma cholesterol of the HC and HCC groups were significantly higher than baseline concentrations. Cholesterol levels increased three-fold from the initial concentration for both groups after two weeks of treatment, as shown in Fig. 2A. In the HC group, total cholesterol levels increased from 2.38 (SD 0.81) to 7.33 (SD 1.93) mmol/l and that of the HCC group increased from 2.35 (SD 0.68) to 7.40 (SD 2.05) mmol/l. Cholesterol level of the control rabbits was unchanged at 1.8 (SD 0.28) before and after the same two-week period.

The mean total cholesterol levels of the offspring of the NC, HC and HCC rabbits were 9.47 (SD 1.56), 6.73 (SD 0.87) and 3.60 (SD 0.66) mmol/l, respectively (Fig. 2B). Statistical analysis showed significant differences between the total cholesterol levels of offspring of the different treatment groups (p = 0.0002, F = 23.1 and df = 2). Bonferroni’s multiple comparison test indicated significant differences between the NC and HC groups (p < 0.05), the NC and HCC groups (p < 0.0001), and the HC and HCC groups (p < 0.05).

Histological sections of the aortic arch of the rabbit offspring showed intima–media thicknesses of 58.5 (SD 6.02) µm for the NC, 146 (SD 18.24) µm for the HC and 99 (SD 4.87) µm for the HCC groups. ANOVA showed significant differences (p < 0.0001, F = 149.2 and df = 2) between the intima–media thickness of the aortic arch between the three groups, as shown in Fig. 3. Bonferroni’s multiple comparison test showed significant differences between the NC and HC groups (p < 0.001), the NC and HCC groups (p < 0.001), and the HC and HCC groups (p < 0.001).

Histological sections of the aortic arch revealed intimal lipid accumulations or lesions on all five sections per pup in the HC group (100%), whereas no lesions were observed on any of the five sections per pup from the NC and HCC groups (Fig. 4).
the descending thoracic segment of the aorta, again no lesions were found on sections from the NC group, as shown in Fig. 5. Lesions were present on 40% of the sections from the HC group and 20% of the sections from the HCC group. In the abdominal aorta, no lesions were present in any section of the three groups of rabbit pups.

**Fig. 3.** (A) shows micrographs of H & E-stained sections of the aortic arch of rabbit pups born to the control rabbits (NC), hypercholesterolaemic rabbits without cocoa (HC) and hypercholesterolaemic rabbits given cocoa (HCC). The value of the scale bar is 140 µm. (B) shows a bar chart of the intima–media thickness of the aortic arch of offspring from the three groups. *p < 0.001 and #p < 0.001 compared to the HC group. Error bars indicate standard deviation.

**Fig. 4.** Micrographs at different magnifications of Oil red O-stained sections of the aortic arch of offspring. (A) shows a whole section of the aortic arch. (B) and (C) show higher magnifications of the aortic arch and lesions (indicated with arrows). Sections from the NC and HCC pups show no lesions, whereas sections from the HC pups show the presence of atherosclerotic lesions.
Increased deposition of collagen and smooth muscle in vascular walls is associated with advanced atherosclerosis, and so collagen and elastic fibre deposition were assessed by staining with VVG. Collagen and elastic fibres within the intima of the blood vessels stain blue-black and red/pink, respectively. Sections with more pink and black stain represent increased collagen and elastic fibres, as shown in Fig. 6.

Sections from the arch and abdominal aorta showed more deposition of collagen and elastic fibres in the intima of pups born to hypercholesterolaemic mothers in the HC group, but less in those born to hypercholesterolaemic mothers fed on cocoa (HCC). Collagen and elastic fibre content of sections from the thoracic aorta did not show obvious differences between the two groups (Fig. 6).

Discussion

The causal role of maternal hypercholesterolaemia in foetal atherogenesis has been established and evidence suggests that both lipid-lowering and antioxidant interventions during pregnancy may inhibit atherosclerosis. Previously, researchers seldom considered maternal hypercholesterolaemia to promote atherosclerosis in offspring because the placenta was thought to be impermeable to cholesterol and also because cholesterol levels of newborns were not correlated with maternal hypercholesterolaemia. However, results from later studies have shown that serum cholesterol level is increased in early foetal life, and the Fate of Early Lesions in Children (FELIC) study showed that increased maternal cholesterol levels enhanced the formation of fatty streaks in foetuses. Although with age, cholesterol levels in foetuses decrease, maternal hypercholesterolaemia increases the progression of atherosclerosis later in life.

In this study, plasma cholesterol levels were significantly high in offspring of normocholesterolaemic as well as hypercholesterolaemic rabbits. Offspring of hypercholesterolaemic mothers that received cocoa powder, however, showed reduced cholesterol levels compared to the other two groups, suggesting that consumption of cocoa during pregnancy may
reduce plasma cholesterol levels in the offspring of rabbits. It is not clear from this study the mechanism by which cocoa reduced plasma cholesterol levels of the offspring, however, this could have been due to antioxidant activity, as has already been shown by Napoli and colleagues.\footnote{6,26}

Interestingly, plasma cholesterol level was highest in offspring of normocholesterolaemic rabbits and this may have been because pregnancy induces a temporary condition of hypercholesterolaemia necessary for the normal development of the foetus.\footnote{18} However, beyond certain physiological levels, this condition may lead to the development of certain pathologies in both the mother and foetus.\footnote{32}

Although the placenta was thought to be impermeable to lipoproteins, Frantz \textit{et al.}\footnote{33} showed that this may not be the case, and results from our study also suggest cholesterol possibly crossed the placenta into the foetus. This is consistent with earlier observations that foetal cholesterol levels showed an inverse correlation with maternal cholesterol level during the first six months of life.\footnote{56} Increased cholesterol levels in offspring of normocholesterolaemic mothers did not, however, result in atherosclerotic lesion formation in this study.

Maternal hypercholesterolaemia in rabbits led to the formation of atherosclerotic lesions in the aortic arch and descending thoracic aorta of offspring, as shown by increased intima–media thickness and the presence of lesions in sections of the aorta. Increased intima–media thickness of arteries, a consequence of pathological vascular remodelling, is associated with several risk factors for atherosclerosis, including age, systolic blood pressure, diabetes, LDL cholesterol, smoking and familial hypercholesterolaemia in humans.\footnote{34} The intima–media thickness is also increased in hypercholesterolaemic patients compared to controls,\footnote{35} and has become a convenient marker for atherosclerosis.\footnote{36}

Oxidation of LDL cholesterol is key in the initiation of atherosclerosis\footnote{7} and may also trigger vascular remodelling by enhancing smooth muscle cell proliferation and collagen deposition, leading to increased intima–media thickness. Increased deposition of elastic and collagen fibres was observed in the present study, shown by VVG-stained sections of aortic arch and thoracic aorta, suggesting increased atherogenesis, as shown previously.\footnote{37} Moreover, maternal hypercholesterolaemia has already been shown to increase the presence of collagen

<table>
<thead>
<tr>
<th>Aortic arch</th>
<th>Thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>20μm</td>
<td>20μm</td>
<td>20μm</td>
</tr>
</tbody>
</table>

![Fig. 6. Histomicrographs of Verhoeff–Van Gieson-stained sections of the arch, thoracic and abdominal segments of the aorta from offspring of NC, HC and HCC rabbits. Elastic fibres are stained blue-black or black and collagen fibres are stained red or pink.](image)
fibres in the intima–media of coronary arteries. The association of cocoa intake with a smaller number of aortic sections with atherosclerotic lesions, as well as reduced collagen and elastic fibre staining in the rabbits may be explained by inhibition of LDL oxidation and reduction in oxidative stress through high antioxidant and anti-inflammatory activity. 

Maternal hypercholesterolaemia may also trigger the formation of fatty streaks and atherosclerosis in offspring by enhancing endothelial dysfunction, a key event in the initiation and progression of atherosclerosis. It is known that flavanol-rich cocoa inhibits endothelial dysfunction, and hence the initiation of atherosclerosis during foetal development.

Conclusion

Regular consumption of cocoa powder during pregnancy reduced atherogenesis and intima–media thickening in pups of hypercholesterolaemic rabbits by inhibiting lesion formation and the deposition of elastic and collagen fibres in the aorta.

We acknowledge the College of Health Sciences, University of Ghana for providing financial support for this study and technicians from the departments of Anatomy, Medical Biochemistry and Pharmacology, University of Ghana, for their assistance. We also acknowledge Noguchi Memorial Institute of Medical Research, Accra for their support.

References

25. Cordero-Herrera I, Martin MA, Goya L, Ramos S. Cocoa flavonoids attenuate high glucose-induced insulin signalling blockade and modu-


