Original Article

Carbohydrate Restriction Reduces Lipids and Inflammation and Prevents Atherosclerosis in Guinea Pigs

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Aim: There is limited information on how dietary carbohydrate restriction (CR) or the combination of dietary cholesterol (chol) and CR may affect atherosclerosis development. Guinea pigs were used to evaluate the effects of chol and CR on aortic cholesterol accumulation, mechanical properties of aortas and cytokine production.

Methods: Ten male guinea pigs were fed either low (L) or high (H) chol in combination with CR or high carbohydrate (control) for 12 wk.

Results: Groups fed the high chol (control-H and CR-H) had significantly higher concentrations of cholesterol in aortas and higher activity of serum phospholipase A₂ than the L groups. CR resulted in significantly lower concentrations of small LDL particles and aortic cytokines and chemokines than the control groups. Aortas from the control-H and the CR-L were stiffer than those from the control-L and the CR-H groups. This finding could be explained by the reduction in arterial stiffness during the early stages of atherosclerotic.

Conclusion: these results demonstrate that CR has a major impact on atherogenicity.

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Key words; Atherosclerosis, Cytokines, Aorta stiffness, Low-carbohydrate diet

Introduction

Atherosclerosis is the most common pathologic process leading to cardiovascular disease (CVD)¹. Although the earliest visible lesion in the development of atherosclerosis is the fatty streak, it is widely recognized that atherosclerosis is a chronic inflammatory disease rather than simply a lipid accumulation problem². The hydrolysis of phospholipids by the enzyme secretory phospholipase A₂ (sPLA₂) is one of the earliest steps in the production of the inflammatory process^{3,4}. This enzyme is responsible for the production of arachidonic acid, which is the precursor of different inflammatory compounds, such as cytokines^{3,4}. These compounds are implicated in the chemoattraction of

monocytes, the proliferation of inflammatory cells and the induction of their own secretion, favoring the onset and progression of atherosclerosis².

In advanced stages of atherosclerosis, the chronic inflammation and the deposition of calcium and atherogenic products, such as lipids and advanced glycosylated end-products (AGEs), lead to the development of atherosclerotic plaques and increase the stiffness of the arterial wall⁵⁻⁷. This increase in arterial stiffness, associated with the atherosclerotic process, has already been described as a predictor of the morbid-mortality of cardiovascular events (myocardial infarction and stroke)⁷. Thus, the arterial stiffness is an important parameter to be considered in studies focusing on the effect of interventions to prevent and/or treat atherosclerotic disease.

One of the most common interventions proposed to prevent the development of atherosclerosis is dietary modification. A number of studies demonstrated that different types of diet can prevent atherosclerosis and reduce the risk for cardiovascular diseases^{8,9}. One

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example is the carbohydrate restricted diet (CR), which has been effective in reducing plasma triglycerides (TG), increasing HDL-cholesterol (HDL-C) and decreasing the formation of the more atherogenic, small dense LDL particles⁸. In relation to inflammation, CR has been shown to reduce plasma levels of different types of cytokines (e.g. tumor necrosis factor- α , interleukin-6 and C-reactive protein)⁹. In summary, it seems that CR has a hypolipemiant and anti-inflammatory effect. Therefore, it is possible that CR can reduce the lipid accumulation in arterial wall and prevent deleterious effects in the aorta's mechanical properties. Our lab group has shown that guinea pigs are an adequate model for the study of atherosclerosis and inflammation¹⁰⁻¹². The aim of this study was to evaluate the effect of CR on the factors that contribute to the development atherosclerosis, such as inflammation, lipid accumulation in aorta, sPLA₂ activity, and arterial mechanical properties (stiffness and energy absorption capability) in guinea pigs.

Materials and Methods

Diets

Four experimental diets designed to meet the nutritional requirements of guinea pigs were prepared by Research Diets (New Brunswick, NJ). Diets varied in the amount of macronutrients and the concentration of dietary cholesterol. Vitamins, minerals, and fiber were adjusted for the increased caloric content of the CR diets. The composition was as follows: the high carbohydrate-low cholesterol diet (control-L) and the high carbohydrate-high cholesterol diet (Control-H) had the following energy contribution: 25.5% protein: 20.2% fat and 54.3% carbohydrate. The carbohydrate restricted-low cholesterol (CR-L) and the carbohydrate restricted-high cholesterol (CR-H) diet had the following energy contribution: 30% protein, 60% fat and 10% carbohydrate. The amount of cholesterol was either low (0.04 g/100 g) or high (0.25 g/100 g), equivalent to 300 mg/d or 1,800 mg/d in the human situation, respectively¹³. The high cholesterol was given to develop atherosclerosis in guinea pigs¹².

Animals

Forty male adult guinea pigs weighing between 800-1,000 g were assigned to the four dietary treatments (10 per group). Guinea pigs were housed individually in a metal cage in a light cycle room (light from 07:00-19:00 h) and had free access to water. Food consumption was monitored every other day, and guinea pigs were weighed weekly to ensure stable weight during the 12 wk of treatment. Guinea pigs

were deprived of food for 12 h and sacrificed by heart puncture after isoflurane anesthesia. Blood was collected and plasma separated by centrifugation (200x g) and aortas were harvested and stored at -80°C for further analysis. Animal experiments were conducted in accordance with U.S. Public Health Service/U.S. Department of Agriculture guidelines. Experimental protocols were approved by the IACUC.

Aortic Lipids

A section of thoracic aorta suspended in formalin was thoroughly cleaned of any excess tissue and fat. Aortic cholesterol concentrations were analyzed as previously reported¹¹. From the cleaned tissue, aortic lipids were extracted from 0.05 g of aortic arch tissue using 10 mL of chloroform: methanol (2:1) overnight at RT. The extraction solution was mixed with acidified water to separate into two phases, which were then filtered by gravity filtration. The lower phase was separated and an aliquot of 200 μL was evaporated completely and reconstituted with 200 μL ethanol for enzymatic determination of free cholesterol¹².

Plasma Lipids and Small Dense LDL

Plasma total cholesterol and LDL-C were measured by enzymatic methods and small dense LDL by nuclear magnetic resonance as previously reported¹⁴.

Aortic Cytokines

The section of ascending aorta, aortic arch, and the upper portion of the descending aorta were processed for cytokine determination. A clean portion of aorta was rinsed with phosphate buffered saline and then cut into small segments and homogenized in 1 mL of lysis buffer in a rotor-stator (VirTis, Gardiner, NY) on ice for 60-90 sec to ensure complete tissue disruption followed by use of a Potter-Elvehjem homogenizer for additional 60 sec. The homogenized tissue was centrifuged at 400x g for 10 min at 4°C and the supernatant collected and stored at -80°C . Twenty-two aortic cytokines were analyzed using a mouse cytokine/chemokine multiplex immunoassay (Lincoplex, Linco Research, St. Charles, MI). Seventeen of the proinflammatory compounds showed good cross-reactivity in the aorta of the guinea pigs including 2 chemotactic cytokines (chemokines): monocyte chemotactic protein-1, (MCP-1) and chemokine C-C motif ligand 5 (RANTES) and 13 inflammatory cytokines: tumor necrosis factor- α (TNF- α), interferon- γ (INF- γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukins 1 β , 2, 4, 6, 7, 9, 12, 13 and 15 (IL-1 β , IL-2, IL-4, IL-6,

IL-7, IL-9, IL-12, IL-13 and IL-15, respectively). Briefly, 25 μL of sample were added into the wells of a microplate and mixed with 25 μL of mixed beads. The microplate was sealed and covered with aluminum foil and incubated with agitation on a plate shaker overnight (16-18 h) at 2-8°C. Next, the fluid was gently removed by vacuum filtration and the microplate was washed two times with buffer, 200 μL /well. Subsequently, 25 μL of antibody detection cocktail was added to each well, and the plate was sealed and covered with aluminum foil and incubated with vigorous agitation at RT for 60 min. Later, 25 μL of streptavidin-phycoerythrin was added to each well and the plate was sealed, covered, and incubated with agitation at RT for 30 minutes. The content of each well was gently removed by vacuum filtration and the plate was washed 3 more times with 200 μL /well of wash buffer. Finally, 100 μL of sheath fluid was added to all wells and the wells were sealed, covered, and shaken for 5 minutes. The plates were read on a Luminex instrument (Luminex® 200™ System, Austin, TX.).

Secretory Phospholipase A₂ (sPLA₂) Activity

The activity of sPLA₂ was measured in plasma using Cayman kits (Cayman Chemicals, Ann Arbor, MI). Briefly, 10 μL of plasma sample were placed with 10 μL of DTNB and 5 μL of assay buffer in a microplate well. Subsequently 200 μL of substrate solution was added and the microplate was carefully shaken. Finally the absorbance at 415 (or 404) nm was read every minute to obtain at least 5 points. Two points on the linear part of the curve were selected and the change in absorbance during that time was determined using the following equation:

$$\Delta A_{414} = (A_{414} (\text{Time } 2) - A_{414} (\text{Time } 1)) / (\text{Time } 2 - \text{Time } 1).$$

The following formula to determine sPLA₂ activity was used:

$$\text{sPLA}_2 \text{ activity} = \Delta A_{414} / \text{min} / 10.66 \text{ mM}^{-1} * 0.225 \text{ mL} / 0.01 \text{ mL} * \text{sample dilution}.$$

$$\text{sPLA}_2 \text{ activity was expressed as } \mu\text{mol/min/mL}^{15}.$$

Aorta Stiffness

Ring specimens for tensile testing were excised from the proximal portion of descending thoracic aorta and were immersed in saline solution at 4°C. The cross-sectional area was calculated as follows: cross-sectional area = 2 × thickness × width. A digital caliper was used to obtain the width of each ring and three-point bend clamps were used in DMA 2980 (Dynamic Mechanic Analyzer) to measure the vessel thickness with a minimum of pre-load force. The artery wall was considered incompressible for this

analysis.

Determination of aorta stiffness was done according to Nosaka *et al.*¹⁶. Briefly, two stainless steel rods were inserted through the lumen of an aorta in a parallel fashion while the vessel was immersed in a saline solution at RT. One rod was attached to a motorized Controller (DMA 2980). This apparatus enabled us to stretch the vessel at a constant tensile force (0.01 N) while vessel tension was recorded via a force transducer until breakage. Stress (tension per cross-sectional area: F/A) vs. strain (fractional change in vessel width: ((w-wo)/wo)) curves were generated. Because the obtained curves were non-linear, the elastic modulus was calculated for each point of the graph and modulus was plotted versus stress obtaining a straight line, such as suggested Hayashi *et al.*¹⁷. Thus the slope of each line demonstrates how much the modulus increases per unit of stress, which is an indirect measurement of aorta stiffness¹⁷. In addition, the area under the stress-strain curve was calculated by the integral of the curve. This area represents the amount of energy that the aorta can absorb until its breakage. The absorbed energy provides an estimate of the maximum volume of blood that the aorta can accommodate before rupture.

Statistical Analysis

Two-way ANOVA was used to evaluate carbohydrate and cholesterol effects and the interactions on lipids accumulated in the aorta, sPLA₂ activity, cytokines and aorta stiffness. $P < 0.05$ was considered significant.

Results

Plasma and Aortic Lipids

The levels of cholesterol accumulated in the aorta were different between the high cholesterol groups (Control-H and CR-H) and the low cholesterol groups (Control-L and CR-L) ($p < 0.05$). The high cholesterol groups had higher concentrations of cholesterol accumulated in the aorta (Control-H: 7.3 ± 3.11 and CR-H: 6.4 ± 2.11 mmol/g) than the low cholesterol groups (Control-L: 5.4 ± 3.16 and CR-L: 4.3 ± 1.06 mmol/g). These results clearly indicate that the cholesterol accumulation in the aorta was affected mainly by dietary cholesterol. The same effect was seen for triglycerides accumulated in the aorta. Thus, the high cholesterol diets caused higher plasma triglyceride (Control-H: 34.1 ± 11.1 and CR-H: 31.9 ± 5.41 mg/g) accumulation than the low cholesterol diets (Control-L: 20.6 ± 14.3 and CR-L: 13.6 ± 7.64 mg/g) ($p < 0.001$).

It is well known that cholesterol accumulation in

Fig. 1. Correlation between plasma LDL cholesterol and cholesterol accumulated in the aorta for all guinea pigs.

the aorta is closely linked to high levels of plasma LDL-C. In general it is well accepted that the higher the levels of LDL-C, the higher the cholesterol accumulated. Plasma LDL cholesterol concentrations were higher in the H groups (2.94 ± 0.70 and 2.47 ± 0.47 mmol/L for Control-H and CR-H, respectively) compared to the L groups (1.37 ± 0.55 and 1.45 ± 0.53 mmol/L, for Control-L and CR-L respectively) ($p < 0.01$). It was also observed that as the LDL-C levels increase in plasma, the cholesterol accumulated in the aorta also increases. They present a positive correlation between these two parameters ($r = 0.63, p < 0.01$ – **Fig. 1**). The concentration of small dense LDL subfractions for the 4 groups is shown in **Fig. 2**. Guinea pigs fed the CR diets had lower concentrations of small dense LDL than those fed the Control diets.

Aortic Cytokines

Fifteen proinflammatory cytokines were detected in the aorta of guinea pigs. All of these inflammatory markers were found to have a significant carbohydrate effect (**Table 1**). The high carbohydrate groups had a higher concentration of aortic cytokines than the CR groups, regardless of the level of cholesterol in the diet.

Secretory Phospholipase A₂ (sPLA₂) Activity

Guinea pigs fed the high cholesterol diets had higher plasma sPLA₂ activities compared with guinea pigs fed the low cholesterol diets ($p < 0.05$) (**Fig. 3**). These results indicated that dietary cholesterol and lipid accumulation in the aorta were the main stimulators of sPLA₂ activity.

Aorta Stiffness

The strain, which is the deformation of the artery,

Fig. 2. Concentrations of small dense LDL subfractions fed 54.6% energy from carbohydrates with low (0.04 g/100 g) (control-L) or high (0.25 g/100 g) dietary cholesterol (control-H) or 10% energy from carbohydrates (carbohydrate restricted, CR) with low (CR-L) or high (CR-H) cholesterol.

*, ** and *** indicate significantly different at $p \leq 0.05$.

and the stress, which is the force applied per cross-sectional area were measured to determine the stiffness of the artery. When we plotted stress vs. strain it was observed that the arteries of the CR-L and Control-H groups were stiffer than the arteries of the CR-H and Control-L. This indicates that in order to cause the same degree of deformation in the arteries, more force needs to be applied in the CR-L and Control-H groups (data not shown). However, the stress vs. strain curve is just a general representation of the mechanical behavior of the arteries. A better representation of the properties of the aortas is generating a curve of modulus vs. the stress because it is easier to visualize the stiffness of soft materials such as the arteries (**Fig. 4**). In concordance with the strain-stress curve, the Control-H and CR-L groups had higher elastic modulus/stress or in other words we may say that were stiffer than the Control-L and CR-H groups ($p < 0.05$). However, aortas from Control-H group demonstrated worse energy absorption capability when compared with the CR groups. In addition, the groups that received Control diet presented significantly lower capability to absorb energy than those that received CR (**Fig. 5**). Finally, the groups that received H presented a trend in reducing the capacity to absorb energy compared with those that received L ($p = 0.17$).

Discussion

Guinea pigs have been shown to be a good ani-

Table 1. Concentrations of stimulating factors and cytokines in aortas

Parameter	Low Carbohydrate (pg/g)		High Carbohydrate (pg/g)		Carbohydrate Effect
	Low Chol	High Chol	Low Chol	High Chol	<i>p</i> -value
IL-1 β	92.1 $\pm$ 19.3	116.1 $\pm$ 7.0	194.6 $\pm$ 52.6	261.6 $\pm$ 24.3	0.007**
IL-2	64.5 $\pm$ 16.7	63.4 $\pm$ 5.9	107.4 $\pm$ 29.1	122.5 $\pm$ 6.5	0.048*
IL-4	116.2 $\pm$ 25.8	137.4 $\pm$ 7.1	253.6 $\pm$ 69.0	290.8 $\pm$ 29.9	0.014*
IL-7	100.3 $\pm$ 21.9	133.9 $\pm$ 10.1	244.9 $\pm$ 67.8	293.5 $\pm$ 34.2	0.006**
IL-6	103.1 $\pm$ 22.0	126.8 $\pm$ 7.7	195.6 $\pm$ 47.6	269.4 $\pm$ 34.9	0.006**
IL-9	100.6 $\pm$ 22.3	110.3 $\pm$ 7.8	193.3 $\pm$ 52.0	257.7 $\pm$ 20.2	0.008**
IL-12	109.5 $\pm$ 22.5	147.8 $\pm$ 9.6	253.3 $\pm$ 68.7	307.5 $\pm$ 27.4	0.009**
IL-13	108.1 $\pm$ 25.4	105.1 $\pm$ 11.1	213.1 $\pm$ 61.2	268.1 $\pm$ 18.1	0.011*
IL-15	99.9 $\pm$ 20.0	122.5 $\pm$ 7.4	225.4 $\pm$ 63.2	290.3 $\pm$ 31.2	0.007**
IFN- γ	85.5 $\pm$ 19.1	86.6 $\pm$ 5.6	149.3 $\pm$ 40.1	196.0 $\pm$ 14.8	0.014*
TNF- α	92.1 $\pm$ 20.4	116.2 $\pm$ 9.0	207.4 $\pm$ 55.6	249.7 $\pm$ 28.4	0.010**
GM-CSF	93.6 $\pm$ 20.7	117.1 $\pm$ 10.5	212.7 $\pm$ 56.4	249.5 $\pm$ 30.9	0.011*
G-CSF	119.8 $\pm$ 26.4	152.1 $\pm$ 10.9	253.4 $\pm$ 70.6	320.9 $\pm$ 31.7	0.014*
MCP-1	73.3 $\pm$ 17.8	90.2 $\pm$ 9.5	153.8 $\pm$ 41.1	190.4 $\pm$ 18.6	0.011*
RANTES	101.7 $\pm$ 22.2	104.0 $\pm$ 10.7	186.5 $\pm$ 48.1	261.0 $\pm$ 23.5	0.006**

Values are presented as mean $\pm$ SEM for $n=10$ guinea pigs per group. *for $p \leq 0.05$ and **for $p \leq 0.01$.

Fig. 3. Concentrations of sPLA₂ of guinea pigs fed 54.6% energy from carbohydrates with low (0.04 g/100 g) (Control-L) or high (0.25 g/100 g) dietary cholesterol (Control-H) or 10% energy from carbohydrates (carbohydrate restricted, CR) with low (CR-L) or high (CR-H) cholesterol.

*, ** and *** indicate significantly different at $p \leq 0.05$.

mal model for the study of atherosclerosis and inflammation¹⁰. Because human studies over the last decade have shown that CR diets improve plasma lipid profiles and reduce atherogenic lipoproteins, we used guinea pigs in the current study to evaluate the effects of CR on the different aspects of the onset and progression of atherosclerosis, such as: lipid accumulation in the aorta, sPLA₂ activity, inflammation, and arterial stiffness¹⁸.

Fig. 4. Curves of modulus/stress of the aortas of guinea pigs fed 54.6% energy from carbohydrates with low (0.04 g/100 g) (Control-L) or high (0.25 g/100 g) dietary cholesterol (Control-H) or 10% energy from carbohydrates (carbohydrate restricted, CR) with low (CR-L) or high (CR-H) cholesterol.

* for $p \leq 0.05$.

Cholesterol Effects

One of the major causes of atherosclerosis is lipid accumulation in the aorta. It is accepted that hypercholesterolemia is related to the onset and progression of atherosclerosis and that plasma LDL-C concentrations is the major contributor¹⁹. In agreement with these observations, in the current study we observed that high cholesterol diets not only caused elevated LDL-C levels but also increased the cholesterol accu-

Fig. 5. Area under the stress-strain curves, of guinea pigs fed 54.6% energy from carbohydrates with low (0.04 g/100 g) (Control-L) or high (0.25 g/100 g) dietary cholesterol (Control-H) or 10% energy from carbohydrates (carbohydrate restricted, CR) with low (CR-L) or high (CR-H) cholesterol.

*, ** and *** indicate significantly different at $p \leq 0.05$.

modulated in the aorta. The latter variables were significantly correlated.

High cholesterol diets also affected TG accumulation in the aorta. Surprisingly, a high carbohydrate intake did not show such effect. Usually, carbohydrate effect on TG accumulation result from a higher caloric intake associated with high carbohydrate diets. In the present study, however, the caloric intake was not different among groups, which may explain the lack of carbohydrate effect. Thus, the explanation for the fact that the highest aortic TG accumulation occurred in H groups is based in the lipoprotein profile. LDL, which is a TG rich lipoprotein, is the most important molecule that accumulates in arteries. Especially after the action of sPLA₂, LDL particles become smaller and denser, presenting higher affinity to the extracellular matrix (ECM). This fact favors the lipoprotein trapping in arterial wall and the formation of foam cells²⁰. Finally, since LDL particles carry both CHOL and TG, the increase in CHOL and TG in aorta is explained by the same factor: the higher concentrations of LDL in plasma of animals that received H.

It is known that sPLA₂ is mainly produced and secreted by the proinflammatory cells macrophages/monocytes, mast cells, and T lymphocytes, which play a critical role in the onset and advance of atherosclerosis²¹. sPLA₂ participates in the hydrolysis of membrane phospholipid-derived inflammatory mediators such as arachidonic acid, which is involved in the synthesis of platelet-activating factor and prostaglandins, important components at the beginning of the inflammatory cascade^{19, 22}. Moreover, sPLA₂ hydrolyzes oxi-

dized phospholipids, generating lysophosphatidycholine and free oxidized fatty acids²³. Thus, this enzyme is responsible for most of the increased lysophosphatidycholine content of oxidized LDL particles. This is the first study in guinea pigs showing increased sPLA₂ activity by high cholesterol diets.

An interesting observation is that the groups with higher sPLA₂ activities also had higher cholesterol accumulated in the aorta, suggesting that sPLA₂ could have been produced by those inflammatory cells located in the area of lipid accumulation.

Although the sPLA₂ activity was greater in H groups, the inflammation is higher in Control groups. Hence, while it seems that the inflammation in H groups was modulated by the action of sPLA₂, Control seems to promote inflammation through other pathways, such as AGEs stimulation²⁴⁻²⁶. This is consistent with the fact that this enzyme hydrolyzes lipids. Thus, the greater the lipid accumulation, the greater the sPLA₂ activity. Future studies should be done to uncover the pathways for increased inflammation promoted by high carbohydrate intake.

Carbohydrate Restriction Effects

One of the major determinants that characterize atherosclerotic inflammation is high production of cytokines and chemokines by cells of the immune system as well as endothelial and smooth muscle cells¹⁹. High levels of cholesterol are associated with the development of atherosclerosis in animal models and in human studies, and it is therefore reasonable to expect the primary trigger of cytokine release could have been linked with the cholesterol accumulated in the aorta^{27, 28}. However, in this study we found that a CR had a robust effect on reducing cytokine expression in the aorta, and the effect was independent of dietary cholesterol. It is well known that different subfractions of LDL have different atherogenic potential²⁹. Smaller LDL particles are more atherogenic than the larger ones due to their longer plasma residence time and because their size allows them to penetrate the intima more readily where the atherosclerosis process begins³⁰. Small dense LDL also has lower antioxidant content and is more susceptible to oxidation³¹. Because of the increased concentration of small dense LDL in guinea pigs fed the high carbohydrate diets (Controls), there could have been greater penetration of these smaller LDL particles into the arterial wall where they became oxidized. There is sufficient evidence that oxidized LDL particles are potent inflammatory agents and possess a chemoattractant effect on monocytes and lymphocytes in addition to promoting the differentiation of monocytes into macrophages^{27, 32}. This could be an

explanation why the high carbohydrate groups had higher levels of cytokines and chemokines in the aorta.

Another possible explanation of why high carbohydrate diets increased cytokines levels in the aorta could be linked to the reactivity of glucose with any proteins or lipoproteins. Glucose forms chemically irreversible glycated products with reactive amino groups of circulating lipoproteins or vessel wall proteins, generating advanced glycosylation end products (AGEs)³³. These glycosylation AGEs can induce modification of LDL particles and the vascular proteins and thus increase their atherogenicity or their normal functionality, respectively.

Finally, the third possible mechanism associated with the increase in inflammatory markers in the Control groups is the glycation of LDL particles. Glycosylation in the apoB of LDL occurs within the lysine residues, where the LDL receptor domain recognition is located²⁶. Thus, glycated LDL are poorly recognized by the LDL receptor and removed from circulation by nonspecific receptor present on macrophages, lymphocytes and smooth muscle cells²⁴. In addition, the binding of AGE and LDL facilitates the oxidation of this lipoprotein, which stimulate the production of inflammatory cytokines^{25, 26, 34, 35}. The different mechanisms aforementioned can account for the observation that Control diets contribute to the increase in aortic cytokine levels.

Effects of Diet on Aorta's Mechanical Properties

In the present study, we also indirectly evaluated the stiffness of the thoracic aorta and found that CR affected this parameter. When strain vs. stress—two important parameters in the determination of the stiffness of any material—was plotted, we observed that the arteries of the different dietary groups had different elastic properties. According to these curves we observed that the arteries from the CR-L and the Control-H groups were stiffer because they required more force per unit of area (stress) than the arteries of the Control-L and the CR-H groups to cause the same deformation (strain). This observation was also seen when a graph of modulus vs. stress was plotted (**Fig. 4**). The lines with the more pronounced slopes indicate stiffer arteries confirming that the Control-H and CR-L are stiffer since they have similar slopes.

The similar artery stiffness of the CR-L and Control-H groups might seem surprising in light of the previously observed finding that greater aorta stiffness is observed in advanced stages of atherosclerosis. However, studies conducted in diabetic rats and hypercholesterolemic-fed rabbits have shown that the relationship between atherosclerosis and arterial stiffness

is not linear^{36, 37}. Both studies mentioned that the normal artery is stiffer than the artery at the beginning of the atherosclerosis process. This is because at the onset of atherosclerosis, the lipid accumulation and the foam cell formation start causing arterial remodeling³⁸. As a result of the remodeling process, there is an increase in the activity of metalloproteinases and collagenases, which in turn degrades the collagen and the elastin of the arteries, increasing arterial compliance^{38, 39}. However, in the event of prolonged diabetes or challenge with hypercholesterolemic diets, there is a higher increase in the rate of collagen synthesis (changing the collagen/elastin ratio) and an increase in the proliferation of smooth muscle cells, which in turn increases the stiffness of the artery^{17, 40}. Therefore, the atherosclerosis process in relation to stiffness follows a parabolic curve as we indicated in **Fig. 5**. Hence, we may conjecture that even when the arterial stiffness of the CR-L and Control-H groups was similar, they are at different stages, with the CR-L group being at the normal stage or at the beginning of the atherosclerosis process, and the Control-H at the advanced stage. In the same way, because the Control-L and CR-H were similar in stiffness but softer than the other two groups, we may surmise that these two groups are located between the initial and the advanced stages of atherosclerosis.

Our data obtained from the other parameters of atherosclerosis supports our speculations. First, the Control-H group had higher levels of lipid accumulation in the aorta than the CR-L. Second, the Control-H group had higher concentrations of aortic inflammatory cytokines and higher sPLA₂ activity than the CR-L group and third the CR-L group had lower concentrations of small dense LDL. Thus, since the primary trigger of cytokine release is linked to the cholesterol accumulated in the aorta, and since higher sPLA₂ accelerates atherosclerosis, we may conclude that the Control-H was at the more advanced stage. In addition, recent studies have found that high plasma glucose increases the chances that glucose will react with collagen, so inducing the formation of intermolecular cross-links, which have a dramatic effect on the physical properties of the vascular system, and this could account for the markedly increase in arterial stiffness observed in diabetic subjects^{41, 42}. Although it had been suggested that AGEs formation was common only for diabetics, it is recognized that prolonged intake of simple sugars also increases the production of AGEs^{6, 16}. This information supports the idea that the Control-H group was at a more advanced stage of atherosclerosis than the CR-L group. It also indicates that the combination of high carbohydrate intake and

high cholesterol had the most adverse effects on the atherosclerotic process.

Even though stiffness was not different between Control-H and CR-L groups, the evaluation of the area under the curve indicates that the Control-H group presented worse mechanical properties characteristics of the aorta. This area is related with the amount of blood that the aorta can accommodate before the rupture. The greater the area, the greater the capability to accommodate the blood without rupture. The results showed that the amount of energy that the aorta from animals subjected to Control-H can absorb was significantly lower compared with CR-L animals. Therefore, the combination of high carbohydrate and high cholesterol diets favored the worsening of the biomechanical properties of the aorta.

In this study we have demonstrated that high concentrations of dietary cholesterol and high carbohydrate result in key modifications in the arteries involved in the initiation and progression of atherosclerosis. High cholesterol increases accumulation of lipids in the arteries plus increased activity of sPLA₂ while high carbohydrate increases concentrations of aortic cytokines and chemokines plus affects the mechanical properties of the aorta that can be associated with different stages of the atherosclerotic process. Our results also show that CR attenuated the development of atherosclerosis even in the presence of high dietary cholesterol.

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