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Research Article

Moringa *oleifera* Leaves do not Alter Adipose Tissue Cholesterol Accumulation or Inflammation in Guinea Pigs Fed a Hypercholesterolemic Diet

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Abstract

We have previously demonstrated in guinea pigs that *Moringa Oleifera* leaves (*ML*) decrease hepatic lipids and inflammatory cytokines while plasma cholesterol and triglycerides are unaffected. To determine the effects of ML on adipose tissue inflammation and lipid accumulation, twenty- four guinea pigs (n = 8/per group) were randomly allocated to consume either a control diet (0% *ML*), a low moringa diet (LM, 10% *ML*) or a high moringa diet (HM, 15% *ML*) for 6 weeks. All guinea pigs were fed a hypercholesterolemic diet (0.25% cholesterol) to induce systemic inflammation. Plasma, liver and adipose tissue were harvested from guinea pigs at the time of sacrifice. Similar to our earlier findings in plasma, in this study, we observed no differences in cholesterol concentrations in adipose or in inflammatory cytokines among the three dietary groups. Further histologic evaluation revealed that except for 2 guinea pigs who developed inflammation in the adipose tissue, the rest of the guinea pigs did not, suggesting that 6 weeks were not enough time for the accumulating cholesterol to induce an inflammatory state in guinea pigs. We conclude that *ML* although very effective in protecting against hepatic steatosis, had no effect on the concentrations of cholesterol in plasma suggesting that cholesterol was delivered with equal efficiency to the adipose tissue of guinea pigs from control, LM or HM groups through macrophage receptors. Further studies are necessary to evaluate whether a longer feeding time would resolve dyslipidemias and thus decrease cholesterol uptake by the adipose tissue.

Keywords: *Moringa Leaves; Adipose Tissue; Cholesterol; Inflammation; Guinea Pig; Hypercholesterolemic Diet*

Abbreviations

ApoE: Apolipoprotein E; CE: Cholesterol Ester; CHD: Coronary Heart Disease; IL: Interleukin; IFN-γ: Interferon γ; LDLR-R: LDL Receptor; LRP-1: LDL Receptor Related Protein; MCP-1: Monocyte Chemotactic Protein 1; Mø: Macrophage, *ML*: Moringa Leaves; ox-LDL: Oxidized LDL; PPARγ2: Peroxisome Proliferator-Activated Receptor γ2; SR-B1: Scavenger Receptor B-1; SREBP-1: Sterol Regulatory Element Binding Protein-1; TG: Triglycerides; TNF-α: Tumor Necrosis Factor α

Introduction

Adipose tissue has a key role in the development of several metabolic-related diseases, such as atherosclerosis, coronary heart disease (CHD) and type 2 diabetes [1]. Adipose tissue is the main site for triglyceride (TG) and cholesterol storage in humans as well as in other animal species [2]. Excessive accumulation of cholesterol can be toxic to tissues, including heart, aorta, and liver [3-5].

Several studies showed that increased cholesterol accumulation in epididymal adipose tissue impaired adipocyte differentiation and maturation, induced insulin resistance and secretion of pro-inflammatory cytokines, and stimulated macrophage (Mø) recruitment in LDL receptor knock-out mice [4]. In addition, in adipose-derived stromal cells, cholesterol accumulation leads to impairment of adipocyte development and function [6]. Release of cytokines, such as tumor necrosis factor (TNF)-α, results in infiltration of Mø, neutrophils, T-cells, and other immune cells [7]. Obesity leads to hypertrophied adipocytes due to high TG and cholesterol accumulation, resulting in abnormal cellular cholesterol distribution [8,9]. Overall, these features triggered by lipid accumulation are hallmarks of dysfunctional adipocytes [10].

In guinea pigs, we have demonstrated that high hepatic cholesterol concentrations induce the development of the initial stages of nonalcoholic steatohepatitis and fibrosis [11]. Our previous study indicated that Moringa leaves (*ML*) prevented hepatic steatosis and reduced the level of pro-inflammatory cytokines compared to a control diet where no moringa was provided [12]. *ML* have also been shown to reduced lipid accumulation in adipocytes in other animal species [13].

Guinea pigs have several similarities to humans in lipoprotein and cholesterol metabolism and are considered a good model to study diet-induced atherosclerosis [14,15]. The objective of the present study was to evaluate the effects of a high cholesterol dietary challenge on cholesterol tissue accumulation and inflammation in guinea pigs and whether *ML* would protect against these metabolic alterations.

We hypothesized that high dietary cholesterol would induce cholesterol accumulation, and inflammation in adipose tissue and that *ML* would protect against these metabolic abnormalities

Materials and Methods

Experimental Design and Diets

Guinea pigs were allocated to one of these diets: Control (0% Moringa), Low Moringa (10% Moringa) or high Moringa (15% Moringa). All guinea pigs were fed 0.25% dietary cholesterol to induce systemic inflammation as previously reported [12]. Moringa leaves (*ML*) were provided by Scientech Health International (Mexico City, Mexico).

Cholesterol in Adipose Tissue

Adipose tissue (0.12g) was homogenized with 200 µl of chloroform/isopropanol/MP40 (7:11:01). The mixtures were centrifuged and the supernatant dried at 50°C overnight. Samples were resuspended in water and cholesterol was measured using enzymatic assays kits (Wako Diagnostics, Mountain View, CA) according to Carr., *et al* [16].

Inflammatory Cytokine Concentration in the adipose tissue

Tissue total protein was extracted using a radioimmunoprecipitation buffer and total protein concentration of the lysates were determined using the BCA Protein Assay Kit (Cell Signaling Technologies Inc, Beverly, MA) [17]. Using the same concentration of protein for all samples, the following cytokines were measured using Luminex technology (Luminex MAGPIX System, Austin, TX) with the MILLIPLEX MAP Rat Cytokine Immunoassay kit (Millipore corporation, Charles, MO, USA): interleukin (IL)-1β, IL-6, IL-10, interferon-γ (IFN-γ) and monocyte chemotactic protein 1 (MCP-1), as previously described [18].

Histologic evaluation

Samples of adipose tissue were immersed in 10% neutral buffered formalin. Formalin-fixed adipose tissues were paraffin embedded and 4 - 5 µm sections were stained with hematoxylin and eosin. Histologic evaluation was performed on two separate occasions by a veterinary pathologist (JAS) blinded to the treatments. The entire tissue was assessed for evidence of inflammation or other abnormality, and crown-like structures and were quantified in 10 fields at 200X.

Statistical Analysis

Differences between groups were analyzed by one-way ANOVA and Tukey's LSD post hoc analysis. $P < 0.05$ was considered to be significant. All analyses were conducted on SPSS for Windows, Version 20 (IBM Corp.).

Results

Cholesterol accumulation and Inflammatory Cytokines in the adipose tissue

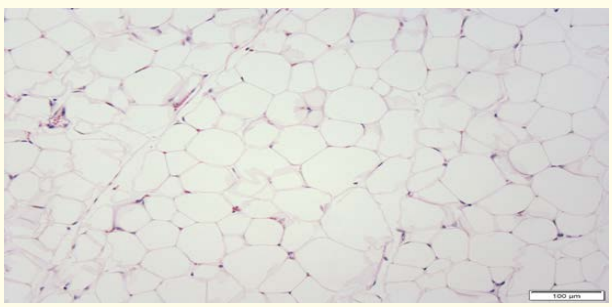
There was no significant difference in the total cholesterol in the adipose tissue among of guinea pigs fed high moringa, low moringa or control group as shown in Table 1. Similarly, there were no significant differences in the inflammatory cytokine concentrations; IL-1β, IL-6, IL-10, IFN-γ, TNFα and MCP-1 among groups of guinea pigs fed high moringa, low moringa or control diets as shown in Table 1.

Parameter	Control	Low Moringa	High Moringa
Total Cholesterol (mg/g)	11.60 ± 1.50	12.77 ± 3.82	10.91± 1.23
Histological CLS scores	0.150 ± 0.18	0.125 ± 0.18	0.175 ± 0.31
IL-1β (ng/g)	30.0 ± 12.37	25.17 ± 7.25	36.17 ± 19.31
IL-6 (ng/g)	16.83 ± 2.32	15.17 ± 2.93	17.33 ± 3.08
IL-10 (ng/g)	40.67 ± 15.31	32.17 ± 12.07	47.50 ± 19.81
IFN-γ (ng/g)	116.33 ± 25.18	95.33 ± 31.87	107.00 ± 24.93
MCP-1(ng/g)	7.00 ± 00.0	7.00 ± 1.10	7.17 ± 0.75
TNFα (ng/g)	14.17 ± 1.17	13.67 ± 2.42	15.00 ± 3.41

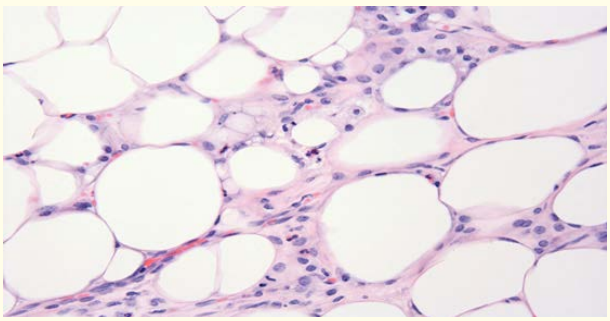
Table 1: *Concentration of cholesterol, and inflammatory cytokines, and CLS scores in adipose tissue of guinea pigs fed a hypercholesterolemic diet (0,25% cholesterol) with no moringa (control), low (10%) or high moringa (15%).*

Histologic evaluation

Adipocytes from all guinea pigs contained abundant lipid irrespective of diet. The majority of the guinea pigs did not have signs of inflammation (Figure 1, Panel A); 2 of the guinea pigs fed the *ML* had inflammation (Figure 1, Panel B). Histological CLS scores are also presented in Table 1.



Panel A



Panel B

Figure 1: *Representative histological section of adipose tissue as observed in the majority of guinea pigs (Panel A). Focal histiocytic inflammation of adipose tissue was observed in two guinea pigs (Panel B).*

Discussion

Cholesterol accumulation in the adipose tissue

Guinea pigs are a good model to study chronic inflammatory diseases. Previous studies showed that a cholesterol challenge can induce atherosclerosis, and hepatic cholesterol accumulation [18,19]. It has been shown that cholesterol synthesis in adipose tissue only accounts for 4% of hepatic cholesterol synthesis [20]. Therefore, most of the cholesterol in the adipocyte is delivered by lipoprotein-mediated mechanisms.

Cholesterol can be delivered into the adipocyte through several receptors including HDL scavenger receptor B1 (SR-B1) [21,22], the LDL receptor and oxidized-LDL (ox-LDL) scavenger receptor [23-25]. HDL-cholesterol removal by SR-B1 takes place in the caveolae, a specific plasma membrane lipid raft where the bound HDL internalizes cholesterol ester (CE) [24]. There is also an SR-B1–independent mechanism via cholesterol ester transfer protein, CE is transferred from HDL to a specific plasma membrane compartment, and then apolipoprotein E (apoE) is secreted into this compartment and acquires CE. Finally, apoE directs CE to the extracellular matrix where it is taken by the LDL receptor–related 1 protein (LRP-1) [23]. Oxidized LDL also enters the adipocyte through cluster of differentiation 36, SR-B1, or ox-LDL receptor 1 [26].

Unexpectedly, no effects of *Moringa* leaves on cholesterol accumulation in the adipose tissue was observed in this study. These results might be related to the lack of effect of ML in reducing plasma LDL cholesterol or oxidized LDL that we previously observed [12], or that the 6-week study period was too short to manifest any beneficial effect.

Inflammatory cytokine concentrations in the adipose tissue

In this study, there were no differences in inflammatory cytokine concentrations in the adipose tissue among groups. In the previous studies, a cholesterol challenge increased levels of Ox-LDL [19,27]. This pro-atherogenic lipoprotein is internalized by SR-B1 and LRP-1 in Mø and smooth muscle cells leading to cell dysfunction and release of pro-inflammatory cytokines and chemo-attractants and formation of foam cells, which are the prominent feature of atherosclerosis [28].

Cholesterol accumulation in adipose tissue has been shown to increase macrophage infiltration associated with high levels of inflammatory cytokines TNF-α and MCP-1. Also, cholesterol accumulation leads to decreased gene and protein expression of sterol regulatory element binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptor γ2 (PPARγ2) resulting in adipocyte dysfunction and blunted cell development [6]. SREBP-1 is known to directly modulate the expression of the PPARγ gene at the transcription level [29], and affect the transcription of several lipogenic genes, important in adipocyte development [30]. Dysfunctional adipocytes are found to be more receptive to TNF-α signaling and subsequent apoptosis, associated with macrophage infiltration and secretion of inflammatory cytokines [4]. None of these metabolic alterations were observed in the 6-week intervention in this study indicating that a longer time of feeding is needed to have the presence of dysfunctional adipocytes associated with the high cholesterol feeding. The increases in cholesterol accumulation and the release of inflammatory cytokines were present in all guinea pigs since the inclusion of *ML* in the diet did not affect the higher cholesterol uptake associated with the dietary intervention [18].

Conclusions

ML had no effect in protecting the adipose tissue against inflammation in guinea pigs fed a high cholesterol diet. It is clear that *ML* exerted its main effects in the liver [12] where substantial reduction in lipid accumulation and in inflammation were observed. The lack of effect of *ML* in reducing plasma cholesterol [12] explains why cholesterol accumulation in the adipose was not reduced leading to inflammation and macrophage infiltration.

Further studies need to be conducted in which *ML* are fed longer periods of time to assess whether plasma lipids could be reduced and therefore cholesterol accumulation in the adipose could also be lowered.

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