In Vivo Effects of Metformin on the Alterations of Bone Microarchitecture Associated with Fructose-induced Metabolic Syndrome in Rats.

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ABSTRACT

Several clinical studies have demonstrated that the Metabolic Syndrome (MS) is associated with a decrease in bone mineral density, and with an increased risk for non-vertebral osteoporotic fractures. We have recently found that orally administered Metformin induces osteogenic effects in rats, promoting osteoblastic differentiation of bone marrow progenitor cells and increasing the repair of bone lesions. In this study we have evaluated the effects of Fructose-induced MS on bone micro-architecture in rats, and the possible modulation of these effects by orally administered Metformin. We utilized young male Sprague-Dawley rats, divided into four groups: C (non-treated controls); C+M (100 mg/kg/day of Metformin in drinking water); F (10% of Fructose in drinking water); and F+M (Fructose+Metformin in drinking water). After three weeks of all treatments blood samples were taken, after which animals were sacrificed by cervical dislocation under anesthesia. Femurs were then dissected for evaluation of metaphyseal micro-architecture after Hematoxilin-Eosin staining of 5 µm histological slices of decalcified bone. In particular, osteocytic density and relative trabecular volume were determined. An increase in serum glucose and triglycerides was observed in Fructose-treated rats, in accordance with the development of MS. In rats treated with Metformin alone (group C+M), the analysis of femoral metaphyses showed an increase in trabecular osteocytic density (118% of control [group C], p<0.05). Treatment with Fructose alone (group F) significantly decreased osteocytic density (79% of control, p<0.05), while co-treatment with Fructose and Metformin partially reverted this decrease (group F+M, 88% of control). Similarly, the relative trabecular volume of femoral metaphysis was increased by treatment with Metformin alone (129% of control), was reduced in Fructose-treated rats (89% of control), and tended to revert back to control values after Fructose-Metformin co-treatment (94% of control). These results show for the first time that (a) Fructose-induced MS in rats alters their femoral metaphysis micro-architecture; and that (b) these deleterious effects can be partially prevented by orally administered Metformin.

Authors declare not having any financial or personal conflicts that may have inappropriately influenced their research.

Key words: Metabolic syndrome, bone micro-architecture, Metformin

INTRODUCTION

Metabolic Syndrome (MS) is a heterogeneous and multifactor entity associated with an increased cardiovascular risk. Clinical findings most commonly observed in patients with MS include insulin resistance, dyslipidemia (specifically high triglycerides and low levels of HDL), central obesity, hypertension, impaired glucose tolerance or diabetes mellitus, and high rates of atherosclerotic disease (1). MS affects approximately 25% of adults in Latin America (range: 18.8 – 43.3% depending on the country) (2). Similar prevalence values are found in the United States of America, and even higher rates are reported in several ethnic groups worldwide (3). The prevalence of MS continues to increase, probably as a result of increased obesity (4,5).

A large number of studies have been performed to study the potential effects of MS on bone tissue and metabolism. Most recent studies have shown that in human subjects
the development of metabolic syndrome is associated with a higher degree of osteopenia and osteoporosis, as well as with a significant increase in the incidence of nonvertebral osteoporotic fractures \(^6\), \(^8\). However, results might not be conclusive, as the Kinjo group \(^9\) showed that femoral neck bone mineral density (BMD) increased with additional components of the MS.

Metformin is one of the most commonly used agents in the treatment of conditions associated with insulin resistance such as type 2 diabetes mellitus and metabolic syndrome. This drug is an insulin-sensitizing biguanide that decreases blood glucose levels without directly affecting insulin secretion \(^\text{10}\).

We have recently reported that metformin exerts direct osteogenic effects on osteoblasts in culture, promoting their proliferation, differentiation and mineralization \(^\text{11}\). We have also found that this biguanide orally administered in rats promotes bone marrow progenitor cell differentiation and increases bone lesions repair \(^\text{12}\).

In this study, our aim was to investigate the potential effects of the fructose-induced MS on bone microarchitecture in rats, hypothesizing that the increased fragility of long bones in MS could be partly due to alterations in the metaphyseal microarchitecture of these bones (namely, decreased relative volume of trabecular bone and/or alterations in cellularity). Another objective of this study has been to evaluate the potential modulation of these effects by orally administered Metformin.

**MATERIALS AND METHODS**

**Animals and Treatments**

We used young male Sprague-Dawley rats (200 – 220 g) house in a biotery at controlled temperature of 23 °C with 12:12 h light:dark cycles, on a standard balanced diet and provided with water ad libitum. All animal studies were conducted in accordance with the Guidelines on the Handling and Use of Laboratory Animals \(^\text{13}\) following the provisions established under national (ANMAT Regulation # 5330/97) and international bioethical regulations. In addition, NIH guidelines for laboratory animals were followed (http://grants.nih.gov/grants/olaw/olaw.htm).

Animals were divided into four groups of 5 animals each: Control group (C), received water ad libitum; Fructose (F), received 10% fructose solution (Biopack, Buenos Aires, Argentina) in drinking water \(^\text{14}\); Metformin (C + M), received 100 mg/kg/day of Metformin (Química Montpellier, Buenos Aires, Argentina) in drinking water and Fructose + Metformin (F + M), received a combination of both treatments.

The treatment schedule was as follows:

<table>
<thead>
<tr>
<th>0</th>
<th>15d</th>
<th>30d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>Metformin</td>
<td>Sacrifice</td>
</tr>
</tbody>
</table>

Animals were sacrificed by cervical dislocation under anesthesia.

These treatment times were selected for two reasons: (a) we have previously reported that oral administration of Metformin to Sprague Dawley rats for two weeks causes significant changes in osteogenic parameters in vivo and ex vivo; and (b) following this treatment period in young Sprague-Dawley rats with 10% fructose in drinking water, other authors have found that rats develop hypertension, high triglycerides and glucose intolerance \(^\text{14, 15}\).
Blood parameters

Blood samples were withdrawn by cardiac puncture before sacrifice under non-fasting conditions. Serum was obtained by centrifugation. Serum glucose, triglycerides and cholesterol were measured using commercially available kits (Wiener-Lab, Argentina).

Collection of samples for bone histomorphometry

Femurs of each rat were dissected to analyze metaphyseal microarchitecture. Bones were fixed for 72 hours in neutral buffered formalin (NBF) and then decalcified with a 10% EDTA solution, pH = 7.0 (Biopack, Buenos Aires, Argentina) in successive washes. Specimens were embedded in paraffin and 5-mm slices were cut using a microtome (Leica SM 2000 R). The slices were stained with Hematoxylin and Eosin (H&E) and images were taken with a Nikon Coolpix 4500 digital camera on a Nikon Eclipse E400 microscope. Images were analyzed with the Image-J software (http://www.macbiophotonics.ca/imagej/) using a plugin to incorporate a micrometric scale [12].

Bone histomorphometry

For images obtained from H&E-stained slices obtained under the various experimental conditions, we determined the relative volume of trabecular bone in femoral metaphysis, defined as:

\[
\text{Trabecular Area} \times 100
\]

\[
\% \text{Trabecular Bone} = \frac{\text{Total Area}}{\text{Total Area}}
\]

where Total Area is the total surface area of the image, i.e. the sum of trabecular bone plus bone marrow cavities.

Osteocyte density was also calculated by counting the number of osteocytes per trabecular bone area unit.

Statistical Analysis

Results are expressed as mean ± SEM. Differences between groups were analyzed by the ANOVA one-way test using the Tukey test as post test. The GraphPad Prism 5 software was used (GraphPad Software, San Diego, CA, USA). Differences were considered as statistically significant when p <0.05.

RESULTS

A high fructose diet leads to a metabolic status consistent with MS, and these findings are partially prevented by metformin.

Table I shows the results of postprandial blood parameters (blood glucose, triglycerides and cholesterol) in the four groups of rats.

Blood glucose levels are elevated in the group of rats treated with fructose (20% higher than in the control group). In the group that received treatment with fructose and with metformin, blood glucose levels returned to normal after 2 weeks on metformin (with no significant difference from control animals). The group that received metformin only did not show changes in blood glucose levels either, which is consistent with the blood glucose normalizing effect of this drug.
Plasma triglycerides also showed a marked increase in the group of rats receiving a high fructose diet (217% in relation to control). This effect was partially prevented by metformin (185% in relation to control, 85% of the group treated with fructose).

No significant differences were found in blood cholesterol values.

The analysis of bone microarchitecture shows a decrease in bone quantity and quality.

Table II shows the results obtained from the analysis of the relative trabecular volume in femoral metaphyses. There is a decrease in the percentage of trabecular bone in fructose-treated rats (11% reduction from control group); this effect is partially prevented by co-treatment with metformin. In addition, in animals that did not receive fructose in drinking water, treatment with metformin led to a significant increase in relation to controls (30%) in the percentage of trabecular bone.

Figure 1 shows the values obtained for osteocyte density of metaphyseal trabecular bone. A behavior similar to that observed for trabecular bone percentage is observed, with a lower number of osteocytes per area unit for the group of rats receiving a high fructose diet, an effect that is prevented by the oral administration of Metformin. In addition, administration of metformin alone (with no fructose) caused an increase in osteocyte density in relation to controls.

TABLE I. Serum (non-fasting) biochemical parameters in the various experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose (mg/dL)</th>
<th>Blood Triglycerides (mg/dL)</th>
<th>Blood Cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>172,0 ± 6,2</td>
<td>65,0 ± 3,8</td>
<td>45,0 ± 1,6</td>
</tr>
<tr>
<td>Fructose</td>
<td>207,3 ± 5,9&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>141,0 ± 6,6&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>50,2 ± 2,0</td>
</tr>
<tr>
<td>Control + Metformin</td>
<td>168,7 ± 6,6</td>
<td>56,3 ± 3,5</td>
<td>47,3 ± 0,9</td>
</tr>
<tr>
<td>Fructose + Metformine</td>
<td>190,6 ± 7,5</td>
<td>120,0 ± 7,4&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>45,6 ± 2,2</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of measurements
<sup>a</sup> p < 0.05 vs. Control
<sup>b</sup> p < 0.05 vs. Control + Metformin

TABLE II - Histomorphometric analysis – Trabecular bone percentage

<table>
<thead>
<tr>
<th>Group</th>
<th>Trabecular bone % (in relation to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Fructose</td>
<td>89 ± 3&lt;sup&gt;a, b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control + Metformin</td>
<td>129 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fructose + Metformine</td>
<td>94 ± 2</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of measurements
<sup>a</sup> p < 0.05 vs. Control
<sup>b</sup> p < 0.05 vs. Control + Metformin
DISCUSSION

Previous studies have shown that high carbohydrate diets, and especially high fructose diets (HFD) in rats induce a status consistent with the metabolic syndrome, which includes hypertension, dyslipidemia, hyperinsulinemia and impaired glucose tolerance (14, 16). In agreement with what has been reported by previous studies, our findings show postprandial hyperglycemia and hypertriglyceridemia in rats fed with a HFD, which evidences an impaired metabolic status consistent with metabolic syndrome. Additionally, we found that co-treatment with metformin totally or partially prevents these alterations.
Several clinical studies report that metabolic alterations such as type 1 and type 2 diabetes mellitus are associated with bone alterations that make patients more prone to fractures (6-8). In this study we found that generating metabolic syndrome induces a decrease in the percentage of trabecular bone, which might be related to a lower trabecular bone density. Furthermore, we found a decrease in osteocyte density, which in the long term might favor accumulation of microdamage in bone, given the role played by osteocytes in maintenance of its bone microenvironment integrity. If our current findings in an animal model of metabolic syndrome could be extrapolated to a clinical situation, they would suggest a link between such syndrome and increased bone fragility.

It is currently accepted that oxidative stress might play an important role in the pathophysiology of metabolic syndrome. Hyperglycemia promotes oxidative stress and generation of reactive oxygen species (ROS) (17). The excessive accumulation of triglycerides induces the generation of free radicals and lead to oxidative damage in adipose tissue (18). This status of increased production of ROS is associated with a decrease in the levels of antioxidant substances (retinyl ester, vitamin C, Vitamin E and several carotenoids) (18-21).

Metformin prevents the formation of reactive oxygen species, thus preventing alterations caused by oxidative stress in several cell types and tissues including osteoblasts (22-24), by mechanisms which have not been completely elucidated yet. In addition, we have found that metformin exerts direct osteogenic effects, both in vitro and in vivo (11, 12). As other authors, we have also found that metformin exerts its effects on osteoblasts by various signal transduction pathways, such as an increase in the expression and subcellular redistribution of iNOS, an increased activation of ERK 1-2 and AMPK, and consequently an increased expression of the osteogenic transcription factor Cbfa1/Runx-2 (12, 25-27). Specifically, Jang et al have shown by means of a series of elegant experiments that metformin may stimulate MC3T3E1 osteoblast differentiation in culture through the transactivation of Runx-2 via AMPK/USF-1/SHP regulatory cascade (27). Thus, metformin may either exert a direct effect on bone cells or act indirectly on them by normalizing blood glucose and triglyceride levels, which would result in a lower production of ROS, thus avoiding the alterations derived from this event. In this study, we have found that oral metformin administration can increase relative trabecular bone volume and osteocyte density in femoral metaphyses of rats not suffering from metabolic syndrome, and to reverse the deleterious effects of a high fructose diet on such bone parameters.

Advanced glycation end-products (AGEs) are the result of non-enzymatic glycosylation reactions of amino groups of proteins, lipids or nucleic acids with reducing sugars or carbonylic intermediates. These reactions occur in an accelerated manner in conditions such as decompensated diabetes, metabolic syndrome and during physiological aging. Proteins with a long half-life such as type I collagen, the main component of the extracellular matrix of bone tissue, are targets for AGE formation. This may cause a pathological cross-linking of glycosylated collagen, leading to a loss of bone flexibility and elasticity, and causing increased tissue fragility (28, 29). Our group has previously shown that AGEs exert direct deleterious effects on osteoblast proliferation and differentiation in culture and that these effects by be prevented by co-incubation with metformin (22).

Thus, in our current model in vivo metformin might be preventing the deleterious effects of a high fructose diet on bone by multiple mechanisms. We are currently designing further experimental approaches that might enable us to clarify which of those mechanisms are relevant to our model, in order to be able to contribute to the optimization of treatment strategies with potential clinical applicability.
REFERENCES


11. Cortizo, A. M.; Sedlinsky, C.; McCarthy, A. D.;


