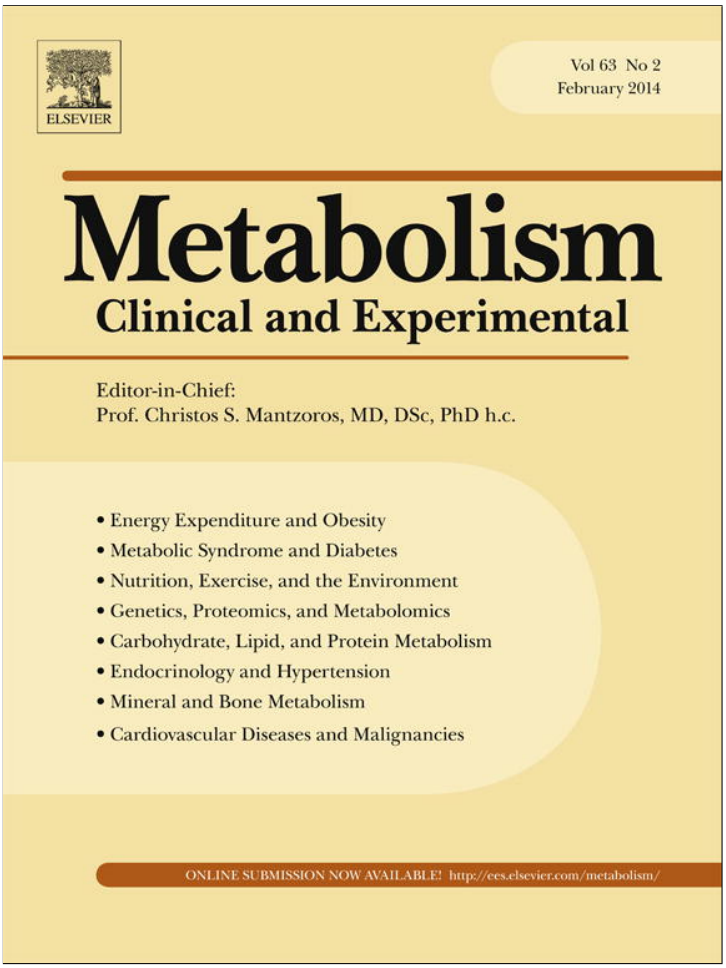

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Effects of a metabolic syndrome induced by a fructose-rich diet on bone metabolism in rats

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ABSTRACT

Objective. The aims of this study were: first, to evaluate the possible effects of a fructose rich diet (FRD)-induced metabolic syndrome (MS) on different aspects of long bone histomorphometry in young male rats; second, to investigate the effects of this diet on bone tissue regeneration; and third, to correlate these morphometric alterations with changes in the osteogenic/adipogenic potential and expression of specific transcription factors, of marrow stromal cells (MSC) isolated from rats with fructose-induced MS.

Materials/Methods. MS was induced in rats by treatment with a FRD for 28 days. Halfway through treatment, a parietal wound was made and bone healing was evaluated 14 days later. After treatments, histomorphometric analysis was performed in dissected femoral and parietal bones. MSC were isolated from the femora of control or fructose-treated rats and differentiated either to osteoblasts (evaluated by type 1 collagen, Alkaline phosphatase and extracellular nodule mineralization) or to adipocytes (evaluated by intracellular triglyceride accumulation). Expression of Runx2 and PPARγ was assessed by Western blot.

Results. Fructose-induced MS induced deleterious effects on femoral metaphysis microarchitecture and impaired bone regeneration. Fructose treatment decreased the osteogenic potential of MSC and Runx2 expression. In addition, it increased the adipogenic commitment of MSC and PPARγ expression.

Conclusions. Fructose-induced MS is associated with deleterious effects on bone microarchitecture and with a decrease in bone repair. These alterations could be due to a deviation in the adipogenic/osteogenic commitment of MSC, probably by modulation of the Runx2/PPARγ ratio.

Abbreviations: AB, Alcian Blue; ALP, alkaline phosphatase; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; FRD, fructose rich diet; H&E, Haematoxylin and Eosin; IBMX, 3-isobutyl-1-methylxanthine; MS, metabolic syndrome; MSC, marrow stromal cells; NBF, Neutral Buffered Formalin; p-NP, *p*-nitrophenol; p-NPP, *p*-nitrophenylphosphate; TRAP, Tartrate Resistant Acid Phosphatase.

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1. Introduction

Metabolic Syndrome (MS) is a heterogeneous and multifactorial human disorder associated with increased cardiovascular risk [1]. Common associated comorbidities observed in MS patients include insulin resistance, dyslipidemia (particularly hypertriglyceridemia and low levels of HDL), central obesity, hypertension, glucose intolerance, diabetes mellitus (DM), and a high proportion of atherosclerotic disease. MS affects approximately 25% of adults in Latin America, ranging from 18.8% to 43.3% depending on the country of origin [2]. A similar prevalence has been observed in the US population, and an even higher prevalence was reported in certain ethnic groups around the world [3]. Importantly, the prevalence of MS is correlated with an increased prevalence of obesity, age, and is also associated with an increased risk for the development of DM with its associated cardiovascular complications [4].

Western diets contain substantial amounts of refined sugars including sucrose, fructose and glucose. Fructose intake has been associated with higher levels of plasmatic triglycerides (VLDL) and a decrease in the HDL-cholesterol [5]. According to USDA Nationwide Food Consumption Survey reports, the average daily fructose intake in the US population was 37 g in 1978. Sugar-sweetened non-alcoholic beverages, such as soft drinks, appeared as the major source of fructose, and adolescents and young adults (19–22 years) of both genders are the main consumers. Recent data from the NHANES 1999–2004 study, have estimated an average fructose intake of 49 g/day, which corresponds to a 30% increase in daily intake [5]. Several studies performed in rats that received fructose or sucrose-rich diets reproduced the severe and adverse metabolic and cardiovascular effects observed in MS patients [5–8], suggesting that this could be an interesting animal model to investigate different aspects of MS.

Bone is a highly dynamic tissue [9]. Marrow stromal cells (MSC) are found in the bone marrow microenvironment and present the ability to differentiate into various cell types such as osteoblasts, adipocytes, and chondrocytes [10–12]. In this context, bone marrow metabolic conditions are determinants of the biologic balance between osteoblast-mediated bone formation and marrow adipogenesis [11]. Diverse factors such as endogenous hormones and drug treatment can affect this delicate balance, modifying the osteoblast–adipocyte ratio in the bone marrow [11]. There is a growing body of clinical evidence reporting the association of skeletal abnormalities that include osteopenia, osteoporosis and/or an increased incidence of low-stress fractures, with MS [13–17] or some of its individual components such as hypertension [18,19] and obesity [20], in elderly patients of both sexes. However, this association has not been found in all studies [21], and its underlying mechanisms have not been elucidated to date.

The aims of this study were: first, to evaluate the possible effects of a fructose-rich diet-induced MS on different aspects of long bone histomorphometry in young male rats; second, to investigate the effects of this diet on bone tissue regeneration; and third, to correlate these morphometric alterations with changes in the osteogenic/adipogenic potential and expression of specific transcription factors, of marrow stromal cells (MSC) isolated from rats with fructose-induced MS.

2. Methods

2.1. Animal Treatments

Young adult male Sprague–Dawley rats initially weighing 200 to 220 g were used. They were maintained in a temperature-controlled room at 23 °C with a fixed 12 h light–dark cycle. All experiments on animals were carried out in conformity with the Guidelines on Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare (1992) [22]. Approval for animal studies was obtained from the institutional accreditation committee (INIBIOLP's Animal Welfare Assurance No A5647-01). The animals were divided into two groups of 6 animals per group: one group received standard chow and water *ad libitum* (control, C) and the other group received standard chow and water containing 10% w/v fructose (Biopack, Buenos Aires, Argentina) *ad libitum* (fructose-rich diet, FRD) [23]. After 28 days, all animals were sacrificed under anesthesia by cervical dislocation. This period of time was chosen because some authors have found that drinking 10% fructose solution for at least 3 weeks causes, in Sprague–Dawley rats, development of hypertension, hypertriglyceridemia and impaired glucose tolerance [24,25]. Prior to sacrifice, body weight was measured and non-fasting blood samples were obtained for determination of serum biochemical parameters. Glucose, triglycerides and cholesterol were measured by commercial kits (Wiener Laboratories, Rosario, Argentina) with a Metrolab 2300plus automated Chemistry Analyzer (Metrolab, Argentina). Insulin was determined by a rat-specific ELISA kit (ALPCO, New Hampshire, USA) and fructosamine was measured with a colorimetric kit (Biosystems, Barcelona, Spain).

2.2. Bone reossification model

The possible effects of a fructose-rich diet-induced MS on the process of bone repair were evaluated by a reossification model previously described by Santana et al. [26]. Briefly, half-halfway through the study (i.e., after 14 days) all animals were anesthetized by intraperitoneal/intramuscular injection of 0.12 mL/100 g body weight of 62.5 mg/mL ketamine hydrochloride and 6.25 mg/mL xylazine (Laboratorios Richmond, Buenos Aires, Argentina). Circular craniotomy defects of 1.0 mm diameter were performed in the right parietal bones of animals with a cylindrical low-speed carbide bur. Animals were then maintained in the previously described conditions for another 14 days to allow bone lesions to heal partially.

2.3. Histological examination of femoral metaphysis

After sacrifice, both femora of each rat were dissected. One femur was used for histomorphometric analysis and the other was used for isolation of MSC, as described below. The length of each femur was measured using a Vernier caliper. Femora were fixed in Neutral Buffered Formalin (NBF) for 72 h and decalcified by consecutive immersions in 10% EDTA (Biopack, Buenos Aires, Argentina), pH = 7.0. After decalcification, they were embedded in paraffin, and 5 μm sections were obtained using an SM 2000R Leica microtome (Leica Microsystems, Wetzlar, Germany). The sections were stained with Haematoxylin and

Eosin (H&E), Alcian Blue (AB) or histochemistry for Tartrate Resistant Acid Phosphatase (TRAP) (Sigma, St. Louis, MO, USA). H&E staining was used to determine relative trabecular bone area, defined as the ratio between trabecular bone area and total tissue area Tb.Ar/T.Ar [%]; and osteocyte density defined as the number of osteocytes per square millimeter of trabecular bone. These parameters were determined in the femoral metaphysis. AB staining was used to evaluate the epiphyseal growth plate height. The measurements were performed parallel to chondrocyte columns in the central two thirds of the proximal growth plate [27].

TRAP histochemistry was performed to specifically identify osteoclasts [28]. Osteoclastic density in the primary spongiosa was determined as the positive TRAP area per square millimeter of trabecular bone. Photographs were taken with a Micrometrics 519CU camera system on an Eclipse E400 Nikon microscope (Nikon, Tokyo, Japan). Images were analyzed using the ImageJ program (http://www.macbiophotonics.ca/imagej) [29].

2.4. Histological examination of bone reossification

After sacrifice, right parietal bones were dissected and processed for histologic and quantitative histomorphometric analysis. Parietal bones were fixed in NBF, decalcified in 10% EDTA, pH = 7.0, and embedded in paraffin. 5 μm sections were obtained using an SM 2000R Leica microtome. The sections were stained with Haematoxylin and Eosin (H&E) or histochemistry for Tartrate Resistant Acid Phosphatase (TRAP). H&E staining was used to determine the newly reossified bone area in the parietal wound, and osteocyte density in the newly formed bone. Osteoclastic density was calculated as the positive TRAP area per micrometer of defect perimeter. Photographs were taken with a Micrometrics 519CU camera system on an Eclipse E400 Nikon microscope (Nikon, Tokyo, Japan). Images were analyzed using the ImageJ program (http://www.macbiophotonics.ca/imagej) [29].

2.5. MSC isolation and incubation

MSC were obtained from the femora of C and FRD rats as previously described [29]. Briefly, immediately after dissection bone marrow cells were collected by flushing one femur from each animal with Dulbecco's modified essential medium (DMEM) (Invitrogen, Buenos Aires, Argentina) under sterile conditions. The resulting suspension was seeded in a 25 cm^2 tissue culture flask and incubated in DMEM supplemented with penicillin (100 UI/mL), streptomycin (100 μg/dL) and 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina) at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. After 24 h, non-adherent cells were removed by changing culture medium, after which medium was changed twice a week. When cells reached confluence (10 to 15 days), the cell monolayer was detached using 0.12% trypsin–1 mmol/L EDTA and sub-cultured in tissue plates.

2.6. Cell proliferation Assay

Cell proliferation was determined using the crystal violet mitogenic bioassay. We have previously shown that the results of this assay correlate linearly with osteoblast cell number counted with a haemocytometer [30]. MSC from C and FRD animals were grown in 24-well plates. After different periods of culture, cell number was estimated for each condition.

2.7. Osteogenic differentiation of rat MSC

MSC were plated at a density of 5×10^4 cells/well in 24-well plates containing 10% FBS–DMEM and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After cells reached confluence, they were induced to differentiate into osteoblasts using an osteogenic medium (DMEM containing 10% FBS, 25 μg/mL ascorbic acid and 5 mmol/L sodium β-glycerol-phosphate) for a further 15 or 21 days [29]. Medium was changed twice a week. Prior to, and after 2-3 weeks of osteogenic induction, the osteoblastic differentiation markers alkaline phosphatase (ALP), type 1 collagen and extracellular calcium deposits were determined in cultures of BMPC as described below.

After 15 days of osteogenic differentiation, cell monolayers were washed with phosphate-buffered saline (PBS) and lysed with 200 μL 0.1% Triton-X100. An aliquot of 100 μL of the lysate was used to evaluate ALP activity by the hydrolysis of *p*-nitrophenylphosphate (p-NPP) to *p*-nitrophenol (p-NP) at 37 °C. The absorbance of p-NP was recorded at 405 nm [30]. Aliquots of the same lysate were used for protein determination by Bradford's technique [31]. Type 1 collagen production was also evaluated after 15 days of culture as reported previously [32]. Briefly, cell monolayers were fixed with Bouin's solution and stained with Sirius red dye for 1 h. The stained material was dissolved in 1 mL 0.1 N sodium hydroxide, and the absorbance of the solution was recorded at 550 nm. Extracellular calcium deposits (mineralization nodules) were evaluated after 21 days of osteogenic differentiation using alizarin S red staining [33]. Stained calcium deposits were extracted with 1 mL 0.1 N sodium hydroxide, recording the optical density at 548 nm.

2.8. Adipogenic differentiation of rat MSC

To induce adipocyte differentiation, MSC were grown to confluence in 24-well plates in DMEM supplemented with 10% FBS, after which the cells were cultured for an additional 10 days with DMEM–10% FBS plus 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone (Decadron, Sidus, Argentina), and 200 nmol/L insulin (Lilly, Buenos Aires, Argentina). At the end of this adipogenic induction, cells were lysed with 0.1% Triton-X100 and intracellular triacylglyceride deposits were analyzed with an enzymatic commercial kit (Wiener, Rosario, Argentina) according to the manufacturer's instructions. Aliquots of the same extract were used for protein determination by Bradford's technique [31].

2.9. Western Blot Analysis

MSC were grown to confluence in 6-well plates in DMEM–10% FBS and then differentiated into osteoblasts or adipocytes for different periods of time as previously described. At the end of the culture periods, cells were lysed in Laemmli's buffer [34]. The lysates were heated to 100 °C, and 30 μg of protein was subjected to 10% SDS-PAGE. The separated proteins were

transferred to PVDF membranes. After washing and blocking, the membranes were incubated overnight at 4 °C with an antibody against Runx2/Cbfa1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for evaluation of osteoblastogenesis, and PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for evaluation of adipogenesis. In order to normalize the results, all blots were stripped and reprobed to assess β-actin (Novus Biologicals, Littleton, CO, USA). Blots were developed by an enhanced chemiluminescence method. The intensity of the specific bands was quantified by densitometry after scanning of the photographic film. Images were analyzed using the ImageJ program (http://www.macbiophotonics.ca/imagej).

2.10. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Differences between groups were assessed by Student's *t*-test using InStat Graph Pad 3.0 software (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Body weight and biochemical evaluation of experimental groups

There was no significant difference in body weight after the evaluation period (253.3 ± 10.2 g C vs. 252.0 ± 10.2 g FRD).

Table 1 shows serum profiles for both experimental groups. FRD group evidenced non-fasting hyperglycemia (52% higher than C), hyperinsulinemia (313% higher than C) and hypertriglyceridemia (108% higher than C), as well as greater levels of serum fructosamine (56% higher than C). No significant difference was observed in cholesterol levels. The parameters for FRD group are compatible with the development of fructose-induced MS.

3.2. Histomorphometric analysis shows a deleterious effect of fructose-induced MS on femoral metaphysis micro-architecture

There was no significant difference in femoral length between C and FRD groups (32.5 ± 0.5 mm for C, versus 31.3 ± 0.8 mm for FRD).

Fig. 1 shows the results obtained from histomorphometric analysis. No differences between groups were observed for bone marrow adiposity. H&E staining (Fig. 1, A–F) shows that there was a significant decrease in osteocyte density in the FRD group (19% reduction versus C), and while the metaphyseal relative trabecular bone area also showed a tendency to decrease in the FRD group (C: 44% ± 4.8% vs. FRD: 39.0% ± 4.5%) this did not reach statistical significance. The results obtained from AB staining did not evidence differences in the height of epiphyseal cartilages between groups (Fig. 1, G–I). Analysis of TRAP histochemistry shows that FRD rats had a significantly lower proportion of osteoclast-covered surface in their femoral primary spongiosa (30% decrease versus C, Fig. 1, J–L).

3.3. Fructose-induced MS impairs bone regeneration

Fig. 2 shows the quantitative histomorphometric analysis of tissue sections from parietal wound areas of both experimental groups. H&E staining showed a significant decrease in reossification area in the FRD group (40% of C), and that there were also less osteocytes per area of newly-formed bone tissue in the FRD group (88% of C) (Fig. 2, C–F). Analysis of TRAP histochemistry shows that FRD rats had a significantly lower proportion of osteoclast-covered lesion perimeter (45% of C, Fig. 2, G–I).

3.4. Fructose-induced MS decreases the osteogenic potential of MSC

We next evaluated the osteogenic potential of MSC isolated from the femora of rats from both experimental groups, in order to explore the possible *ex vivo* effect of a fructose-rich diet on MSC. After 28 days, MSC were obtained from C and FRD rats. After the first passage, ALP activity and type 1 collagen production were determined for all cultures (i.e., prior to osteogenic differentiation). Cells obtained from FRD rats showed a significantly lower ALP activity (28% decrease) and type 1 collagen production (32% decrease) than cells obtained from C rats (Fig. 3, A, C).

We also evaluated the osteoblastic differentiation of MSC from both groups after 15 or 21 days of culture in an osteogenic medium. After 15 days of incubation, MSC derived from FRD rats expressed lower levels of ALP (37% decrease) and produced lower amounts of type 1 collagen (25% decrease) than MSC obtained from C rats (Fig. 3, B, D). Similarly, after 21 days of osteogenic induction, a significant decrease in the formation of mineralization nodules was observed for MSC from the FRD group (Fig. 3, E).

Lower measures of osteoblastic markers in FRD-derived MSC under basal or osteogenic induction could be explained by poorer proliferation of these cells. To clarify this issue, we investigated the in vitro proliferative capacity of MSC from both experimental groups. As can be seen in Table 2, we found

Table 1 – Non-fasting serum profiles after 28 days with or without fructose in drinking water.

Group	Glucose [mg/dL]	Insulin [ng/mL]	Fructosamine [μmol/L]	Triglycerides [mg/dL]	Cholesterol [mg/dL]
C	164 ± 6	1.26 ± 0.17	147 ± 26	71.5 ± 6.3	47.9 ± 1.7
FRD	250 ± 19 †	5.22 ± 1.35 ‡	229 ± 20 ‡	148.4 ± 11.2 *	49.3 ± 1.1

Results are expressed as the Mean ± SEM, n = 6.
Differences versus C: * $p < 0.001$, † $p < 0.002$, ‡ $p < 0.05$.

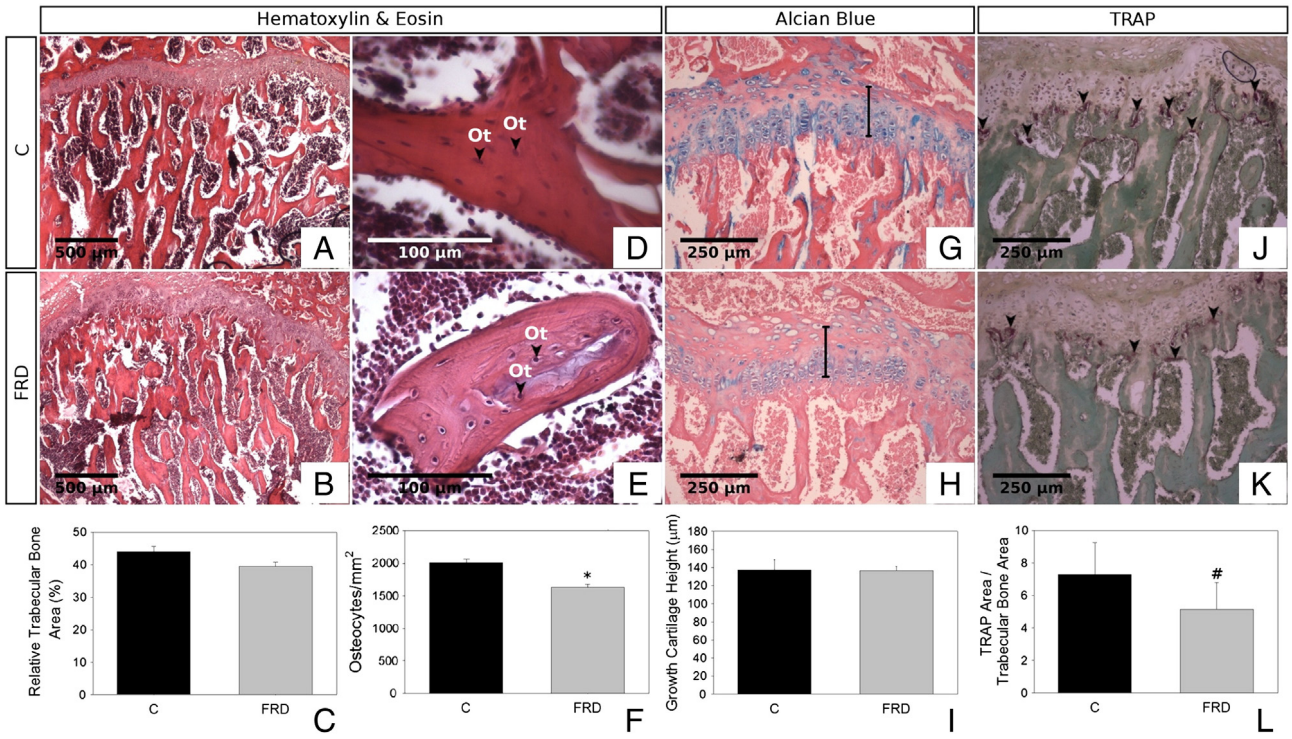

Fig. 1 – Effects of a Fructose-induced MS on femoral microarchitecture.Representative photographs of histological sections from the proximal femora of control (A, D, G, and J) and FRD rats (B, E, H, and K), and quantitation of histomorphometric parameters (C, F, I, and L). Femora were dissected, processed and stained with Haematoxilin & Eosin (A, B, D, and E), Alcian Blue (G and H) or histochemistry for TRAP (J and K).Differences versus C: * $p < 0.0001$, # $p < 0.05$.

that MSC obtained from control and FRD animals proliferate at similar rates, discarding this as a possible cause for our observed decrease in osteoblastic differentiation of FRD-derived MSC.

3.5. *Fructose-induced MS increases the adipogenic commitment of MSC*

We evaluated the *ex vivo* effect of a fructose-rich diet on MSC adipocytic differentiation (by quantitation of triacylglycerides in cell lysates), prior to and after 10 days of adipogenic induction (Fig. 4). In their basal state (i.e., before induction) and after 10 days of adipogenic induction MSC from FRD rats showed higher levels of triacylglyceride accumulation than MSC from C rats.

3.6. *Fructose-induced MS affects the expression by MSC of osteoblast- and adipocyte-specific proteins*

We next evaluated possible changes in the expression of Cbfa1/Runx2, a marker of osteoblastogenesis, and of PPARγ to evaluate adipocytic commitment (Fig. 5). MSC obtained from C and FRD groups were cultured for different periods of time in basal, osteogenic or adipogenic media, after which cell lysates were obtained and submitted to Western immunoblotting as described in Methods. Both in their basal (undifferentiated) state and after 15 days of osteogenic induction, MSC from FRD rats showed lower levels of the osteoblastic transcription factor Runx2 (Fig. 5, A) when compared with MSC from C rats. Analysis of PPARγ expression in MSC from C rats, showed a significant increase in the levels of this adipocytic inducer after they were submitted to 10 days of adipogenic induction. However, MSC from FRD rats already expressed high levels of PPARγ in their basal condition, which did not further increase after 10 days of adipogenic induction (Fig. 5, B). Runx2/PPARγ expression ratio in basal (undifferentiated) MSC from FRD group was 25-fold lower than in undifferentiated MSC from C rats.

4. Discussion

The prevalence of obesity and the Metabolic Syndrome (MS) has been continuously increasing over the past decades. Additionally, MS can predispose to chronic conditions such as type 2 Diabetes mellitus and cardiovascular disease [1]. There is also a strong correlation between the continuous increase in obesity and MS, and an increase in fructose intake [5]. Although incompletely known, several mechanisms have been proposed for fructose-induced deleterious effects [35]. For instance, oxidative stress and production of inflammatory cytokines have been implicated as mediators of fructose and/or obesity-induced insulin resistance. Recently, several studies have reported the association of Diabetes and MS with alterations in bone and mineral metabolism [36,37], although the effect of Diabetes on bone appears to be consistently greater than that of MS.

In this study we have evaluated long-bone micro-architecture, bone tissue regeneration and the adipogenic/osteogenic

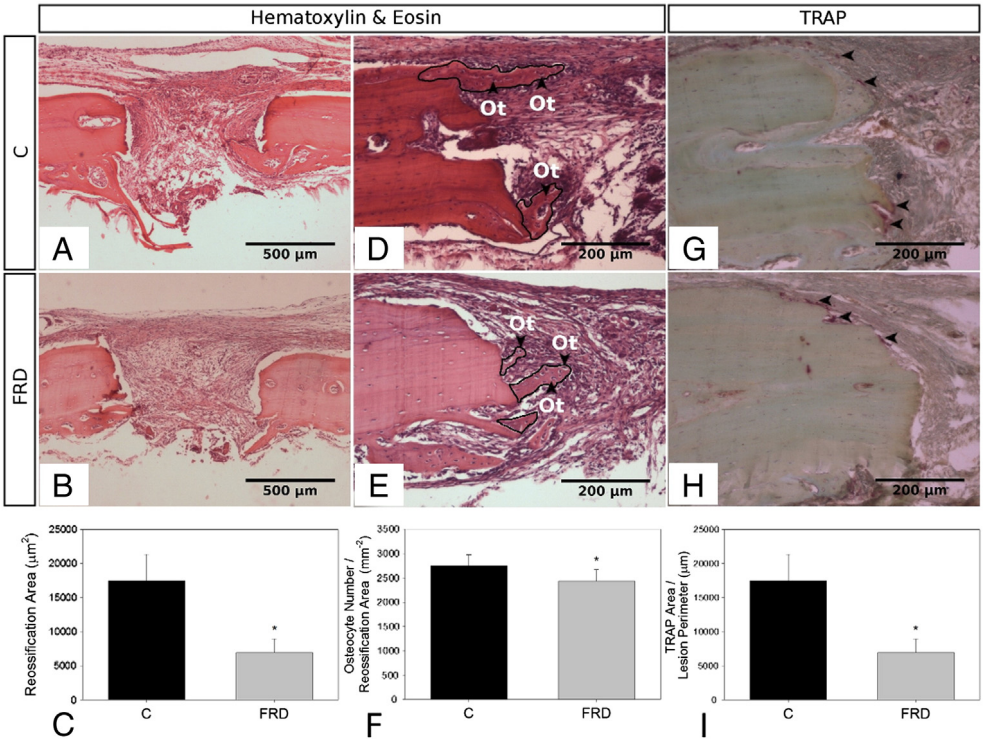

Fig. 2 – Effects of a Fructose-induced MS on bone repair of a parietal wound.Representative photographs of histological sections of a parietal wound performed in control (A, D and G) and FRD rats (B, E and H). A 1 mm circular parietal wound was performed under anesthesia on day 14 of the in vivo study. After a further 14 days all animals were sacrificed, and the parietal bones were dissected, processed and stained with Haematoxilin & Eosin (A, B, D, and E) or histochemistry for TRAP (G and H) to asses new bone formation, osteocyte density, and TRAP positive surface. Quantification of the parameters is shown in panel C, F and I.

commitment of marrow stromal cells (MSC), in a rat model of MS induced by a fructose-rich diet (FRD). This widely used model correlates very well with the human MS phenotype [8], showing insulin resistance, dyslipidemia, hypertension and glucose intolerance. In the present study, FRD animals exhibited post-prandial hyperglycemia with elevated fructosamine levels (a marker of non-enzymatic glycosylation), hypertriglyceridemia and hyperinsulinemia, suggesting overt insulin resistance.

Our selection of this animal model could help us to further understand how alterations of insulin metabolism may induce deleterious effects on bone tissue and functionality. Considering that the main pathogenic mechanisms of type 2 Diabetes are insulin deficiency and/or insulin resistance, it could be interesting to evaluate each mechanism separately by using models of partial insulin deficiency or of insulin resistance. In this context, we have recently found that partially insulin-deficient Diabetes in rats induces deleterious effects on long-bone micro-architecture that are due to a decrease in MSC osteogenic potential [38].

In the present study, we found that MS induced slight though significant alterations in bone architecture that were less evident than those observed in our previous study with insulin-deficient diabetic rats. Thus, in the FRD group we found a 20% decrease in the osteocytic density of femoral trabecular bone and a 30% reduction of osteoclast- covered (TRAP-positive) bone surface in the primary spongiosa. However, we were unable to find significant changes in femoral length, trabecular bone area or growth cartilage height. Nevertheless, this was a 28-day trial of FRD and a longer study may reveal additional femoral alterations to rigorously test the hypothesis of a reduced capacity for bone maintenance. To our knowledge this is the first report demonstrating cellular alterations in trabecular bone as a consequence of a fructose-induced MS. Altogether, our previous and present results could be indicating that the degree of micro-architectural alterations of bone leading to increasingly poorer bone quality could depend on the severity of the underlying metabolic condition. This is supported by reports of other authors that the incidence of low-impact bone fractures in type 1 DM is 7–12 times that of matched non-diabetic individuals [39,40], but is increased approximately 2-fold versus controls both in type 2 DM [41] and in MS [13].

Several cross-sectional and longitudinal clinical studies have reported skeletal abnormalities associated with MS [13–17] or with some of its individual components such as hypertension [18,19] and obesity [20], although this association has not been found in all studies [21]. On the other hand, more severe metabolic conditions such as Diabetes could be associated with an even higher trabecular bone loss and/or poorer bone quality. In this context, other authors using a model of streptozotocin-induced type 1 Diabetes, found significant and progressive trabecular bone loss [42]. With the more moderate model of partially insulin-deficient Diabetes induced by streptozotocin–nicotinamide, we only found a slight decrease in long-bone trabecular area together

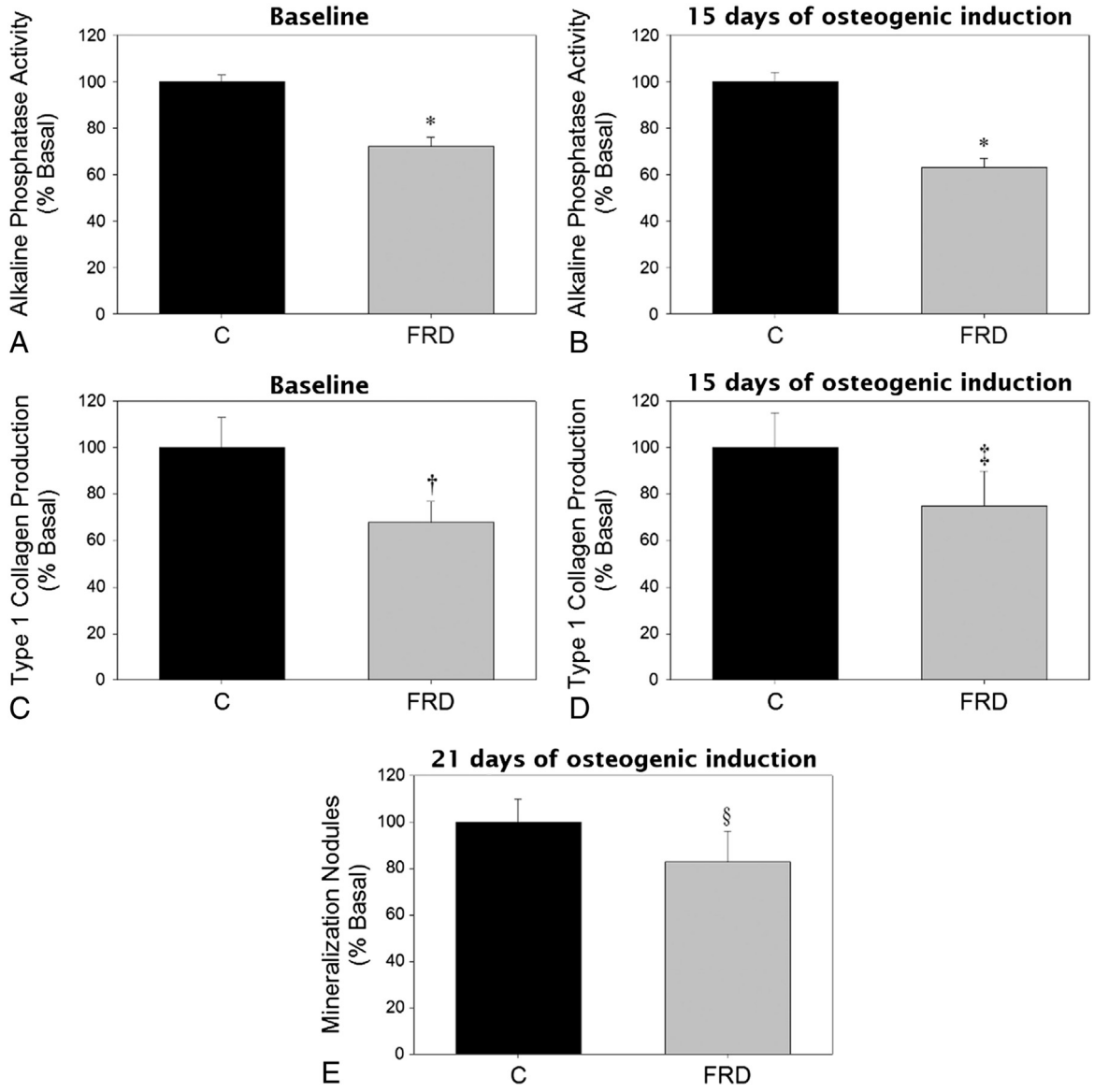

Fig. 3 – Effect of fructose-induced MS on MSC osteogenic potential.MSC obtained from the femora of Control (C) or Fructose-rich diet (FRD) rats were submitted (or not) to an osteogenic induction, and then evaluated for alkaline phosphatase (ALP) activity, type 1 collagen secretion and formation of extracellular nodules of mineralization. (A) Basal ALP activity (prior to osteogenic induction). (B) ALP activity after 15 days of osteogenic induction. (C) Basal type 1 collagen production. (D) Type 1 collagen production after 15 days of osteogenic induction. (E) Extracellular mineral nodule deposition after 21 days of osteogenic induction.Differences versus C: * $p < 0.05$, † $p < 0.02$, ‡% $p < 0.01$, § $p < 0.001$.

with an important reduction in osteocyte density, growth plate height and TRAP activity in the primary spongiosa [38]. Those results were in turn more severe than the bone alterations we are reporting in our present model of MS, suggesting that insulin-resistance may affect bone to a lesser degree than insulin deficiency.

Recent studies show that osteocytes play a crucial, central role in regulating the dynamic nature of bone in all its diverse functions. Osteocytes are known to be the principal sensors for mechanical loading of bone [43]. A reduction in osteocyte density can be explained by a decrease in osteocyte formation from active osteoblasts and/or an increase in osteocyte apoptosis. Both situations could develop as a consequence of oxidative stress induced by MS, DM or even

Table 2 – Marrow Stromal Cell Proliferation Assay.

Time [hours]	C [cell number × 10^4/well]	FRD [cell number × 10^4/well]
0	4.1 ± 0.58	3.9 ± 0.77
24	4.2 ± 0.63	4.2 ± 0.55
48	6.9 ± 0.85	7.9 ± 0.84
96	9.5 ± 0.14	10.2 ± 0.89

MSC from control (C) and FRD animals were plated on 24 well plates (approximately 4×10^4 cells/well). After different incubation periods, Cell number/well was estimated by the Cristal Violet assay.
Results are expressed as the Mean ± SEM.

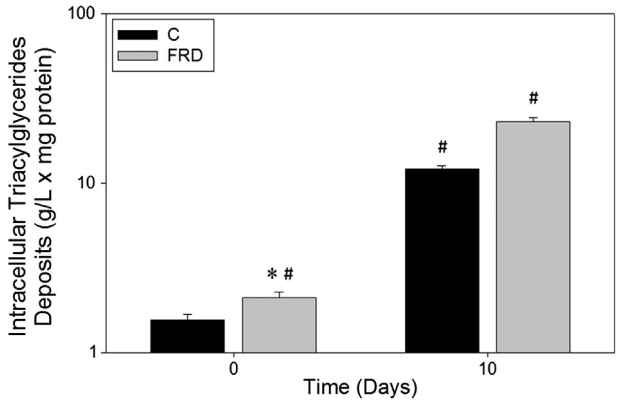

Fig. 4 – Effect of fructose-induced MS on MSC adipocytic differentiation.MSC were obtained from the femora of C and FRD rats and submitted (or not) to an adipocytic induction, and then evaluated for intracellular triacylglyceride levels. Data are mean ± SEM. Differences were:* $p < 0.05$ C-0d vs FRD-0d.# $p < 0.001$ for: C-0d vs C-10d; C-0d vs FRD-10d; FRD-0d vs C-10d; FRD-0d vs FRD-10d and C-10d vs FRD-10d.

aging [44]. In rat models, we have found a progressive reduction in osteocyte density associated both with partial insulin-deficiency [38] and with insulin resistance (our present results).

Other authors have been unable to demonstrate changes in the mechanical properties or mineral density of bone, after a 12-week treatment with a fructose rich diet [45]. We believe this to be in agreement with the concept that in the short term, MS induces slight alterations in bone micro-architecture but no measurable changes in macroscopic structure. However, after sufficient time the deleterious microscopic defects can add up, reducing bone quality enough to increase bone fracture incidence. This is supported by our current results showing (1) a decrease in trabecular bone osteocyte density that could be interpreted as a reduced capacity for bone maintenance at a micrometric level; and (2) a reduction in TRAP activity of the primary spongiosa together with no changes in the height of the epiphyseal growth plate, which in combination would be expected not to affect bone longitudinal growth at a macroscopic level.

We used a model of bone reossification to evaluate the effect of fructose-induced MS on bone tissue regeneration. To this effect, we performed uniform parietal wound as described previously [29] and allowed them to heal for 14 days. Fructose treatment was found to simultaneously decrease reossification, slightly but significantly reduce osteocytic density and diminish osteoclastic activity in the lesion site, suggesting a concerted decrease in bone formation and remodeling. These alterations in bone healing could be the consequence of insulin resistance and/or deranged glucose metabolism. Diabetes has been shown to have negative effects on fracture healing [46]. In previous experiments we found that streptozotocin-induced Diabetes significantly delayed bone healing of a parietal defect, with a 75% decrease in bone reossification area, a 60% decrease in osteocytic density and an increase in TRAP activity in the reossification area [29]. Thus, both insulin deficiency and insulin resistance appear to affect bone tissue regeneration, although different mechanisms could be involved in each case.

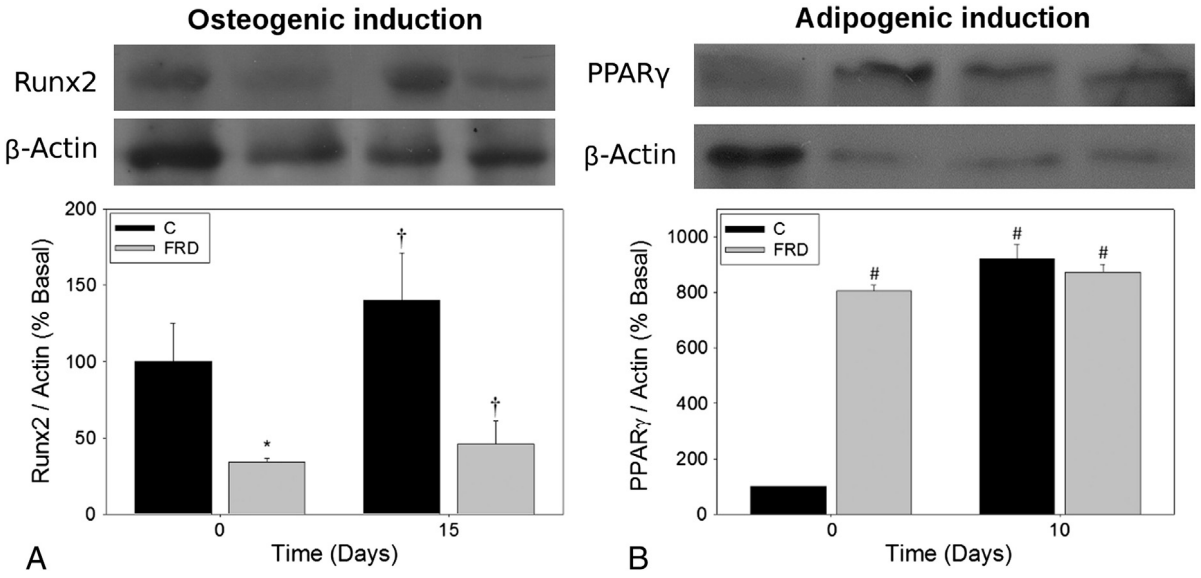

Fig. 5 – Effect of fructose-induced MS on MSC expression of Runx2 and PPARγ.MSC were obtained from the femora of C and FRD rats and submitted (or not) to an osteogenic or adipogenic induction. Cell lysates were obtained in all cases, and evaluated by Western blotting to determine expression of (A) the osteoblastic transcription factor Runx2, or (B) the adipogenic protein PPARγ. Data are express as mean ± SEM.Differences for panel A were:N.S. C-0d vs C-15d; C-0d vs FRD-15d and FRD-0d vs FRD-15d.* $p < 0.05$ C-0d vs FRD-0d.† $p < 0.01$ FRD-0d vs C-15d; C-15d vs FRD-15d.Differences for panel B were:N.S. FRD-0d vs C-10d; FRD-0d vs FRD-10d and C-10d vs FRD-10d.# $p < 0.001$ C-0d vs FRD-0d, C-0d vs C-10d and C-0d vs FRD-10d.

The presence of different factors such as cytokines, hormones or reactive oxygen species in the bone marrow microenvironment has the ability to promote the commitment of MSC to different cell types, such as osteoblasts, adipocytes, and chondrocytes [47]. The present work sought to evaluate the effect of a fructose-rich diet on the ability of MSC to differentiate and express specific markers of the osteoblastic or adipogenic phenotype. Our results show that fructose-induced MS decreased the ex vivo osteogenic potential of MSC (Fig. 3) as evaluated by type 1 collagen production, ALP activity and extracellular matrix mineralization; and increased the ex vivo adipogenic potential of MSC (Fig. 4) determined by intracellular triglyceride accumulation. These findings were associated with a decrease in Runx2 and an increase in PPARγ expression in basal (undifferentiated) conditions. Consequently, a reduction in the Runx2/PPARγ ratio of MSC obtained from animals with fructose-induced MS was observed (Fig. 5). Furthermore, when FRD-derived MSC were induced to differentiate to osteoblasts, Runx2 did not increase to the same degree as it did in control MSC. Additionally, under adipogenic induction MSC from control animals increased their levels of PPARγ. On the other hand, adipogenic induction of FRD-derived MSC did not further increase their already high levels of PPARγ expression, although cells became differentiated to adipocytes (Figs. 4 and 5B). These observations could be due to the possible presence of PPARγ agonists, such as polyunsaturated fatty acids, in the microenvironment of FRD animals [48]. We have previously reported a similar pattern using an insulin-deficient diabetic model [29]. Thus it appears that insulin resistance or deficiency associated with Diabetes or MS can modulate the reciprocal regulation of Runx2 and PPARγ in MSC, promoting their adipogenesis and decreasing their osteogenic capacity, which in turn could affect bone structure and function.

In conclusion, we have demonstrated that fructose-induced MS is associated with deleterious alterations in bone microarchitecture and in the reossification of bone lesions. These alterations could be due to a deviation in the adipogenic/osteogenic commitment of MSC, probably by modulation of the Runx2/PPARγ ratio.

Author contributions

Study design: MSM, ADM, AMC. Study conducted by: JIF, MVG, MSM, ADM, AMC. Data collection: JIF, MVG, MSM. Data analysis: JIF, MVG, MSM, ADM, AMC. Data interpretation: JIF, MVG, MSM, ADM, AMC. Drafting manuscript: JIF, ADM, AMC.

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Conflict of interest

There are no conflicts of interest.

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