

# Apoptosis of endothelial progenitor cells in a metabolic syndrome experimental model

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## ABSTRACT

**Aim:** This study tests the hypothesis postulating that metabolic syndrome induced by chronic administration of fructose to spontaneously hypertensive rats (FFHR) generates impairment in vascular repair by endothelial progenitor cells (EPC). **Materials and Methods:** To characterize the vascular adverse environment present in this experimental model we measured: NAD(P)H oxidase activity, eNOS activity, presence of apoptosis in the arterial wall, all these parameters were most affected in the FFHR group. Also, we found decreased level and proliferative capacity of EPC measured by flow cytometry and colonies forming units assay in cultured cells, respectively, in both groups treated with fructose; FFHR (SHR fructose fed rats) and FFR (WKY fructose fed rats) compared with their controls; SHR and WKY. **Results:** The fructose-fed groups FFR and SHR also showed an incremented number of apoptotic (annexinV+/7AADdim) EPC measured by flow cytometry that returns to almost normal values after eliminating fructose administration. **Conclusion:** Our findings suggest that increased apoptosis levels of EPC generated in this experimental model could bein part the underlying cause for the impaired vascular repair by in EPC.

**Key words:** Apoptosis, endothelial progenitor cells, metabolic syndrome

## INTRODUCTION

Postnatal bone marrow contains a subtype of progenitor cellsthat have the capacity to migrate to the peripheral circulationand to differentiate into mature endothelial cells, taking part in postnatal neovascularization. Therefore,these cells have been termed endothelial progenitor cells (EPC).<sup>[1]</sup>

EPC are characterized by the expression of the surface

markers: CD34; a protein involved in the interaction between progenitor cell and the stromal mesenchyme from bone marrow, whose expression is lost with cell differentiation<sup>[2]</sup> and FLK-1, a tyrosine kinase receptor present in both progenitor and endothelial cells, whose function is to mediate cellular functions of vascular endothelial growth factor (VEGF).

Cells localized in bone marrow presented this phenotype without expressing vascular endothelial (VE) cadherin or von Willebrand factor. Mature endothelial cells show a high expressionof FLK-1, VE-cadherin, and von Willebrand factor but CD133 expression isnot detectable.<sup>[3,4]</sup>

Spontaneously hypertensive rats (SHR) provide a genetic model to study essential hypertension.<sup>[5]</sup>Moreover, feeding carbohydrate-enriched diets to rats has been

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shown to induce insulin resistance (IR), hyperinsulinemia, and dyslipidemia associated with an increase in blood pressure.<sup>[6]</sup> Fructose-fed rats (FFR), is a model that has been used to assess the pathophysiological mechanisms and cardiovascular changes associated with metabolic syndrome (MS).<sup>[7]</sup>

The EPC average lifespan has been reported to be shortened in presence of oxidative stress and regulated by anti-oxidative mechanisms. Oxidative stress accelerates the senescence of progenitor cells. It is thought that oxidative stress induces cardiovascular damage by shortening lifespan and impairing EPC functions.<sup>[8]</sup>

A reciprocal relationship between insulin resistance and vascular endothelial dysfunction has recently been identified as an important factor in the development of cardiovascular disease.<sup>[9,10]</sup>

In this study, we investigated changes in EPC behavior that could be related with hypertension and oxidative stress in the context of a metabolic syndrome experimental model, and we trying to associate these changes to the high apoptosis EPC levels found in this experimental model.

## MATERIALS AND METHODS

### Animals and experimental design

All procedures were performed according to institutional guidelines for animal experimentation. Male Wistar Kyoto (WKY) rats and spontaneously hypertensive (SHR) rats (30 days old) were fed a standard commercial chow diet *ad libitum* and housed in a room under conditions of controlled temperature (20°C), humidity, and a 12-h light/dark cycle. Animals were randomly distributed into four groups ( $n=8$  each) and treated during a 10 week experimental period. Group I: WKY; group II: FFR (fructose-fed rats) WKY receiving 10% fructose solution (Parafarm, Buenos Aires, Argentina) as drinking water; group III: SHR; group IV: FFHR (fructose-fed hypertensive rats) SHR receiving 10% fructose solution as drinking water. At the end of the experimental period, rats were anesthetized with ketamine (35 mg/kg i.p.) and xilacine (15 mg/kg i.p.) and euthanized.

The femurs and tibiae from the hind legs were harvest to extract the bone marrow. The ends of the femurs were cut with a scalpel to remove the hip and knee joints and to expose the marrow. In a similar manner, tibiae were cut to remove knee and ankle adjacent regions. Then a 5 ml syringe filled with RPMI culture media supplemented with 10% fetal calf serum (FCS) with a 26-gauge needle was

inserted into one end of the bone using tweezers to hold the bone over a 15 ml Falcon tube. The media was then flushed while moving the syringe needle up and down inside the bone to break up the tissue into a cell suspension. This procedure was repeated for each bone.

### Systolic blood pressure measurement

Systolic blood pressure (SBP) was monitored indirectly in conscious pre-warmed slightly restrained rats by the tail-cuff method, and recorded on a Grass Model 7 polygraph (Grass Instruments Co., Quincy, MA, USA). The rats were trained on the apparatus several times before measurement.

### Biochemical determinations

Fasting plasma insulin was assayed by ELISADSL-10-1600 Active<sup>TM</sup>, (Diagnostic System Laboratories, Inc. Webster TX USA) in a ELISA microplate reader (Rayto RT-2100C, China).

Plasma glucose, triglycerides and HDL-cholesterol levels were assayed using a commercial colorimetric method (Wiener Lab., Argentina). Homeostasis model assessment (HOMA) was used as an index to measure the degree of insulin resistance and was calculated by the formula:  $[\text{insulin} (\mu\text{U/ml}) \times \text{glucose} (\text{mmol/l}) / 22.5]$ .<sup>[11]</sup>

### NAD(P)H oxidase activity in homogenates of arterial tissue

The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in aorta homogenates. To assess NAD(P)H-oxidase activity, segments were dissected from the abdominal aorta of the animals immediately after slaughter and were incubated in Jude-Krebs buffer.  $\beta$ -NADPH was then added as substrate and chemiluminescence was measured continuously for 3 min on a micro plate fluorometer (Fluoroskan Ascent FL, Thermo LabSystems, Waltham, MA, USA). Enzymatic activity was adjusted to the weights of the arteries and was expressed in counts per minute per milligram of tissue, as relative luminescence units (RLU).

### eNOS activity in homogenates of arterial tissue

The activity of the  $\text{Ca}^{2+}$ /calmoduline-dependent endothelial nitric oxide synthase (eNOS) enzyme was measured in homogenates of mesenteric arteries (resistance bed vessel) by conversion of L-[<sup>3</sup>H] arginine in L-[<sup>3</sup>H] citruline, as previously described.<sup>[12]</sup> Calcium dependent NOS activity was calculated as the difference between activities in the presence or absence of  $\text{Ca}^{2+}$ /calmoduline. Values were

corrected according to protein content (Bradford method) in the homogenates and incubation time and are expressed as dpm/mg protein/min. The material obtained from each animal was processed independently.

### Immunohistochemistry

Arteries were fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin and then sectioned (5  $\mu$ m) with a microtome (Microm HM325; Thermo Scientific, Walldorf, Germany). After these were dewaxed and treated with 10mM sodium citrate buffer (pH 6.0) for antigen retrieval of the tissue. Slides were incubated with 0.3% (wt./vol.)  $H_2O_2$  in absolute methanol for 30 minutes to inhibit any endogenous peroxidase activity for immunohistochemistry (IHC) or were treated for 20 minutes in 50 mM  $NH_4Cl$  at room temperature to reduce the auto-fluorescence. Thereafter were incubated for 30 min in blocking solution (phosphate buffer solution containing 0.2% BSA and 0.01% saponin) to avoid secondary antibodies unspecific signal. The primary antibodies used were: mouse monoclonal anti caspase-8 p18 (D-8) (Santa Cruz Biotechnology, USA) 1:100; rabbit polyclonal anti caspase-3 activated (Sigma, USA) 1:100 overnight at 4°C diluted in 10 mM phosphate buffer, 0.9% NaCl and 0.1% Triton. For detection, the following secondary antibodies were used: anti-rabbit biotinylated (Sigma, USA) 1:200 with peroxidase-labeled streptavidin (Histofine SAB-PO kit), and for fluorescence anti-mouse labeled with Cy-5 (Jackson ImmunoResearch) 1:200. Primary antibodies were routinely omitted to determine the level of non-specific label. The same procedure was performed for the cells obtained from magnetic cell separation, using. For IHC, the visualization of a positive reaction was developed with a peroxidase substrate solution containing 0.02% (wt./vol.)  $H_2O_2$  and 0.1% (wt./vol.) 3,3'-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany) in PBS giving the reaction product a brown color counterstained with hematoxylin. Photographs were captured with a Nikon Optiphot-2 microscope and a CCD-Nikon (Japan) Camera. For immunofluorescence analysis image were detected with an Olympus FV-1000 (Japan) microscope.

### Flow cytometry

Flow cytometry was performed using a FACS Area III flow cytometer (BD Bioscience, San Jose CA, USA). Samples from bone marrow and peripheral blood were treated with FACS lysing solution diluted 1/10 for lyses erythrocytes under gentle hypotonic conditions preserving the leucocytes. After washing with PBA (phosphate buffer solution 1 $\times$ , 0.01% BSA, 0.02 % sodium azide) and centrifuged at 1400 rpm for 10 minutes, the cells were

stained with CD34 FITC (BD Bioscience, San Jose, CA, USA) and anti-PE FLK-1 (BD Bioscience, San Jose CA, USA) antibodies. Their corresponding isotypes were used to avoid non-specific binding. Flow cytometry analysis was performed using FACS Diva 6.1.3 Software (BD, Bioscience, San Jose, CA, USA).

### Magnetic cell separation by positive selection

The bone marrow cell suspension was centrifuged at 1500 rpm for 10 min. The mononuclear fraction was separated by centrifugation with Ficoll-Paque PREMIUM 1.084 gradient (GE Healthcare Bio-Sciences AB, Sweden). The mononuclear fraction was stained with a PE anti-FLK-1. The FLK-1<sup>+</sup> cells were then incubated with magnetic particles coated with anti-PE (BD Bioscience, IMag Particles) and were placed in a magnetic field. The labeled cells migrated toward the magnet attaching to the tube walls leaving a fraction of negative cells in suspension which were removed by successive washes with PBA. After the third wash, the supernatant was eliminated, and the positive fraction (cells attached to the tube walls) was removed with culture medium and placed on a culture flask.

The effectiveness of the immunomagnetic separation was determined by immunostaining of these separated cells with CD34 and FLK-1, which could check the purity of the cell population obtained. We note that 100% were positive for EPC [Figure 2d] markers confirming the effectiveness of the immunomagnetic separation method employed.

### EPC colony formation assay

A modified EPC colony formation assay was performed. From the cell suspension obtained by flushing the bone marrow cell counts were made to adjust the concentration of one million cells per milliliter in order to plant the same number of cells per flask with RPMI 1640 medium (Invitrogen Corporation, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS), 100 ng/ml stem cell factor, 10 ng/ml vascular endothelial growth factor, 10 ng/ml Flt-3, 10 ng/ml SCF, 45  $\mu$ g/ml heparin, 100 U/ml 100  $\mu$ g/ml penicillin--streptomycin and seeded into a pre-coated culture flask (EPP, Switzerland) with 2% gelatin. We consider a colony of endothelial progenitor cells to a central cluster of rounded cells surrounded by multiple flat cells. The average number of colonies was calculated manually on the fifth day under a Nikon ELWD (Japan) inverted microscope using 0.3/OD75 objective at 40 $\times$ , and 20 fields were counted for each culture flask.

### Apoptosis study

After erythrocyte lyses, bone marrow cells were stained with

anti PE-CD34 (BD Bioscience), 7-amino-actinomycin D (Sigma, USA) and annexinV-FITC (BD Bioscience) in annexinV binding buffer and analyzed by flow cytometry. For each sample 200.000 events were acquired. Based on the settings of EPC population, first EPC were gated, follow by gating for EPC that bound annexinV. After this, these cells were plotted on a gate and were analyzed for their 7AAD expression. Apoptotic EPCs were defined as CD34<sup>+</sup>/annexinV<sup>+</sup>/7AAD<sup>dim</sup>. The intermediate 7AAD staining is named “dim”, indicating that cells take up small amounts of the dye, which is characteristic for apoptosis, fully viable cells with complete and intact cell membranes keep all of the 7AAD out of the cell and are 7AAD negative. Dead cells take up large amounts of 7AAD, resulting in a so-called “bright” signal. Isotypes-stained samples were used as negative controls.<sup>[13]</sup>

In order to reveal whether the increased apoptosis of EPC is really due to the changes caused by metabolic syndrome, we studied data from two additional groups (fifth and sixth group) of animals to which was suspended the administration of fructose after completing the original protocol. Apoptosis was measured after 10 days to stopping fructose administration.

### Statistical and data analysis

Data are expressed as mean  $\pm$  SEM. The statistical significance comparing data between groups was assessed by one-way ANOVA or Kruskal–Wallis test followed by Bonferroni and Dunn post-test, whichever were applicable. Significance was established at a *P* value less than 0.05.

## RESULTS

### Biochemical and systolic blood pressure determinations

Chronic administration of fructose induced several alterations included in the cluster of risk factors characterizing

MS. The insulin resistance index (HOMA-IR) was higher in FFR and FFHR. The comparison between HOMA index evidenced that FFR and FFHR developed insulin resistance [Table 1] (*P*<0.05 vs. WKY and SHR). Table 1 also shows the time-course of metabolic changes and SBP changes values at the end of the experimental period. By the sixth week, SBP of FFHR and SHR showed a significant increase compared to the control group (*P*<0.05 vs. WKY), and the increase in pressure in the FFR group was lower, but significant.

### Characterization of vascular environment

#### Oxidative stress determinations

The oxidative stress state was measured by the NAD(P)H oxidase and eNOS activity. We observed a significant increment in the NAD(P)H oxidase activity (cfm/mg of tissue) in FFR and FFHR compared to their controls; WKY and SHR [Figure 3a]; while arterial eNOS activity (dpm/mgp/min); decreased in both groups treated with fructose [Figure 3b] (\**P*<0.05 vs. WKY and # vs. SHR).

#### Apoptosis in the arterial wall

Caspase-8 activated measured by immunofluorescence was higher in FFHR \**P*<0.05 expressed as AUF (arbitrarier units of fluorescence)/mm<sup>2</sup>, [Figure 4b].

Caspase-3 activated measured by immunohistochemistry was expressed as percentage of positives arteries, both markers were increased in the FFHR group, indicating the presence of apoptotic cells in the arterial walls of these animals [Figure 4a].

### Epc characterization

#### Morfology

Cultured cells were stained by Hematoxylin-eosin showing

**Table 1: The above values correspond to metabolic variables**

|                                | Metabolic variables evaluation |                   |                    |                     |
|--------------------------------|--------------------------------|-------------------|--------------------|---------------------|
|                                | WKY                            | FFR               | SHR                | FFHR                |
| Fasting glucose (mmol/L)       | 4.88 $\pm$ 0.14                | 6.44 $\pm$ 0.18*  | 5.00 $\pm$ 0.19    | 6.50 $\pm$ 0.19**   |
| Fasting triglycerides (mmol/L) | 0.80 $\pm$ 0.01                | 0.9 $\pm$ 0.01    | 1.80 $\pm$ 0.02**  | 1.90 $\pm$ 0.10**   |
| HOMA index                     | 4.32 $\pm$ 0.10                | 7.20 $\pm$ 0.10*  | 10.93 $\pm$ 0.10** | 14.10 $\pm$ 0.40*** |
| HDL Cholesterol (mg/dl)        | 22.50 $\pm$ 0.46               | 19.30 $\pm$ 0.90* | 12.20 $\pm$ 0.80** | 13.60 $\pm$ 1.20**  |
| Insuline (ng/ml)               | 3.895 $\pm$ 0.97               | 6.401 $\pm$ 2.41* | 8.671 $\pm$ 1.56*  | 8.849 $\pm$ 3.15*   |
| Systolic blood pressure (mmHg) |                                |                   |                    |                     |
| Baseline                       | 105 $\pm$ 3                    | 102 $\pm$ 1       | 103 $\pm$ 1        | 106 $\pm$ 3         |
| 3 week                         | 113 $\pm$ 2                    | 131 $\pm$ 3*      | 161 $\pm$ 3        | 162 $\pm$ 2**       |
| 6 week                         | 120 $\pm$ 1.3                  | 136 $\pm$ 3*      | 177 $\pm$ 1**      | 181 $\pm$ 1**       |

Symbols indicate: \**P*<0.05 vs. WKY; +*P*<0.05 vs. SHR; #*P*<0.05 vs. FFR



a round growth until form a monolayer, and then they acquire the cobblestone morphology [Figure 2b and c].

#### EPC colony formation assay

Typical EPC colony formation is shown in Figure 2a. The number of EPC colonies formed in culture was decreased in both fructose feeding groups [Figure 2]. This result could indicate that chronic fructose administration altered the EPC growth by shortening their proliferation.

#### Flow cytometry analysis of EPC levels

Progenitor cells belong to the mononuclear cell fraction, which can be identified using a morphological gate, for their low side scatter. On this cell fraction, we separately analyzed one gate for the CD34<sup>+</sup> cells and another gate for the FLK-1<sup>+</sup> cells. At the intersection of these two gates, we identified CD34<sup>+</sup>/FLK-1<sup>+</sup> in the upper-right quadrant. [Figure 1]. EPC levels were decreased in the groups treated with fructose measured by flow cytometry (as a percentage) in bone marrow and peripheral blood, compared to their control WKY rats. These data indicate that fructose feeding affected the EPC levels even in the most immature cell stages in bone marrow.

#### Apoptosis is increased in EPC from fructose fed rats

In order to determine the apoptosis levels of EPC, we analyze by flow cytometry the annexinV expression in bone marrow cells. Fructose fed rats showed more apoptotic EPC (CD34<sup>+</sup>/annexinV<sup>+</sup>/7AAD<sup>dim</sup> cells) than controls, quantified by flow cytometry [Figure 5].

#### Apoptosis decrease by stopping the fructose administration

After ten days of stopping fructose the number of CD34<sup>+</sup>/annexinV<sup>+</sup>/7AAD<sup>dim</sup> cells was lower in the FFR-SF

(fructose fed rat-stop fructose) and FFHR-SF(fructose fed hypertensive rat-stop fructose) groups respect to the values measured at the end of the protocol [Figure 6].

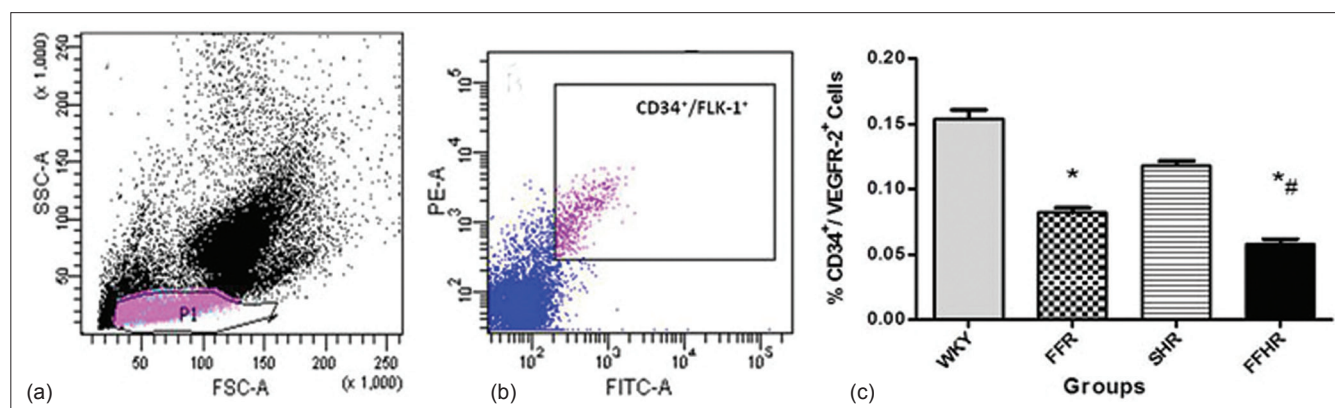
To confirm that metabolic syndrome was reversed in the groups to which was stopped fructose administration, values of glucose, insulin and HOMA index were analyzed [Table 2] finding this parameters decreased almost like the control values.

## DISCUSSION

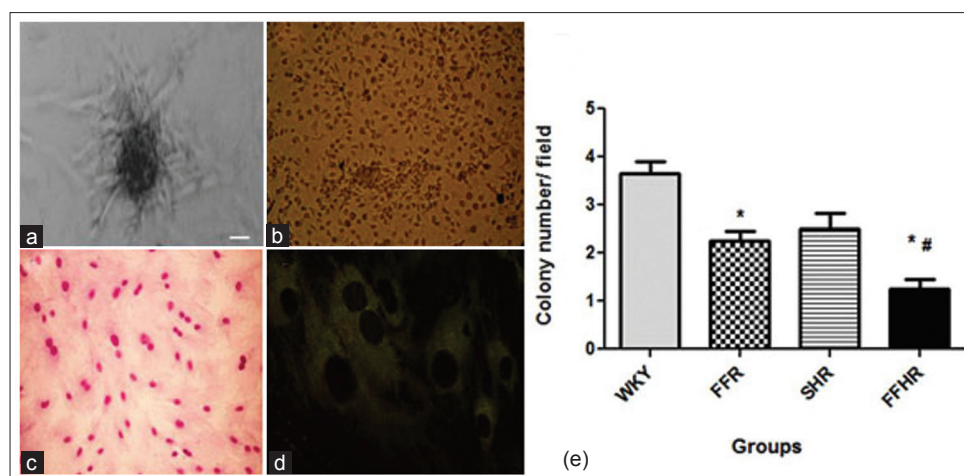
Consistent with previous data reported by our group fructose-fed rats provide a useful model of diet-induced metabolic syndrome.<sup>[7,12,14]</sup> The combination of these two experimental models (FFR and SHR) allows us to simultaneously assess genetic and environmental factors, a very common clinical condition. These obtained results suggest that both pathologic states: high-fructose diet and genetic predisposition to hypertension may contribute to the development of metabolic syndrome.

Endothelial progenitor cells (EPC) derived from bone marrow, identified as CD34<sup>+</sup>/FLK-1<sup>+</sup> can be further differentiated into endothelial cells. Hill *et al.*<sup>[13]</sup> reported that dysfunction of EPC was clearly correlated with vascular injury in the case of various risk factors such as hypertension, dyslipidemia, diabetes mellitus and smoking. Fadini *et al.*<sup>[15]</sup> showed a close negative correlation between EPC levels and metabolic syndrome severity.

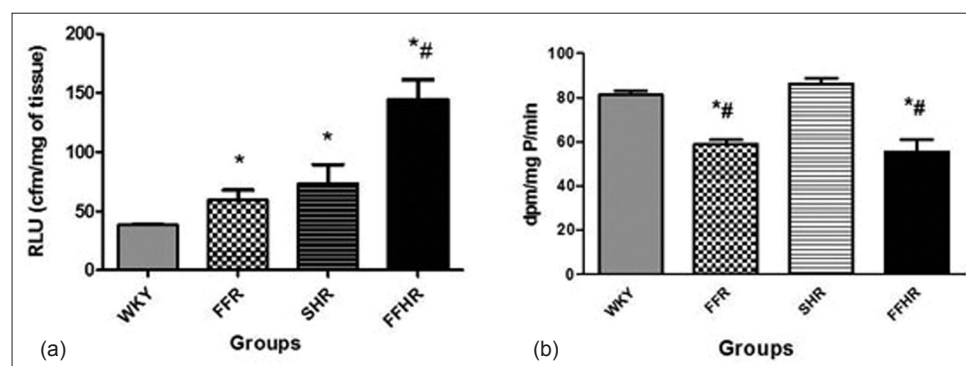
Previous attempts to culture EPC gave rise to highly variable results due, primarily to divergent culture conditions,<sup>[16,17]</sup> and in addition, the absolute number of



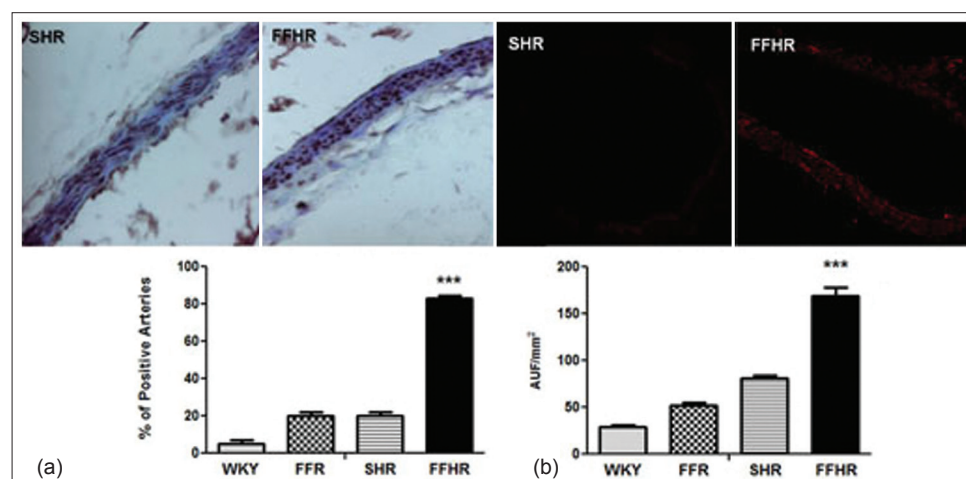
**Figure 1:** Representative cytograms used for the quantitative enumeration of EPCs by flow cytometry. (a) Endothelial progenitor cells belong to the mononuclear cell fraction, which can be identified using a morphological gate for the low side scatter. (b) Dot plot of CD34<sup>+</sup>/FLK-1<sup>+</sup> cells. (c) Quantification by flow cytometry of EPC levels in bone marrow and peripheral blood of the four groups of animals. A significant difference was assessed by Kruskal-Wallis test and Dunn's post test \**P* < 0.05 vs. WKY and # vs. SHR



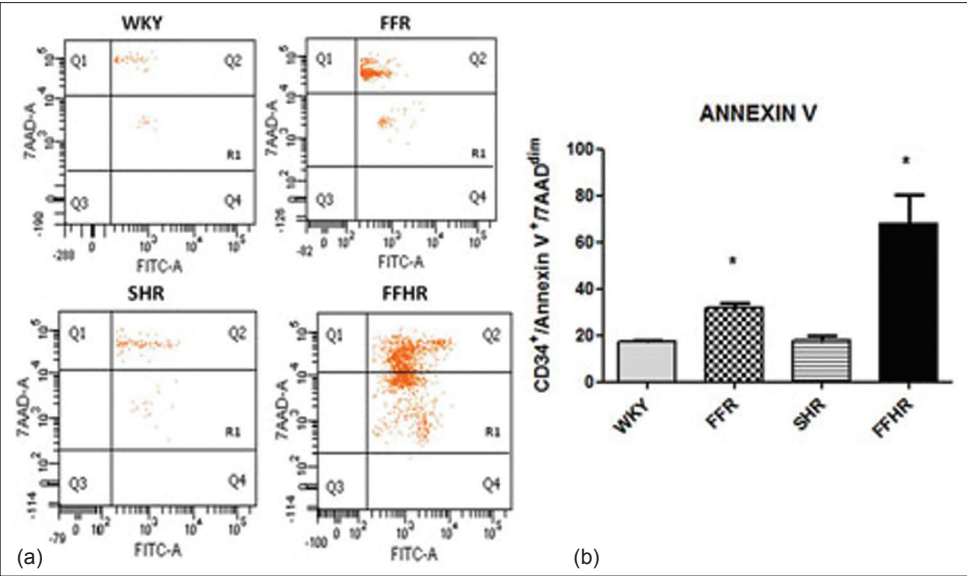
**Figure 2:** EPC Morphology. (a)  $\times 100$  one colony on fifth day. Scale bar 10  $\mu$ m. (b) 10 $\times$  confluent cells on tenth day. Scale bar 20  $\mu$ m (c) H and E staining. Scale bar 20  $\mu$ m ( $\times 10$ ). (d) Immunofluorescence of CD34 $^{+}$ /VEGFR-2 $^{+}$  cells obtained by immunomagnetic separation. Scale bar 5  $\mu$ m ( $\times 40$ ). Colonies count. (e) The number of colonies per field was diminished in the fructose treated groups \* $P < 0.05$  vs. WKY and # vs. SHR



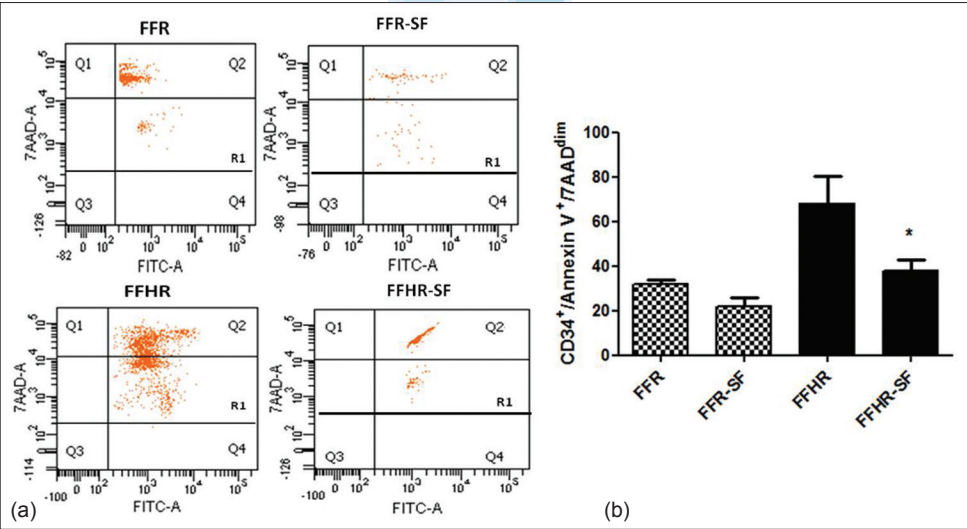
**Figure 3:** Effects of fructose feeding on oxidative stress state. (a) NAD(P)H oxidase activity in aorta homogenates. (b) eNOS activity in aorta homogenates. A significant difference was assessed by One-way ANOVA and Bonferroni's Multiple Comparison post-test. \* $P < 0.05$  vs. WKY and # vs. SHR



**Figure 4:** Apoptosis in the arterial wall. (a) Caspase-3 activated (immunohistochemistry) WKY:  $5 \pm 1.98$ , FFR:  $20 \pm 1.9$ , SHR:  $20 \pm 2.23$ , FFHR:  $83 \pm 1.83$  (percentage of positive arteries). (b) Caspase-8 activated (immunofluorescence) WKY:  $29.60 \pm 1.63$ , FFR:  $52.60 \pm 3.01$ , SHR:  $81 \pm 3.0$ , FFHR:  $169 \pm 9.0$ , \* $P < 0.05$  arbitraries units of fluorescence/mm<sup>2</sup>. Both markers were increased in the FFHR group, suggesting the presence of apoptotic cells in the arterial walls of these animals



**Figure 5:** Gating strategies for flow cytometry. (a) In the lower right quadrant the % of apoptotic cells (annexin V<sup>+</sup>/7AAD<sup>dim</sup>) was higher in the fructose feeds rats with no differences in the SHR group. A significant difference was assessed by Kruskal-Wallis test and Dunn's post test \**P* < 0.05 vs. WKY. (b) EPC with decreased membrane integrity were identified based on intermediate 7AAD staining. To evaluate if these cells were apoptotic, the binding of annexin V (FITC) to EPC was measured, and these cells were identified as apoptotic cells when they also had a 7AAD<sup>dim</sup> staining. CD34<sup>+</sup>/annexinV<sup>+</sup>/7AAD<sup>dim</sup> cells (R<sub>1</sub>) are increased in both groups with fructose



**Figure 6:** Apoptosis decrease by stopping the fructose administration. (a) In the flow cytometry dot plot CD34<sup>+</sup>/annexinV<sup>+</sup>/7AAD<sup>dim</sup> cells (represented in R<sub>1</sub>) are compared. (b) Bars show a diminished number of apoptotic EPC levels in FFR-SF (fructose fed rat-stop fructose) and FFHR-SF (fructose fed hypertensive rat-stop fructose) compared with FFR and FFHR respectively. A significant difference was assessed by One-way ANOVA and Bonferroni's Multiple Comparison post-test. \**P* < 0.05 vs. FFHR

**Table 2: The table shows how the most representative parameters of metabolic syndrome are modified after discontinuation of fructose administration**

|                          | Metabolic variables evaluation |                |              |                |
|--------------------------|--------------------------------|----------------|--------------|----------------|
|                          | FFR                            | FFR-SF         | FFHR         | FFHR-SF        |
| Fasting glucose (mmol/L) | 6.44 ± 0.18                    | 4.94 ± 0.17*** | 6.50 ± 0.19  | 5.12 ± 0.14*** |
| HOMA index               | 7.20 ± 0.10                    | 4.44 ± 0.30*** | 14.10 ± 0.40 | 6.21 ± 0.10*** |
| Insuline (ng/ml)         | 6.40 ± 2.41                    | 3.74 ± 0.30*   | 8084 ± 315   | 6.60 ± 0.16    |

Symbols indicate: \**P* < 0.05 and \*\*\**P* < 0.001 vs. FFR; \*\*\**P* < 0.001 vs. FFHR



EPC could not be quantified due to the lack of EPC-related cell markers. Recently, the identification and quantification of circulating EPC using antibodies against EPC-related cell surface antigens and flow cytometry analysis have been developed.<sup>[18]</sup>

In our study, there was a marked decrease in EPC levels from peripheral blood and bone marrow measured by flow cytometry from the animals treated with fructose compared to their controls, which was greater in the FFHR.

Increased production of reactive oxygen species is one of the most relevant factors involves in hypertension, and this phenomenon directly affects EPC function. Ingram *et al.*,<sup>[19]</sup> demonstrated that EPC are sensitive to oxidants, and treated with H<sub>2</sub>O<sub>2</sub> undergoes increased apoptosis and decreased tube formation. It has also been postulated that candesartan, an angiotensin II receptor blocker, improves EPC dysfunction through an anti-oxidative mechanism.<sup>[20]</sup>

Vascular oxidative stress has been demonstrated in SHR,<sup>[21]</sup> this genetic model exhibit increased NAD(P)H driven superoxide generation in resistance (mesenteric) and conduit (aortic) vessels, associated with NAD(P)H oxidase subunit overexpression and enhanced oxidase activity.<sup>[22,23]</sup>

It has been known that vascular tissue is a rich source of reactive oxygen species (ROS).<sup>[24]</sup> In this scenario, we measured the NAD(P)H oxidase and eNOS activity and we found an increase in the first and a decrease in the second in both groups treated with fructose in comparison to the control groups, leading FFHR in the worst condition due to the lower eNOS and the highest NAD(P)H activities.

It has been recognized several stimuli for apoptotic cell death. One possible explanation for changes in EPC may be an increased apoptosis levels in these cells, which could be triggered by the hyperglycemia,<sup>[26,27]</sup> the insulin resistance<sup>[28]</sup> and the oxidative stress present in this experimental model.<sup>[25]</sup> In our experiments we could observe a higher percentage of EPC in the experimental groups that binds annexin V; a specific marker for apoptosis, which also showed a higher 7-AAD uptake, confirming the increased apoptosis in EPC.

We could observe that this phenomenon is reversed upon discontinuation of administration of fructose lowering the percentage of apoptotic cells.

In conclusion, the metabolic syndrome caused by chronic

administration of fructose in spontaneously hypertensive rats (FFHR) has proven to generate a decrease in EPC levels, as well as in their proliferation potential. Our findings suggest that the increased apoptosis levels are the probably underlying cause for this phenomenon.

## ACKNOWLEDGMENT

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# Lemboa, *et al.*: Apoptosis of endothelial progenitor cells in a metabolic syndrome experimental model

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