High-fat diet increases STAT-3 and c-Myc expression and induces VEGF production in bone marrow mesenchymal stem cells in a rat model

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Excessive intake of a high-fat diet (HFD) results in overweight, obesity and the development of insulin resistance, adipose tissue macrophage infiltration and significant increases in inflammatory biomarkers. Bone marrow mesenchymal stem cells (MSCs) are directly involved in hematopoiesis and angiogenesis, and are currently receiving considerable attention due to their remarkable applications to cell therapy. In obese people, hematopoiesis and angiogenesis can be affected as a result of the unbalanced production of several kinds of mediators, such as VEGF. VEGF production is regulated by transcription factors such as Stat-3, which can also activate other transcription factor regulators such as c-Myc, which is closely correlated to cell proliferation. Two-month-old male Wistar rats were fed an HFD and bone marrow MSCs were isolated. The cell cycle, Stat-3 and c-Myc expression, and VEGF production were evaluated. HFD animals showed greater adipose tissue mass as well as higher blood cholesterol, leptin, and C-reactive protein levels. MSCs from the HFD group showed a higher percentage of cells in the S/G2/M phase, increased production of VEGF and higher expression of c-Myc and Stat-3. These data led us to infer that HFD induces alterations in bone marrow MSCs, which could modify their modulatory capability and affect their use in cell therapies.

Keywords: Cell cycle, Cell therapy, Cholesterol, Hematopoiesis, Obesity, Transcription factors, Transcription regulator

The recent rise in human obesity is caused by increased energy intake and decreased energy expenditure, resulting in a massive increase in adipose tissue, insulin resistance, hyperinsulinemia, and dyslipidemia. These factors are linked to metabolic syndrome¹,², which is thought to be a major contributor to the development of diabetes as well as cardiovascular complications³,⁴.

There is a general consensus that metabolic diseases are commonly linked to the inflammatory process, characterized by low-grade and chronic activation of the inflammatory response⁵,⁶. In our earlier studies, we demonstrated that ingestion of a high-fat diet (HFD) in a rat model induces changes in the hematopoietic system, leading to leukocytosis and neutrophilia as a result of the greater capacity of bone marrow hematopoietic cells regarding proliferation and differentiation into granulocytic cells⁷,⁸.

It is well-known that adequate hematopoiesis depends on the bone marrow microenvironment, which is formed mainly of mesenchymal stem cells (MSCs) and cells derived from their differentiation. MSCs have the capability to differentiate along multiple mesenchymal lineages; thus, they are not only important for hematopoiesis but are also used for several procedures in cell therapy⁹. MSCs have received considerable attention due to their potential benefit in the treatment of diseases that result in a loss of viable tissue; for example, an ischemic myocardium is commonly observed in obese individuals⁰. MSCs have been shown to protect myocardial function from ischemic injury through paracrine actions by increasing the local production of vascular endothelial growth factor (VEGF), which has important functions in hematopoiesis and is the principal regulator of blood vessel formation¹¹,¹².

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It has been reported that VEGF expression is associated with the activation of signal transducer and activator of transcription 3 (Stat-3)\textsuperscript{13,14}. Stat-3 has been implicated in a variety of cellular functions, including cell survival, proliferation, inflammation, and angiogenesis. Activated Stat-3 provokes the upregulation of genes encoding cell cycle regulators such as c-Myc, which is closely correlated with increased cell proliferation rates and contributes to the modulation of VEGF expression\textsuperscript{14-16}.

Knowing that MSCs are important cells for angiogenesis and hematopoiesis and have important applications in cell therapy, and due to the sparse data available on the effects of HFD intake on MSC cell cycling, VEGF production, and Stat-3 and c-Myc expression, the purpose of this study was to evaluate the effects of HFD ingestion on these parameters.

Materials and Methods

Animals and Treatments

Male Wistar rats (initial weight: 298 ± 27 g), aged 2 months old, were obtained from the Animal Laboratory of the School of Pharmaceutical Sciences at the University of São Paulo. Rats were housed in plastic cages (2-3 rats per cage) in an atmosphere of 55 ± 10\% relative humidity, at 22 ± 2°C, with a 12h light/dark cycle (lights on at 07.00 hours). Rats were given free access to food and water. Body mass and diet intake were recorded three times a week. The study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences at the University of São Paulo according to the guidelines of the Brazilian College on Animal Experimentation. After acclimatization for 10 days to a semi-purified diet based on the American Institute of Nutrition’s recommendations for the adult rodent (AIN-93M)\textsuperscript{17}, rats were randomly assigned into two groups: the HFD group and the control (CON) group. For 12 weeks, the CON group received the AIN-93M diet (total energy: 75.8\% carbohydrates, 9.3\% fat, and 14.9\% protein), while the HFD group received an AIN-93M-based diet that was enriched with lard (total energy: 24.2\% carbohydrates, 60.9\% fat, and 14.9\% protein). In a pilot study, it was observed that the rats in the HFD group consumed approximately 30\% less diet than the rats in the CON group. However, given the higher energy density of the HFD (5.55 kcal/g) compared to the CON diet (3.99 kcal/g), the daily energy intake did not differ between the groups. The insufficient intake of micro- and macronutrients by the HFD group rats, caused by the lower consumption of diet, could significantly affect the outcomes of the study. Therefore, to ensure comparable intakes of micro- and macronutrients between the groups, diet ingredients (except for starch and lard) were normalized according to their energy densities, as shown in Table 1. Additionally, because HFD feed is highly susceptible to oxidation, its tert-butylhydroquinone content was increased\textsuperscript{18}.

Blood and adipose tissue mass

Animals were anesthetized with xylazine chlorohydrate (Rompum\textsuperscript{18}, 10 mg/Kg, Bayer S.A., São Paulo, SP, Brazil) and ketamine chlorhydrate (Ketamina\textsuperscript{18}, 100 mg/Kg, Cristália Ltda., Itapira, SP, Brazil), and whole blood samples were collected in tubes without anticoagulant, samples serums were separated and cholesterol was quantified using standard method. Quantification of the leptin and C-reactive protein levels in the serum was performed by the Luminex-based bead-array method LINCOplex simultaneous multi-analyte detection system (Linco Research Inc., St. Charles, MO, USA) according to the manufacturer’s instructions. After euthanasia, adipose tissue was collected, and the epididymal and retroperitoneal fat pads were isolated and weighed.

Bone marrow MSC isolation

Femurs were removed under aseptic conditions, and bone marrow cells were flushed using Dulbecco’s Modified Eagle’s Medium (DMEM) (Vitrocell, Campinas, SP, Brazil) supplemented with 10\% fetal calf serum (Vitrocell, Campinas, SP, Brazil). Cells were washed by adding complete media, centrifuging for 5 min at 300 rpm at 24°C, and removing the supernatant.

Table 1 — The composition of the experimental diets. According to AIN-93M\textsuperscript{18}

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CON diet\textsuperscript{18}</th>
<th>HFD diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>155.40</td>
<td>29.65</td>
</tr>
<tr>
<td>Sucrose</td>
<td>25.04</td>
<td>25.04</td>
</tr>
<tr>
<td>Casein</td>
<td>35.05</td>
<td>35.05</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>10.01</td>
<td>10.01</td>
</tr>
<tr>
<td>Lard</td>
<td>55.44</td>
<td>55.44</td>
</tr>
<tr>
<td>Cellulose</td>
<td>12.52</td>
<td>12.52</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>8.76</td>
<td>8.76</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
<td>0.002</td>
<td>0.007</td>
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</table>

Average body weight gain during the 12 weeks was 16.1 ± 2.1 g in the CON group and 6.1 ± 2.1 g in the HFD group.
The bone marrow cell pellet was resuspended and cultured in 75 cm² culture flasks with complete media at 37°C with 5% CO₂ in the air. MSCs preferentially attached to the polystyrene surface. After 48h, non-adherent cells in suspension were discarded. Fresh complete media was added and replaced every three or four days thereafter. Cells were confirmed to be MSCs by checking for cell surface markers CD14−, CD34−; CD 271+, CD90+, and Sca-1+. Cultures were passaged when they reached 90% confluency by detaching them with 0.25% trypsin–EDTA (GIBCO Invitrogen, Carlsbad, CA) and replating them in culture flasks. Cells were utilized for experimentation in passage 3.

Bone marrow MSC culture and VEGF Quantification

MSCs were isolated as described above and cells at passage 3 were cultured in DMEM medium (Vitrocell, Campinas, SP, Brazil) supplemented with 10% fetal calf serum at a concentration of 1 × 10⁶ cells/mL. These cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24h. Supernatants were subsequently collected for VEGF quantification, which was performed using the Luminex-based bead array method LINCOplex simultaneous multi-analyte detection system (Linco Research Inc., St. Charles, MO, USA) according to the manufacturer’s instructions. The entire procedure was executed under aseptic conditions, and all of the materials were previously sterilized and were pyrogen-free.

MSC cell cycle: 7-Aminoactinomycin D (7-AAD)

The bone marrow MSCs from CON and HFD groups were obtained as described above. The MSCs were fixed in 2% paraformaldehyde for 30 min, permeabilized with 0.01% saponin for 15 min and treated with 4 µg/mL of RNase at 37°C for 1h. After this period the DNA was labeled with 7AAD (Sigma Chemical Co., St. Louis, MO, USA) 10 µg/mL. The analysis of the cell cycle was assessed by quantifying the percentage of cells in the G0/G1 and S/G2/M phases, which data acquisition was performed in a FACS Calibur (Becton Dickinson) cytometry, using the Cell Quest® software package. For data analysis, ModFit LT™ software was used.

Western Blot Analysis: Stat-3 and c-Myc quantification

To determine the protein levels of total and phosphorylated Stat-3 and c-Myc, MSCs were isolated and washed three times with sterile cold PBS and were lysed with RIPA buffer (0.1% SDS, 1% Igepal CA-630, 1% sodium deoxycholate, 10 µM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5 mM EDTA) in the presence of a protease and phosphatase inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA). After centrifugation at 14000 rpm and 4°C for 15 min, the supernatant was collected, mixed with 5X Laemmli buffer (1M Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 10% SDS, 50% glycerol, and 0.01% bromophenol blue) and boiled for 5 min. The protein content of cell homogenates was determined using a BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA), and equal amounts of protein (30 µg per well) were separated on 7.5% SDS-polyacrylamide mini gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). After overnight incubation with the appropriate primary antibodies (1:1000), including Stat-3 (cat no. sc-482, Stat-3 phosphorylated (sc-8001R) c-Myc (cat no. sc-788, ) and c-Myc Phosphorylated (cat no. sc-8000R) all of them from Santa Cruz Biotechnology, Dallas Texas, USA), the membranes were washed three times and incubated for 1h with a secondary antibody conjugated to horseradish peroxidase (cat no. 7074S, Cell Signaling Beverly, Massachusetts, USA). After three washes with TBST, the immuno reactive bands were visualized using the ECL detection system (GE health care ECL™ SELECT Western Blotting Detection Kit, Little Chalfont, Bu kinghamshire, UK). To standardize and quantify the immuno blots, a digital detection system (IMAGE QUANTTM 400 version 1.0.0, Amersham Biosciences, Pittsburgh, PA, USA) was used. The results were expressed in relation to the intensity of β-actin (1:40000 for anti-β-actin, cat A3854 Sigma, Inc., Saint Louis USA) and as a percentage of the control value.

Statistics

Dependent variables were normally distributed. Results were subjected to statistical analysis (Student’s t-test) using the GraphPad Prism® software (version 5.03 for Windows; GraphPad Software, San Diego, CA, USA), and data were expressed as the mean values with their standard deviations. Differences were considered statistically significant when the P value was ≤0.05.

Results

Diet consumption, body weight, epididymal adipose tissue mass, serum cholesterol, leptin and C-reactive protein levels

The HFD group consumed about 30% less chow than CON group, however, both groups consumed the
same amount of calories. It must be highlighted that HFD group increased adiposity in the absence of excess energy intake (Table 2). The increased adiposity of HFD group was not associated with hyperphagia since there were no differences concerning energy intake between the groups. In fact, food intake (g) in HFD group was lower than in CON group during the entire protocol, however, since there was no difference in energy intake, the consumption of protein, fibre and micronutrients were similar between the groups. In addition, HFD groups showed higher weight gain ($P \leq 0.05$) when compared to CON groups with increased fat depots ($P \leq 0.001$); also HFD groups showed higher serum levels of cholesterol ($P \leq 0.01$), leptin ($P \leq 0.05$) and C-reactive protein ($P \leq 0.05$) (Table 2).

**In Vitro determination of VEGF production by MSCs**

The MSCs morphological aspects did not show difference between groups (Fig. 1A-D). However, the VEGF production by MSCs, measured in sample supernatants after 24h of culture, were significantly higher ($P \leq 0.05$) in the HFD group in comparison to the CON group (Fig. 1E).

**Bone marrow MSCs cell cycle assay**

The cell cycle analysis of bone marrow MSCs showed an increased percentage of cells in the S/G2/M phases in the HFD group compared to the CON group (Fig. 2) concomitantly with a reduced percentage of cells in the G0/G1 phase of the cell cycle.

**Western Blots (total and phosphorylated Stat-3 and c-Myc expression)**

The evaluation of the expression of total Stat-3 (Fig. 3A) in both groups did not show significant differences, however when it was evaluated the phosphorylated form of Stat-3 (Fig. 3B), MSCs from HFD group showed increased expression in comparison to the CON group ($P \leq 0.05$). In addition, the total c-Myc expression did not show the difference between groups (Fig. 3C), however, the phosphorylated form of c-Myc (Fig. 3D) was higher in MSCs from HFD group in comparison to CON group.

**Discussion**

This study shows that the HFD leads to an increase in the fat depots associated with increased cholesterol, leptin and C-reactive protein levels. Additionally, the

<table>
<thead>
<tr>
<th>Groups</th>
<th>CON (n=8)</th>
<th>HFD (n=14)</th>
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<tbody>
<tr>
<td>Diet consumption (g/day/animal)</td>
<td>24.93 ± 3.03</td>
<td>17.39 ± 2.19***</td>
</tr>
<tr>
<td>Kcal consumption (day/animal)</td>
<td>99.48 ± 12.1</td>
<td>96.54 ± 12.17</td>
</tr>
<tr>
<td>Lipid consumption (g/day/animal)</td>
<td>1.03 ± 0.12</td>
<td>6.53 ± 0.82***</td>
</tr>
<tr>
<td>Body weight variation (%)</td>
<td>+66.5 ± 6.48</td>
<td>+80.1 ± 14.10*</td>
</tr>
<tr>
<td>Epididymal adipose tissue mass</td>
<td>9.21 ± 1.71</td>
<td>15.17 ± 3.03 ***</td>
</tr>
<tr>
<td>Retroperitoneal adipose tissue mass</td>
<td>8.57 ± 2.27</td>
<td>19.11 ± 3.03 ***</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>72.44 ± 9.93</td>
<td>86.29 ± 10.88 **</td>
</tr>
<tr>
<td>Leptin (pg/mL)</td>
<td>4099 ± 2336</td>
<td>5888 ± 3492 *</td>
</tr>
<tr>
<td>C-reactive protein (ng/mL)</td>
<td>611 ± 260</td>
<td>1002 ± 168*</td>
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</tbody>
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The numbers in brackets denote the number of animals used in the experiment. *($P \leq 0.05$), **($P \leq 0.01$) and ***($P \leq 0.001$) where there was a significant difference between the HFD and the CON groups.

Fig. 1 — Bone marrow mesenchymal stem cells (MSCs) morphology of control group (CON) and high fat-diet group (HFD). Bright field microscope, optical magnitude 10X (A & B) and optical magnitude 40X (C & D). In vitro production of VEGF by bone marrow MSCs of the CON and the HFD (E). The results are expressed as the mean ± SD of the level of VEGF in the supernatant of bone marrow MSCs culture from the CON (n=6) and HFD (n=6) groups. * Indicates that there was a significant difference between the CON and HFD groups ($P \leq 0.05$).
Bone marrow MSCs from HFD was driven to a proliferative state involving the activation of Stat-3 and c-Myc in parallel to a higher VEGF production.

Obesity is associated with insulin resistance, dyslipidemia, and hypertension which are well-known risk factors for most chronic diseases and represent major challenges for basic and clinical research. In our study, it was used a well-established model of HFD to study obesity in rodents. The main advantages of this model lie on its physiological properties as well as its similarity to human disease etiology. Studies have shown that the ingestion of high levels of lipids resulted in increased body weight with a significant increase in the fat depots associated to elevated serum cholesterol levels, which are in accordance with the results obtained in the current work.

Several studies have shown that the nutritional status can influence the function of MSCs, specially their capability to differentiate. The functions of MSCs can vary depending on the cell source, which may be regulated in part by increased levels of free fatty acids, but also may involve other obesity-associated cytokines. MSCs biology comprehension, in different biological models, is important to get a deeper knowledge about the mechanisms that control the fate of these cells as well as how they can modulate different kind of systems, in order to use them in cellular therapies on a better way.

MSCs are a potent source of VEGF, which is an important growth factor able to stimulate vasculogenesis and angiogenesis. Among various tissues that produce VEGF, adipose tissue can act as an endocrine organ producing VEGF in a considerable amount. Previous studies reported that overweight and obese people display elevated serum VEGF levels. Obese patients face several...
specific challenges related to diagnosis and treatment of cancer. Obese patients with cancer have a worse prognosis, decreased disease-free interval after initial treatment and increased risk of metastatic disease suggesting that angiogenesis may be enhanced in obesity.\textsuperscript{20,31,32}

In the current work, MSCs from HFD animals showed the higher capability to proliferate which was evidenced by the higher percentage of cells in the S/G2/M cell cycle phase associated with increase expression of phosphorylated Stat-3 as well as phosphorylated c-Myc and higher production of VEGF. The literature reports that p-Stat3 expression is strongly correlated with VEGF expression, suggesting that dysregulated Stat3 activation may play an important role in VEGF overexpression and an elevated angiogenic phenotype.\textsuperscript{33} The up-regulation of Stat-3 may stimulate the neovascularization as well as the VEGF production\textsuperscript{34} while c-Myc is a key regulator of cell proliferation and its up-regulation is associated with increased proliferation rates.\textsuperscript{35}

Furthermore, VEGF was reported to have hematopoietic effects, inducing colony formation by mature subsets of progenitor cells.\textsuperscript{16} Our group has previously demonstrated the possible effect of VEGF on hematopoiesis in HFD animals since these animals displayed increased bone marrow cellularity and granulopoiesis associated with peripheral leucocytes and neutrophilia and higher capability to form granulocytic colonies in vitro\textsuperscript{6}.

Additionally, some studies report that TNF-stimulated MSCs release VEGF which is mediated by Stat-3 signalling.\textsuperscript{37,38} This finding is in accordance with another previous report of our group\textsuperscript{25} in which we demonstrated that MSCs from HFD animals produce higher amounts of TNF, which could infer another mechanism by the increased VEGF levels as reported in the current work. Our results may also infer that increased expression of Stat-3 is able to mediate the increased VEGF production, and the up regulation of Stat-3 is able to increase the expression of c-Myc which is a factor regulated by Stats and associated with the G1 to S cell cycle transition.\textsuperscript{14,15,39}

Altogether, our data shows that nutritional status, specifically the high ingestion of lipids, increases cholesterol, leptin and C-reactive protein levels and alters the proliferation rates of bone marrow MSCs and the expression of Stat-3 and c-Myc as well as the VEGF production, in which can affect the MSCs modulatory properties and their application on cell therapies.

Acknowledgement

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