

Impact of glucotoxicity induced *in vivo* and *in vitro* in *Psammomys obesus*

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ABSTRACT

Objective: Chronic hyperglycemia characteristic of type diabetes 2 is responsible for the accelerated atherosclerosis with increased cardiovascular risk. In this study, we will propose to analyze the effect of a long-term of glucotoxicity *in vivo* in *Psammomys obesus* by addition of sucrose to 30% for 11 months and *in vitro* study of adventitial fibroblasts in the presence of D-glucose 0.6% for 7 days. **Materials and methods:** Evaluation of plasma biochemical parameters was carried out at the initial time and at the end of experiment. At autopsy, a morphological study of the aorta was performed after fixation in aqueous Bouin and staining with Masson's trichrome. The experimental glucotoxicity is induced by incubation of fibroblasts in DMEM enriched with D-glucose at 0.6% for 7 days. The impact of glucotoxicity is assessed in the intracellular compartments through dosage of total nitrite and malondialdehyde, a product of lipid peroxidation, and thanks to a morphological assay after fixation of cells with aqueous bouin and blood staining with May Grünwald Giemsa. The evaluation of cell proliferation is accomplished by cell counting. **Collagens I and III of the extracellular compartment are characterized by SDS-PAGE. Results:** Animals subjected to sucrose showed hyperglycemia associated with hyperinsulinemia, dyslipidemia, hyperproteinemia, increased CPK and VLDL-LDL and decreased HDL. Histology of aortas revealed endothelial cells hypertrophy, severe disorganization of intima and media. In the presence of glucose, the proliferation of fibroblasts increases very significantly ($P = 2.34 \times 10^{-5}$), the rate of

malonaldehyde, nitrite and total density of chains $\alpha 2$ (I) and $\alpha 1$ (I + III) extra-cellular collagens I and III increased significantly. After staining, the cells showed hypertrophy, vacuolation of cytoplasm and chromatin condensation with nuclear fragmentation, indicative of apoptosis. **Conclusion:** The glucotoxicity induced *in vivo* and *in vitro* is responsible for major structural and metabolic alterations leading to the acceleration of the atherosclerotic process.

Keywords: *Psammomys obesus*; Aorta; Adventitial Fibroblasts in Culture; DT2; Glucotoxicity; Extracellular Matrix; Oxidative Stress

1. INTRODUCTION

Chronic hyperglycemia characteristic of type diabetes 2 is responsible for the accelerated atherosclerosis and increased cardiovascular risk [1], it is established that cardiovascular complications are the major causes of diabetic patients morbidity and mortality worldwide [2,3]. Chronic hyperglycemia induces a modulation of the expression of many key proteins involved in glucose toxicity [4]. It induces a dysfunction of the intracellular signal transduction in modulating the activity of kinase C protein, generating oxygen reactive species, activating ER-stress, generating advanced glycation end products, activating polyol pathway and hexoamines, increased release of proinflammatory cytokines as well as growth factors. All these alterations lead to endothelial dysfunction which may be regarded as a sign of vascular disease being a key factor in the development of atherosclerosis [1, 4,5].

Given our interest in cardiovascular complications risk factors and knowing that *Psammomys obesus*, is a choice

model for diabetes and atherosclerosis, we found it interesting to analyze the effect of a long-term glucotoxicity *in vivo* by adding 30% sucrose during 11 months period of experimentation on aortic vascular cells of *Psammomys obesus* and analyzing the effect of glucotoxicity *in vitro* on fibroblast aortic subculture.

2. MATERIAL & METHODS

2.1. Biological Material

Our study is conducted on an experimental model of gerbillid class (*Psammomys obesus*), gopher, a deserticolous rodent from the region of Beni-Abbes, south west of Algeria, in city of Bechar (30°7 northern Latitude and 2°10 western longitude). In its natural environment, this gerbil eats halophilic Chenopodiaceae poor in calories (0.4 Kcal/g for *Salsola foetida*). The Chenopodiaceae are very rich in water and mineral salts especially the sodium salt. *Psammomys* is an animal primarily diurnal, living alone or in small groups in burrows offering shelter from external temperature with high moisture (50% - 80%) [6].

After familiarization time, animals are divided into two pools, one for control and another experimental: a control pool (n = 6 animals) with average body weight of 94.83 ± 2.99 g receiving 50 g of halophilic plants daily, which corresponds to 20 - 22 calories of energy intake per animal and an experimented pool (n = 6 animals) with average body weight of 86.16 ± 4.87 g receiving daily 50 g of halophilic plants with added 30% sucrose, corresponding to 80 - 82 Calories of energy intake per animal (1 g of sucrose equals 4 calories).

2.2. Methods

2.2.1. Analytical Methods

During the experiment which lasted 11 months and in order to monitor changes in body weight, animals were weighed on a weekly basis during the 11 months of experiment. For metabolic monitoring, monthly blood samples were performed on wake animals through puncture at the retroorbital sinus of the eye using a Pasteur pipette [7]. This technique avoids the use of anesthetics that may impact biochemical parameters. The immediately blood sampled on heparinized and dry tubes is centrifuged (3000 rpm). Plasma is collected for the measurement of biochemical parameters (glycemia, triglyceride, cholesterolemia and serum proteinemia) performed using RANDOX kits and the sera for assay of lipoprotein on agarose gel by the method of Kalwakami (1989) REP-controller kit on lipid Lp (a) of the KPC (cassette COBAS INTEGRA) and insulin (INSULIN-CT kit, CIS Bio International).

2.2.2. Organs Harvesting

At the end of the experiment (11 months), animals were

sacrificed after anesthesia by intraperitoneal injection of urethane at 25%, at 0.4 ml/100 g body weight. Some aortas were harvested under sterile conditions for cell culture and others for the histological study, the rest of organs are fixed and/or frozen at -80°C for further study.

2.2.2.1. Histology of the Aorta

The aortas were fixed in Bouin's aqueous fluid for three days and then washed with running water for 48 hours. Dehydration is carried out in alcohol baths with increasing concentration of 30 min (50° , 70° , 90° , 100°). For complete removal of traces of alcohol, the body is immersed in two baths of butanol for 30 minutes, then immersed in two baths of paraffin for 2 hours at 6°C . Paraffin blocks are made using the Leuckart bars and sections of 5 microns thick are made using an American Optical type microtome. For the structural study, the aorta was stained with Masson trichrome [8].

2.2.2.2. Culture of Adventitial Fibroblasts

The adventitial fibroblasts of *Psammomys obesus* were cultured by the explant technique [9]. The control aorta was removed and immediately plunged in a petri dish containing Dulbecco's modified Eagle's medium DMEM with 10% fetal calf serum (FCS) supplemented with 1% antibiotics (streptomycin 50mg/ml, penicillin 50 IU/ml, Sigma), 1.2% glutamine (Sigma) and 5% Hepes to maintain pH. The aortic lumen is then emptied of its blood. The aorta is incubated for 20 minutes at 37°C in 0.1% collagenase, to remove the endothelium and facilitate the separation between the adventitia and media, and cut into 1 mm explants, 8 - 10 explants were placed in flasks and incubated in the presence of DMEM 20% FCS, 1.2% glutamine and 1% antibiotics and plunged into the incubator at 37°C under humidified atmosphere with 95% air and 5% CO_2 . The cultivation of explants is the primary culture.

On secondary culture and at the ninth passage and at confluence, they were suspended after trypsinisation. They were sown in flasks at 1.5×10^6 cells per flask in DMEM supplemented with 10% FCS, 1.2% glutamine, 1% TBA and incubated in the presence of D-glucose at different dosages, 0.6% for 7 days. At confluence, the medium is removed, the control fibroblasts and fibroblasts subjected to glucose are reincubated in 1.5 ml DMEM without FCS for 24 h. The milieu represents the extracellular compartment; the intracellular compartment is recovered in 1.1 ml of DMEM after scratching with a Pasteur pipette. The intra and extracellular compartments are collected for determinations of total protein, lipid peroxidation products and total nitrite.

2.2.3. Proliferation Study

At the 9th passage and after exposure to D-glucose,

the cells are trypsinized, the evaluation of the rate of proliferation was performed on 100 μ l of cell suspension by counting on Malassez cell.

2.2.4. Morphometric Study

To analyze the state of adventitial fibroblasts of *Psammomys obesus* submitted to D-glucose, these were, at their ninth pass, sown in 6-well plates at 2×10^5 per well in the presence of D-Glucose at 0.6% for 7 days. The milieu is eliminated and the cells washed in 1x PBS and fixed in Bouin's aqueous for 30 minutes. After rinsing with PBS and alcohol (96%), cells were stained for 10 minutes with a solution of May-Grunwald Giemsa (V/V, 1/1) diluted to 1/3 in distilled water. The excess of stain is removed with 1X PBS. The nucleolus count is conducted on 100 cells in different fields and on several wells.

2.2.5. Determination of Malondialdehyde (MDA)

The MDA measured in the intracellular compartments of control fibroblasts and subjected to D-Glucose 0.6% for 7 days, after reaction with TBA [10]. Intracellular compartments of control fibroblasts and those subjected to D-Glucose were centrifuged at 10,000 g for 20 min at 4°C in a buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) 0.2 M, pH 6.5. The MDA content in the supernatant in the presence of 10% TCA reacts with TBA and causes the formation of a complex read at 532 nm.

2.2.6. Determination of NO

NO formation is typically and indirectly assessed by determining the concentrations of nitrites and nitrates that are products of oxidative degradation of NO. The intracellular compartments of control cells and those subjected to D-Glucose were deproteinized by centrifugation at 10,000 g for 10 minutes at -20°C. The determination of nitrite and nitrate is produced directly from the obtained supernatant. The Griess reaction only allows the measurement of nitrite. Nitrates should be reduced to nitrite prior to be quantified. The concentration thus measured represents the sum of nitrites and nitrates. The conversion of nitrate to nitrite is based on a reduction reaction by cadmium and regenerated using a solution of CuSO_4 in 5 mM glycine-NaOH buffer, in contact for 5 minutes. The nitrite contained in the intracellular compartments of control cells, and those subjected to D-Glucose, deproteinized and regenerated are quantified after addition of Griess reagent [0.1% N-(1 naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% phosphoric acid]. OD reading is made at 543 nm [11].

2.2.7. Determination of Total Protein and Collagen

Samples (extra-and intracellular compartments of con-

trol and of cells subjected to D-Glucose) are recovered in the dialysis tubes (Serva, 8/32). These undergo two successive 24 h dialysis, respectively against running water and acetic acid 0.5 M at 4°C with shaking. After dialysis, an aliquot is taken to quantify the total protein and the rest underwent a third dialysis in the presence of pepsin (Merck) at 200 mcg/ml, against 0.5 M acetic acid for 24 h at 4°C. Total protein and pepsin-resistant protein (collagen) contained in the ECC and the ICC will react with the Bradford reagent resulting in the formation of a blue colored complex legible at a wavelength of 595 nm [12].

2.2.8. Electrophoresis of Total Collagen

Collagens were separated by electrophoresis on vertical polyacrylamide gel in 10% in denaturing conditions [13]. Each of the lyophilized samples (500 μ l) of extra-and intracellular compartments of control cells and incubated in the presence of D-glucose are shown with 200 μ l sample buffer and placed under denaturing conditions (incubation 3 to 5 minutes at 100°C) to separate the α chains of collagen by breaking disulfide bonds.

2.3. Statistical Analysis

Our results presented correspond to the means impacted with standard deviation and analyzed by Student's test.

3. RESULTS

3.1. In Vivo Study

3.1.1. Change in Body Weight

In animals submitted to the natural diet supplemented with 30% sucrose, we recorded a very highly significant increase in body weight at the end of the experiment 142 ± 10.98 g vs. 86.17 ± 4.87 g at the initial time (T0) (See **Figure 1**).

3.1.2. Analysis of Biochemical Parameters

3.1.2.1. Study of Glycemia

In our experiment, during the first 3 months, we noted a non significant variation between control animals and those submitted to 30% sucrose, but from the 4th month, the increase in glycemia levels in animals submitted to sucrose became significant (See **Figure 2**).

3.1.2.2. Study of Triglycerides

Our results showed an increase in triglyceride levels in animals submitted to sucrose from the 2nd month of experimentation, this increase was very significant in the 4th and 7th month, it was observed till the end of the experiment. At T3 and T11 statistical analysis revealed little insignificance due to the large individual variations sometimes (See **Figure 3**).

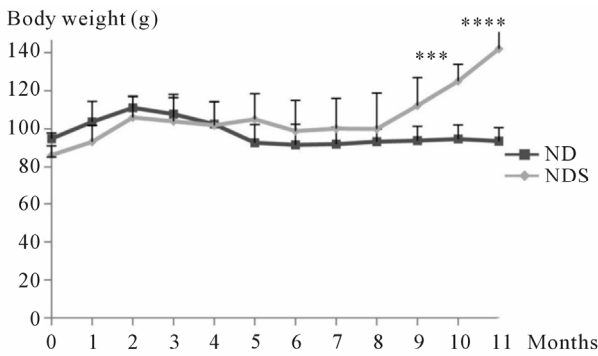


Figure 1. Evolution of body weight in both control and *Psammomys obesus* submitted to 30% sucrose for 11 months of experimentation.

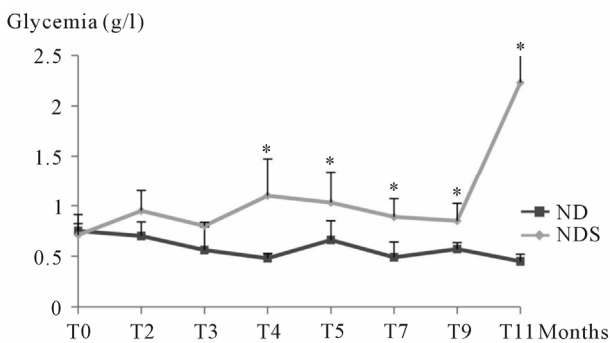


Figure 2. Glycemia levels changes in both control and *Psammomys obesus* submitted to 30% sucrose. The values are the averages impacted by standard deviation. $P > 0.05$, $*P \leq 0.05$. The level of significance is calculated for animals subjected to sucrose vs. corresponding controls.

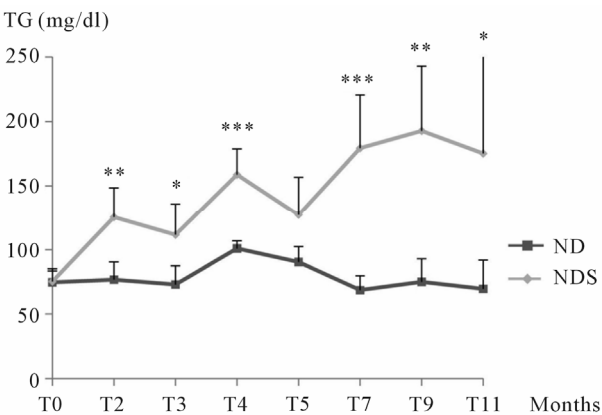


Figure 3. Changes in triglyceride levels in both control and *Psammomys obesus* submitted to 30% sucrose for 11 months. The values are averages affected with the standard deviation. $P > 0.05$, $*P \leq 0.05$, $**P \leq 0.01$; $***P < 0.001$. The level of significance is calculated for animals subjected to sucrose vs. corresponding controls.

3.1.2.3. Study of Cholesterol

During our experiments, we noted a cholesterolemia increase in animals submitted to sucrose compared to

corresponding controls, but statistical analysis revealed no significant difference during the first 4 months of treatment. From the 5th month until the end of the experiment, the increase in cholesterol in animals submitted to sucrose was significant compared to corresponding controls. (See **Figure 4**)

3.1.2.4. Study of Proteinemia

Results obtained showed little variation of proteinemia in *Psammomys* batch submitted to sucrose compared to corresponding controls. However, we noted an increase in this parameter from the 3rd month of experimentation that was significant at the end of experimentation. (See **Figure 5**)

3.1.2.5. Plasma Lipoproteins

Analysis of plasma lipoproteins in *Psammomys* submitted to natural diet supplemented with sucrose at 30% per day revealed that the latter affects all plasma lipoproteins. Our results showed little variation in the atherogenic lipoproteins rates (LDL-VLDL) till the 9th month

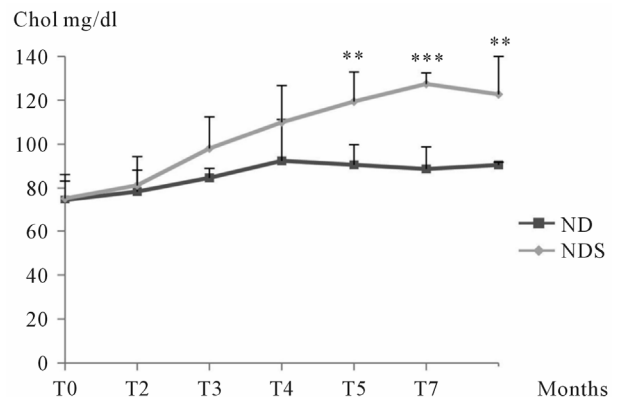


Figure 4. Changes in cholesterolemia levels in both control and *Psammomys obesus* submitted to sucrose during 11 months of experimentation.

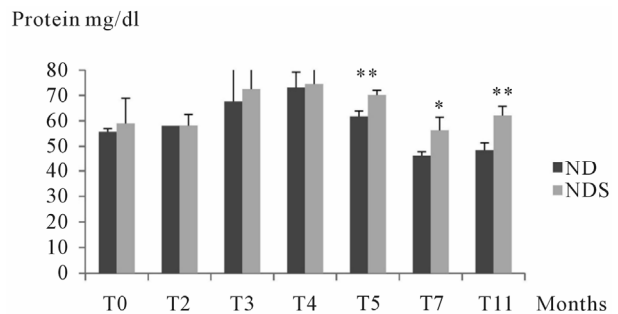


Figure 5. Variations in proteinemia in both control and *Psammomys obesus* submitted to 30% sucrose for 11 months of experimentation. The values are averages affected with the standard deviation. $P > 0.05$, $*P \leq 0.05$, $**P \leq 0.01$. The level of significance is calculated for animals subjected to sucrose vs. corresponding controls.

when we recorded rates of $48.74 \pm 11.63\%$ in the experienced group vs. $36.87 \pm 4.22\%$ at T0; statistical study revealed a significant increase. At the end of the experiment, we noted a very significant increase of $53.35 \pm 0.75\%$ in the experiment group vs. $36.87 \pm 4.22\%$ at T0. On cardioprotective lipoprotein (HDL), we noted a very significant decrease in the 9th month of experimentation, but at the end of experiment, we recorded a very significant decrease which corresponds to 46.64 ± 0.75 vs. $63.13 \pm 4, 22\%$ at T0 (See **Figure 6**).

3.1.3. Determination of Cardiac Marker CPK

Our results showed a significant increase in CPK after 4 months of treatment, compared to its value at initial time (396.2 ± 161.7 vs. 183.6 ± 68.74 U/l). At the end of the experiment, we noted a very significant increase: 4361.25 ± 1321.99 vs. 183.6 ± 68.74 U/l at T0 (See **Figure 7**).

3.1.4. The Insulinemia

During the experiment, we noted during the first 4 months, a non significant change in plasma insulin of animals submitted to the natural diet supplemented with 30% sucrose compared to T0. From the 5th month, insulin increased significantly and reached 321.25 ± 86.41 μ UI/ml at the end of the experiment vs. 20.24 ± 9.38 μ UI/ml at the initial time and statistical analysis revealed

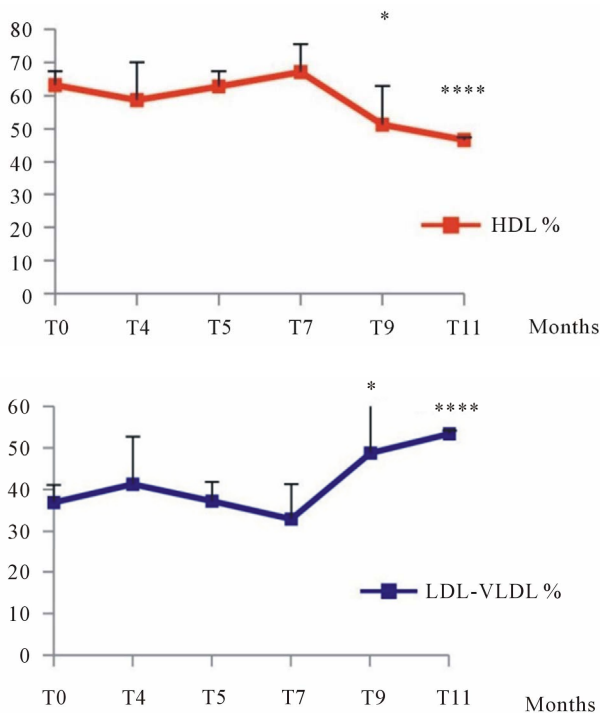


Figure 6. Changes in plasma lipoproteins in *Psammomys obesus* submitted to natural diet supplemented with sucrose at 30% for 11 months. The values are averages affected with standard deviation. * $P \leq 0.05$, **** $P < 0.0001$. The level of significance is calculated for animals subjected to sucrose vs. initial time.

a very significant change (See **Figure 8**).

3.1.5. Morphological Study

At the autopsy, the observation of these sections helped to highlight structural and functional alterations in the three layers of the aortic wall. These alterations are evidence of endothelial dysfunction, an extracellular matrix remodeling of the vascular wall (See **Figures 9, 10, 11**).

3.2. In Vitro Study

3.2.1. Study of Proliferation

Our results showed a very significant increase in the rate of proliferation of adventitial fibroblasts incubated in the presence of D-glucose compared to their corresponding controls. Indeed, after 7 days of incubation, proliferation increased with 23.9% in cells incubated in the presence of D-Glucose at 0.6% vs. the corresponding controls. Statistical analysis revealed highly significant difference (See **Figure 12**).

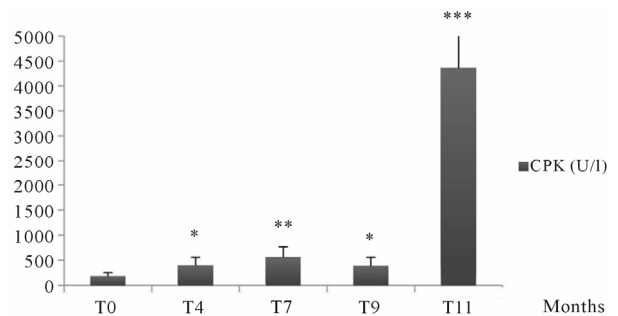


Figure 7. Changes of CPK in *Psammomys obesus* submitted to natural diet supplemented with sucrose at 30% for 11 months. The values are averages affected with standard deviation. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$. The level of significance is calculated for animals subjected to sucrose vs. initial time.

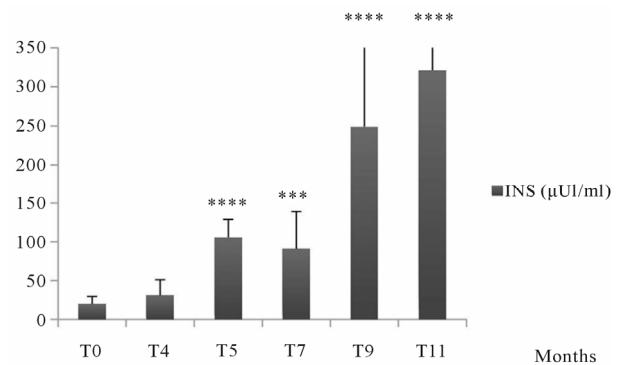


Figure 8. Changes in insulin levels in *Psammomys obesus* submitted to natural diet supplemented with sucrose at 30% for 11 months. The values are averages affected with standard deviation. $P > 0.05$, *** $P \leq 0.001$, **** $P < 0.0001$. The level of significance is calculated for animals subjected to sucrose vs. initial time.

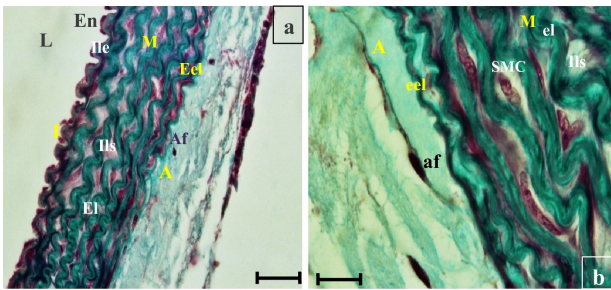


Figure 9. Histological section of aorta *Psammomys obesus* submitted to natural diet with 30% sucrose for 11 months. Masson Trichrome staining. Light (L), intima (I), endothelium (En), internal elastic limiting (Ile), elastic lamina (El), interlamellar space (ILS), smooth muscle cells (SMC), external elastic lamina (Eel), adventitia (A), adventitial fibroblats (AF).

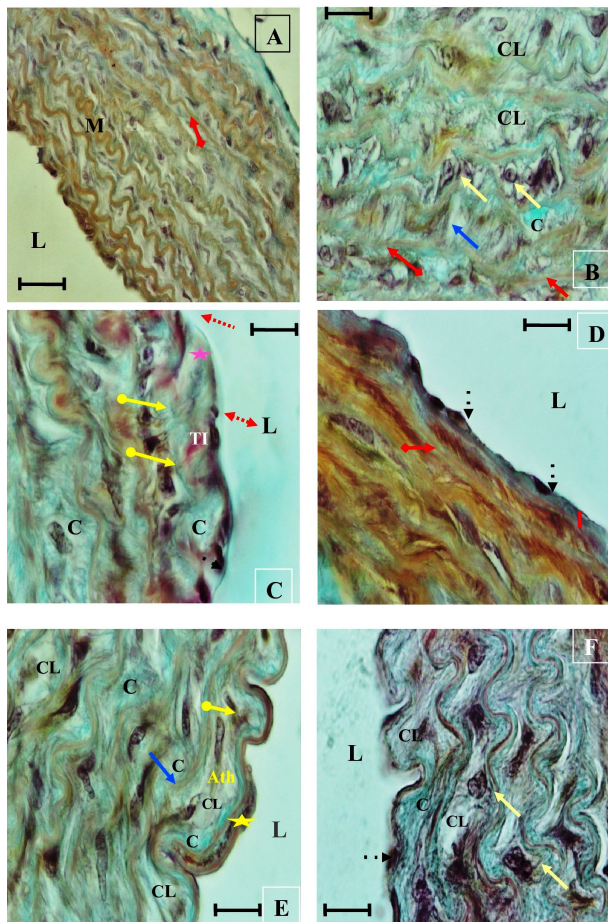


Figure 10. Histological section of aorta *Psammomys obesus* submitted to natural diet supplemented with 30% sucrose for 11 months. Masson Trichrome staining. (→) Hypertrophy of endothelial cells, Increased space in endothelial (—), Endothelia Disruption (↔), Intimal thrombus (TI), Absence of the internal elastic lamina (✱), Thickening of the intima (↔), rupture of elastic plates (→), Ripple loss, thinning of the elastic strips (↔), apoptotic nuclei (→), SMC migration (→), Duplication of internal elastic lamina (→), (C) accumulation of collagen, (CL) Core lipid, (Ath) early atheroma.

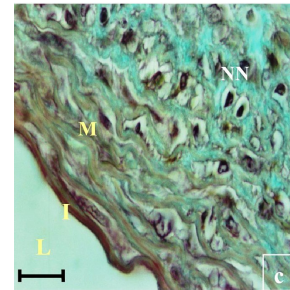
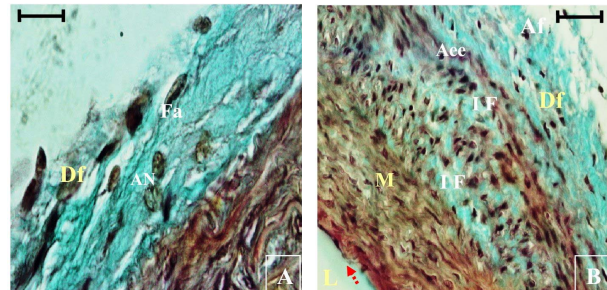


Figure 11. Histological section of aorta *Psammomys obesus* submitted to natural diet supplemented with 30% sucrose for 11 months. Masson Trichrome staining. Apoptotic nuclei (AN), Increased density of Fibroblasts (DS), Infiltration of fibroblasts (IF), Necrotic nuclei (NN).

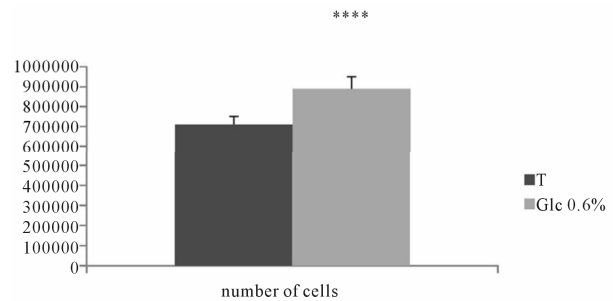


Figure 12. Rate of proliferation of adventitial fibroblasts of *Psammomys obesus* incubated in the presence of D-Glc to 0.6% for 7 days.

3.2.2. Morphological and Morphometric Study

Measuring the number of nucleoli performed on 100 cells in different fields showed an increase of 204.3% in cells incubated in the presence of D-Glucose 0.6% compared to corresponding controls. The aortic fibroblasts submitted to glucotoxicity (0.6%) for 7 days showed large morphological and functional variations marked mainly by the size that appeared to be enhanced; cytosolic vacuolization due to oncosis; densitification chromatin and a fragmented nuclei, indicating apoptosis (See Figures 13, 14).

3.2.3. Determination of Total Nitrite

The rate of total nitrite found in the intracellular compartment of adventitial fibroblasts submitted to D-Glucose at 0.6%, showed a significant increase compared to

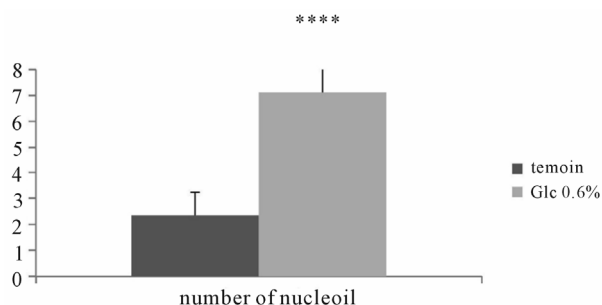


Figure 13. Number of nucleoli in adventitial fibroblasts of *Psammomys obesus* incubated in the presence of D-Glc to 0.6% for 7 days.

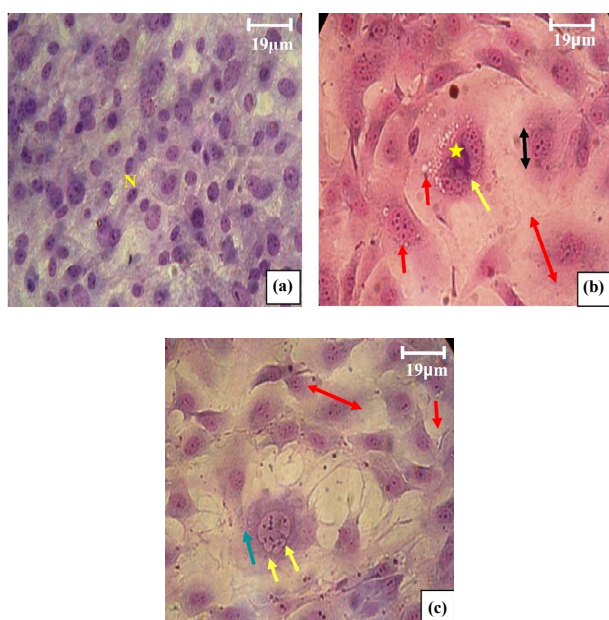


Figure 14. (a) Aortic Fibroblasts of control *Psammomys obesus* ($G \times 525$); (b) and (c) aortic Fibroblasts of *Psammomys* submitted to 0.6% D-Glc for 7 d ($G \times 525$). (↗) Increase size of fibroblast, (↔) nuclei large, (↖) number of nucleoli very important, (↘) vacuolization of the cytoplasm, (★) hypercondensation of chromatin, (↗) nuclear fragmentation.

corresponding controls ($P < 0.0001$) (See **Figure 15**).

3.2.4. Assays of Lipid Peroxidation Products

Our results showed a very significant increase in the rates of lipid peroxidation products in the intracellular compartments of adventitial fibroblasts submitted to D-Glucose at 0.6% compared to corresponding controls ($p < 0.0001$) (See **Figure 16**).

3.2.5. Assays of Total Protein and Collagen

3.2.5.1. Determination of Total Protein

The assessment of total protein in the intra- and extracellular compartment of adventitial fibroblasts incu-

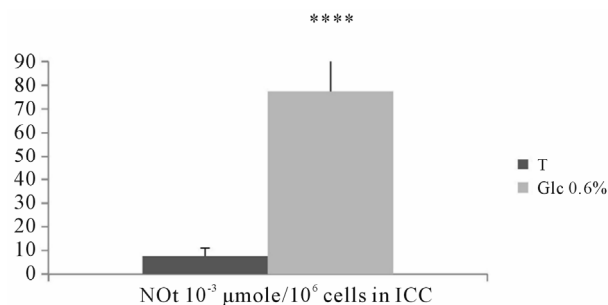


Figure 15. Evaluation of total nitrite ($\times 10^{-3} \mu\text{mole}/10^6 \text{ cells}$) contained in intracellular compartments of aortic fibroblasts of *Psammomys obesus* in culture incubated in the presence of 0.6% D-Glc for 7 days.

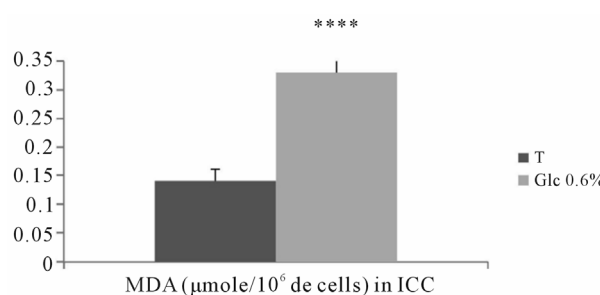


Figure 16. Evaluation of MDA levels ($\mu\text{mole}/10^6 \text{ cells}$) contained in intracellular compartments of aortic fibroblasts of *Psammomys obesus* in culture, incubated in the presence of 0.6% D-Glc for 7 days.

bated in the presence of D-glucose at 0.6% for 7 days showed a significant increase compared to corresponding controls ($P < 0.0001$) (See **Figures 17, 18**).

3.2.5.2. Dosage of Total Collagens

Assessing the amount of total collagen in the extra- and intracellular compartments of adventitial fibroblasts incubated in the presence of D-Glucose at 0.6% for 7 days show a significant increase compared to control ($P < 0.0001$) (See **Figures 19, 20**).

3.2.6. Typage of Collagen Content in the ECC

Electrophoresis profiles showed the presence of 4 bands in the extracellular compartment, just as in treated control fibroblasts but at a different absorbance. The collagen III fibers were mainly represented by three chains $\alpha 1$, the collagen I fibers were represented by 2 chains $\alpha 2$ and 1 chain $\alpha 1$. In our experimental conditions, we noted an increase in the bands absorbance corresponding to $\alpha 1$ chains of collagens I and III relative to the $\alpha 2$ chain of collagen I in the extracellular control fibroblast such as that of fibroblasts submitted to D-Glucose. The evaluation of the absorbance in the ECC fibroblasts submitted to D-Glucose 0.6% for 7 days indicated an increase in the absorbance of the bands as well as an increase in the absorbance of $\alpha 1$ chains of collagen I and III compared

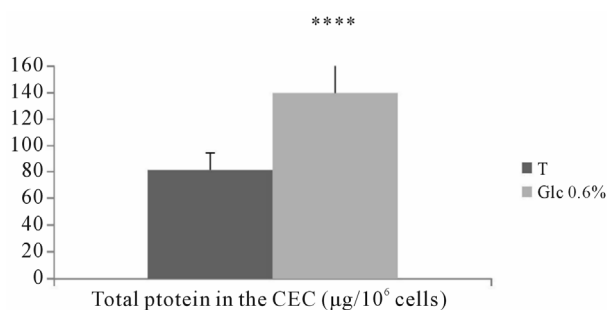


Figure 17. Assessing the amount of protein ($\mu\text{g}/10^6$ cells) in the extracellular compartment of aortic fibroblasts of *Psammomys obesus* incubated in the presence of 0.6% D-Glc for 7 days.

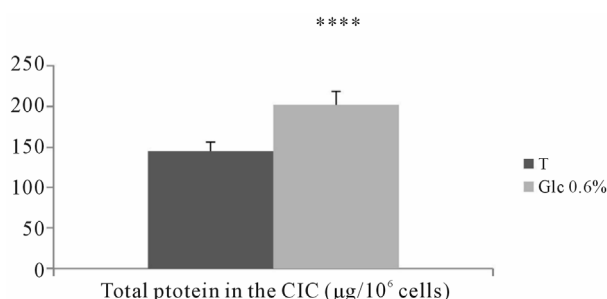


Figure 18. Assessing the amount of proteins ($\mu\text{g}/10^6$ cells) in the intracellular compartment of aortic fibroblasts of *Psammomys obesus* incubated in the presence of 0.4%, et 0.6% D-Glc for 7 days.

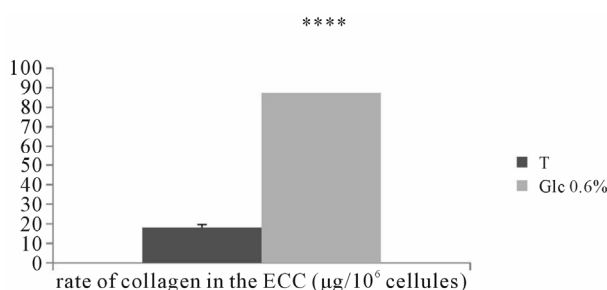


Figure 19. Evaluation of the total amount of collagen ($\mu\text{g}/10^6$ cells) in the extracellular compartment of adventitial fibroblasts in *Psammomys obesus* incubated in the presence of 0.6% D-Glc for 7 days.

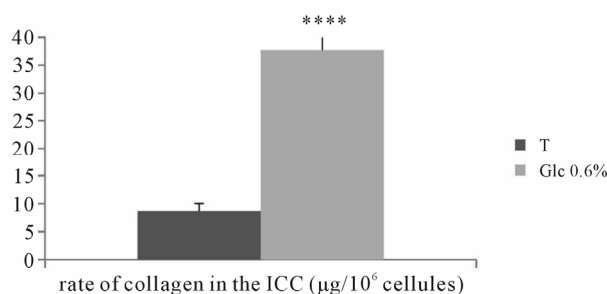


Figure 20. Evaluation of the total amount of collagen ($\mu\text{g}/10^6$ cells) in the intracellular compartment of adventitial fibroblasts in *Psammomys obesus* incubated in the presence of 0.6% D-Glc for 7 days.

to $\alpha 2$ chain of collagen I. Collagen III was thus more represented than collagen I (See **Figure 21**).

4. DISCUSSION

The long-term administration (11 months) of a high-carbohydrate diet caused both plasma and tissue disorders in *Psammomys* confirming its high sensitivity to caloric overload [14]. The elevation of body weight is associated with hyperinsulinemia that is lipogenic, the latter appeared in our animals after 3 months. Our results confirm the work of Aouichat *et al.* (2004) [7] in *Psammomys* subjected to a high carbohydrates diet, those of Shafrir. (2001) [15] and Ikeda *et al.* (2001) [16] in *Psammomys* subjected to a high-calorie diet.

In our study, we found a hyperglycemia from month 4 associated with hypertriglyceridemia, hypercholesterolemia and hyperinsulinemia characteristic of type 2 diabetes [7,15,16]. According to Stentz *et al.* (2005) [17] hyperglycemia is related to alterations in biochemical pathways responsible for micro-and macrovascular injuries related to endothelial dysfunction. Chronic hyperglycemia is associated with an increase of ROS by activating polyols pathways, hexoamines, activation of PKC family, formation of glycation end products that activate the transcription factor nuclear, NF κ B [18]. According to Goel *et al.* (2007) [19], chronic hyperglycemia generates ROS such as superoxide ions via activation of PKC, which alters vascular permeability by decreasing the bioavailability of NO.

Our results show hyperinsulinemia in animals subjected to sucrose, associated with hyperglycemia. According to Marquie *et al.* (1984) [14], Shafrir (2001) [15], Ikeda *et al.* (2001) [16], progression of diabetes in *Psammomys* subjected to high-calorie diet increased from hyperinsulinemia to hyperinsulinemia associated with hyperglycemia then to a deficiency of insulin or insulinopenia due to apoptosis and necrosis of β cells. Insulin increases oxidative stress by overproduction of superoxide radical and decreased vascular NO. It activates the transcription factor NF κ B by activating the PI $_3$ K pathway and plays a role in the induction of proinflammatory gene expression and promotion of the inflammatory process in the vascular tissue, the initial event being the recruitment monocytes in lesions where MCP-1 plays a key role [20]. Shafrir's work (2001) [15] showed an increase in PKC ξ in normoglycemic *Psammomys*, a marker of pre-diabetes compared to *Psammomys* resistant that do not develop diabetes.

Our results show the occurrence of dyslipidemia. Indeed, electrophoresis profile analysis of *Psammomys* plasma lipoproteins subjected to a high-carbohydrate diet showed a significant increase in LDL-VLDL at the end of the experiment, known as highly atherogenic lipopro-

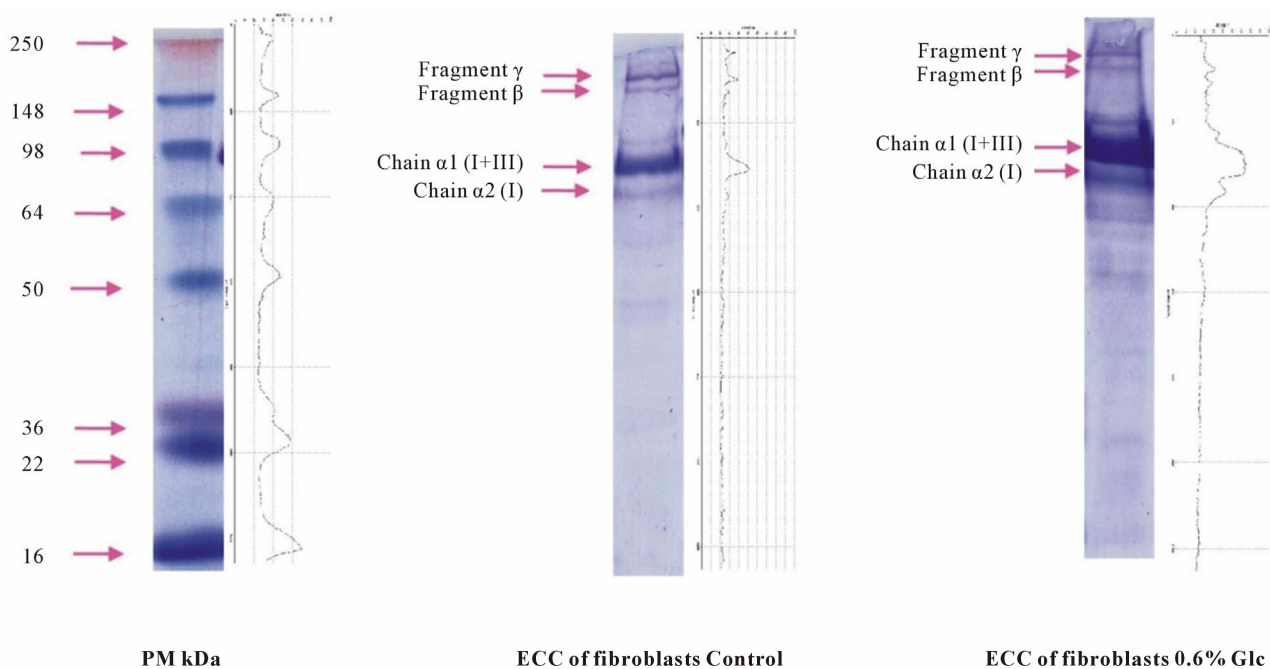


Figure 21. SDS-PAGE of collagen content in the extracellular compartment of both control and treated fibroblasts submitted to 0.6% D-Glc for 7 days.

teins since they carry esterified cholesterol to parietal cells [18]. Besides, we observed a decrease of HDL, which allows the efflux of cholesterol to the liver and are classified as cardioprotective molecules [21]. According to Shafrir (2001) [15], dyslipidemia may be responsible for insulin resistance via increased diacylglycerol (DAG), an intermediate product of esterification of fatty acids into triglycerides and the catabolism of the latter into fatty acids and glycerol. Hyperactivation and increased expression of PKC appear to be the consequence of hyperglycemia and the main cause of diabetic complications associated with hypertriglyceridemia [22] and hyperinsulinemia [23].

According Navab *et al.* (2002) [24], HDL decreases atherosclerosis by protecting LDL from oxidation. Epidemiological studies show a reverse correlation between the level of HDL-cholesterol and cardiovascular risk [25]. However, chronic hyperglycemia can induce glycation of HDL and impair their protective functions with regard to peroxidation, impair cholesterol transport and increase oxidation threshold, thus enhancing the atherosclerotic process [25].

According to Oldegren *et al.* (2003) [26], elevation of CPK is an indicator of cardiac complications in particular, marked by cellular necrosis. Our histological sections show SMCs and apoptotic or necrotic nuclei fibroblasts. In a state of necrosis, SMCs release enzymes and proteins in particular creatine phosphokinase into the blood stream. During a cardiac episode, increased CPK is systematic [27]. The latter remains a very sensitive marker

since in myocardial infarction, its rate rises at the 4th hour, reaching 7 to 12 times its basic rate. Increased CPK reflecting cell lysis could be related to the increase of NO due to hyperglycemia and hyperlipidemia. NO induces apoptosis depending on cell type and concentration. At low doses, it is pro-apoptotic, whereas at higher concentrations, it induces necrosis [28].

In the intima, we observed a thickening of this layer, a hypertrophy of endothelial cells thus an alteration of their structure and functioning, a thickening of the sub-endothelial space presenting a fibrous material, indicating the formation of young plaques rich notably with collagen. These findings corroborate those of Orbe *et al.* (2003) [29] suggesting that chronic hyperglycemia induces endothelial dysfunction through reduced bio-availability of NO. Endothelial damage and ruptures of elastica interna are observed, which would increase intimal permeability allowing an increased passage of macromolecules into the intima thus contributing to its thickening [20].

In the media, we notice alterations characterized by thinning, loss of ondulation, disorganization and disruption of elastic laminae, reflecting vascular remodeling. These results are consistent with those of Lusic (2002) [30]. The elastolysis of the elastic plates and the the elastica interna may be due to the activation of several proteases. According to Lusic (2002) [30], degradation of elastin peptides may contribute to increased proteinemia, and to this end, our results showed that proteinemia in animals subjected to sucrose is significantly increased at

the end of experimentation. Moreover, in regions close to the intima, we noted CML migration zones between interlamellar spaces within a completely disorganized matrix, extended interlamellar spaces with accumulations of collagen and probably lipid bodies. Hyperglycemia, by inducing endothelial dysfunction promotes the synthesis of adhesion molecule such as ICAM-1, VCAM-1, E-selectin and fibronectin, which facilitate the adhesion of monocytes to endothelium [30].

In the adventitia, we noticed a matrix rich in collagen and an important fibroblasts densification. These appear to undergo a phenotypic change associated with their migration to the media. Lesions of the vascular endothelium are responsible for the proliferation and dedifferentiation of fibroblasts and CLM's via activation of NADPH oxidase, for the release of ROS and that of the growth factors in particular PDGF, β FGF, IGF [31].

Our results on the proliferation of adventitial fibroblasts show those of Aouichat *et al.* (2004) [7] on aortic SMCs of *Psammomys* in the presence of 15 mM D-Glucose for 48h and 4 passages. According to Neumann *et al.* (2002) [32], Heo *et al.* (2008) [33], the proliferative effect of D-Glucose is not related to changes in osmolarity, but rather to the activation of MAPKinase via the pathway ERK1/2, which regulates the activity of Erg-1 and which plays a role in proliferation and dedifferentiation of vascular cells [34].

In addition, our morphometric results show a significant increase in the number of nucleoli. This increase is indicative of increased synthesis of cellular protein. Benazzoug, (1998) [35] noted little change in total protein synthesis of incubated human fibroblasts 15Mm Glc. Hoffman *et al.* (1998) [36] noted that hyperglycemia stimulates the synthesis of many growth factors including TGF- β in mice mesangial cells. According to Ryoo *et al.* (2004) [37], activation of PKC θ after hyperglycemia activates the p38 pathway and the production of IL-8 via activation of AP-1 and the involvement of NFkB which increase DNA synthesis. Our morphologic results show an hypertrophy of fibroblasts submitted to D-Glucose. According to Grimm *et al.* (2002) [38], hyperglycemia induces a phenotypic change of SMC into hypertrophied phenotype. Liu *et al.* (2006) [39] have also observed a phenotypic change in cardiac myocyte into hypertrophied phenotype in the presence of D-Glucose, it is linked to increased ROS and would correspond to an adaptation phenomenon.

The hypercondensation of chromatin and nuclear fragmentations that we observed are signs of apoptosis. According to Babich *et al.* (1996) [40], ROS are responsible for hypercondensation of chromatin. ROS in particular superoxide ion (O_2^-) allows interaction with the NO^- and the genesis of peroxynitrite (ONOO-) a cause for damage to DNA. These are detected by the P53 tu-

mor suppressor, to be overexpressed [41]. P53 is a molecular switch that detects DNA breaks and commits cells in the process of repair or apoptosis [41].

Cytoplasmic vacuolization that we observed likely to be linked to increased reactive oxygen species following glucotoxicity is the oncosis and it precedes apoptosis. Babich *et al.* (1996) [40] noted an important vacuolization characteristic of oncosis in fibroblasts exposed to ROS-producing agents. According to Babich and al. (2007) [42], induction of oncosis and apoptosis in fibroblasts treated with an agent that releases ROS is very important. Chen *et al.* (2000) [2] noted the increase in apoptosis in the myocardium of diabetic rats. Boumaza, (2011) [43] observed the oncosis and apoptosis in aortic fibroblasts of cultured *Psammomys* treated with hydrogen peroxide to 1.2 mM for 7d.

The protein contained in the CEC shows an increase compared to corresponding controls. Our findings corroborate those of Chen *et al.* (2000) [2], who noted an increase in matrix proteins, Benazzoug *et al.* (1998) [35] noted an increase in total collagen in human cutaneous fibroblasts incubated in 15 Mm Glc for 48 h. Aouichat *et al.* (2004) [7] also noted an increase in total collagen in SMCs of *Psammomys* incubated in 15 mM Glc for 48 h and 4 passages. The Typing of collagen showed an increase and intensification of bands corresponding to the γ fragments representing the trimer chain $\alpha 1$ (coll I and III), the β fragments representing the dimeric chain $\alpha 1$ (coll I and III), $\alpha 1$ chains (coll I and III) and $\alpha 2$ chain (coll I). Chen *et al.* (2000) [2] noted an increase in $\alpha 1$ chain after diabetes onset, Grimm *et al.* (2001) [38] have shown that hyperglycemia increases the extracellular matrix remodeling of cardiomyocytes. Andreea *et al.* (2008) [44] showed an increase of procollagen $\alpha 2$ (I) and procollagen $\alpha 1$ (III) at D-Glc concentrations of 11mM and 22mM but decreased procollagen $\alpha 1$ (III) at 33 mM of D-Glc. Hyperglycemia activates PKC β , involved in the increased expression of TGF betal which initiates the accumulation of ECM [45].

In our study we assessed the effect of a glucotoxicity on the production of MDA, a marker of lipid peroxidation in the intracellular compartments of *Psammomys* adventitial fibroblasts in culture. According to Hayek *et al.* (2007) [46], D-Glc induces, in macrophages, oxidative stress by activating NADPH oxidase, thus increasing the production of superoxide ion which increases lipid peroxidation, including LDL. According to Whiteside and Dulgasz, (2002) [45], hyperglycemia increases the genesis of ROS via activation of the polyol pathway and glycation, leading to lipid peroxidation [46]. Lipid peroxidation mediated by ROS, mainly targets the polyunsaturated fatty acids located at the cell membrane fluidity modifying and altering membrane permeability, which also affects the functions of membrane proteins, inacti-

vation of receptors of which and activation of some proteases lead to cell damage [47].

Glucotoxicity induced on the adventitial fibroblasts in the presence of 0.6% D-Glc for 7 days induces an increase in production of total nitrite in the intracellular compartments. According to Stentz *et al.* (2005) [17], hyperglycemia induced on T cells, increased synthesis of proinflammatory cytokines, which are responsible for the increase of NO by activation of iNOS. Kolb *et al.* (2001) [41], iNOS, present in all cell types require transcriptional induction, particularly by cytokines; iNOS, once synthesized, smoothly binds to calmodulin and produces larger quantities of NO and within a longer period of time (hours to days) than constitutive NOS which synthesize NO through short bursts in a response to rapid changes in intracellular Ca^{2+} . The redox state of the cellular environment plays a key role in the anti- or proapoptotic NO effect.

5. CONCLUSION

The induction *in vivo* of long term glucotoxicity by addition of sucrose to 30% in *Psammomys obesus* establishes a type 2 diabetes, characterized by increased VLDL-LDL, atherogenic factors, CPK, a marker of cardiovascular complications, as well as functional and structural alterations characterized by the remodeling of the intima and the media. Furthermore, the induction *in vitro* of glucotoxicity by addition of D-Glucose at supraphysiological dosages creates an oxidative stress characterized by an overproduction of MDA, marker of membrane lipid peroxidation and total nitrites. Morphological changes highlight the deleterious effects of glucotoxicity on adventitial fibroblasts in culture. The increased proliferation and the number of nucleoli and protein synthesis and collagens are linked to cell activation following glucotoxicity. The increase in collagen may be related to extracellular matrix remodeling in response to glucotoxicity. All these changes are factors inducing cardiovascular complications and accelerated atherosclerosis.

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