Supplementary material

Effects of selenium in the microcirculation of fructose-fed hamsters.

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SUPPLEMENTARY METHODS

Experimental animals

This study was approved by the State University of Rio de Janeiro Committee for Animal Experimentation (CEA/215/2007) and conducted according to the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), as well as with rules established by the Brazilian College of Animal Experimentation.

One hundred and forty-nine male Golden hamsters (*Mesocricetus auratus*) aged between 4 and 5 months were housed in a 12:12 h light/dark facility at controlled room temperature (21.5±0.5 °C) with experimental chow and water provided *ad libitum*.

Experimental chows

All chows used during experimental procedures were prepared at the Experimental Laboratory of Nutrition of the Fluminense Federal University (UFF, Niterói, Rio de Janeiro, Brazil), and followed the American Institute of Nutrition Rodent Diets (AIN-93) (Reeves et al, 1993).

Three types of chows (composition described on Table 1) were made according to the following parameters: zero, normal and high content of selenium (3-times higher).

Experimental design

Animals were randomly assigned into 6 groups: Group 1, received filtered water and rodent chow without selenium (CTRL; n=25); Group 2, had their drinking water substituted by 10% fructose solution and rodent chow without selenium (FRU; n=25); Group 3, received filtered water and rodent chow with normal levels of selenium (CTRL+Se; n=26); Group 4, had their drinking water substituted by 10% fructose solution and rodent chow with normal levels of selenium (FRU+Se; n=24); Group 5, received filtered water and rodent chow with 3-times higher level of selenium (CTRL+HiSe; n=26) and Group 6, had their drinking water substituted by 10% fructose solution and rodent chow with 3-times higher level of selenium (FRU+HiSe; n=23). All diets were given for sixty days.

For microcirculatory analysis, the animals from each group were divided in three smaller groups: (1) ischemia/reperfusion and topical application of acetylcholine (ACh) followed by topical application of histamine; (2) ischemia/reperfusion and topical application of sodium nitroprusside (SNP) followed by topical application of histamine; and (3) ischemia/reperfusion and topical application of histamine.

Animal preparation and blood glucose levels

On the day of the experiment, anesthesia was induced by an intraperitoneal injection of sodium pentobarbital (pentobarbital sodique, 60 mg/ml, Sanofi Santé Animale, Paris, France) and maintained with α-chloralose [100 mg/kg body weight (Sigma Chemicals, St. Louis MO, USA)] through a femoral vein catheter.

One hour after anesthesia, blood was withdrawn by periorbital puncture and glycemia was analyzed with a glucometer (One touch ultra - Johnson & Johnson, Medical Brazil).

Cheek pouch preparation

The hamster cheek pouch is an appropriate preparation to study the microcirculation. Arteriolar reactivity to acetylcholine (ACh) [18] and spontaneous arteriolar vasomotion [19] in the hamster cheek pouch are stable for 5-6 h.

The cheek pouch of each hamster was everted and mounted in an experimental chamber as previously described by Duling [18] and modified by Bouskela and Grampp [19].

Images were obtained with an intravital microscope (Leitz, Wetzlar, Germany, optical magnification x 210, NA 0.22) coupled to a closed-circuit TV system and were recorded in sVHS for future analysis.

Macromolecular permeability after ischemia/reperfusion

To quantify the increase on macromolecular permeability, fluorescein isothiocyanate (FITC)-dextran, 150000 Dalton, was administered intravenously. Permeability for large molecules was quantified by counting the number of leaky sites (=leaks) in the prepared area using an UV-light microscope (Suppl. Fig. 1).

Ischemia was achieved with a cuff made by thin latex tubing mounted around the neck of the pouch vascular pedicle with an intratubular pressure of 200-220 mmHg obtained by air compression. After deflating the cuff, the number of leaks was counted at baseline and every 2 min after the onset of reperfusion. The maximum number of leaks was obtained at 10 min after the onset of reperfusion.

Microvascular reactivity after ischemia/reperfusion

Acetylcholine (Ach) and sodium nitroprusside (SNP) were freshly prepared for each experiment and applied topically. For consecutive measurements, three arterioles were selected in each preparation. Experiments were performed by taking 2-min videotape recordings of selected microvessels under initial control conditions (before the addition of any drug) and 10 min after each experimental intervention.

To determine the vascular reactivity, microvessel luminal diameter was determined using an Image Shearing device (Vista Electronics, model 908, San Diego, CA, USA) (Suppl. Fig. 2). After these procedures, a topical application of $5x10^{-6}$ M histamine (Sigma Chemicals, St. Louis, MO, USA), 200 µl/min, for 5 minutes was made and the leaky sites quantified.

Serum insulin concentrations

Immediately after the experiments, approximately 2.5 ml of blood was collected by cardiac puncture, centrifuged at 1157 ×g for 10 min at 4°C and the serum stored at −80°C. Total insulin concentration was determined by radioimmunoassay using an insulin kit (BioTrak, Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Statistical analysis was performed initially by Kolmogorov–Smirnov normality and homoscedasticity tests (Bartlett criterion). Data are expressed as means±SEM, unless otherwise noted and analyzed by either one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test or Two-way ANOVA for repeated measures followed by Bonferroni's posttest using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA). Statistical significance was inferred at a 2-sided value of $p<0.05$.

SUPPLEMENTARY RESULTS

Body weight

To evaluate the effect of fructose ingestion and selenium administration on body weight of hamsters, they were weighted every fifteen days until the end of the experiments. An increase in body weight was observed in all groups along the 60 days of diet implementation. The weight increased between day 0 and day 60 of CTRL, FRU, CTRL+Se, FRU+Se, CTRL+HiSe and FRU+HiSe groups by approximately 17%, 29%, 20%, 30%, 21% and 32%, respectively. At 60 days we only observed significant difference between CTRL and FRU+HiSe groups (Suppl. Fig. 3).

Blood glucose levels and insulinaemia

Blood glucose levels of all animals that had their drinking water substituted by 10% fructose solution were higher compared to their controls which were drinking filtered water from 30th day to the 60th day. In animals that kept drinking 10% fructose solution, selenium levels did not affect glycemia. Also, no significant difference was observed between groups that were kept drinking filtered water (Suppl. Fig. 4A).

An increase in serum insulin levels was observed in animals drinking fructose solution compared to their controls. However, no significant differences were observed regarding selenium dietary contents (Suppl. Fig. 4B).

Table heading and Figures legends

Suppl. Table 1: Dietary formulations.

Suppl. Fig. 1: Figure showing macromolecular permeability after ischemia/reperfusion using an UV-light microscope. White arrows indicate leaky sites (=leaks) in the prepared area.

Suppl. Fig. 2: Figure showing microvascular reactivity after ischemia/reperfusion. A: microvessel luminal diameter in control condition. B: microvessel luminal diameter increase after experimental intervention.

Suppl. Fig. 3: Body weight of hamsters drinking water substituted by 10% fructose solution (FRU) or filtered water by itself (CTRL) with different levels of selenium supplementation over 60 days of experimentation. Data are expressed as means ± SEM and were analyzed by two-way ANOVA for repeated measures followed by Bonferroni's posttest. (*) indicates a significant difference of $p<0.05$ between the CTRL group and FRU+Se and FRU+HiSe groups at 60 days.

Suppl. Fig. 4: Blood glucose and serum insulin levels of hamsters that had their drinking water substituted by 10% fructose solution (FRU) or were kept drinking filtered water (CTRL) both with different selenium contents over the 60 days of experimentation. A: Blood glucose levels. B: Serum insulin levels. Data are expressed as means ± SEM represented by vertical bars. (***) indicates a significant difference from the respective control group ($p<0.001$).