Gonzalez-Cadavid Laboratory - MATERIALS AND METHODS

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2-year toxicology study conducted at NCTR and the grantee studies can be found in **(1)**.

Animal procedures.

Dynamic Infusion Cavernosometry (DIC): DIC was performed as previously described **(2-5).** Briefly, basal intracavernosal pressure **(ICP)** was recorded and 0.1ml papaverine hydrochloride (20 mg/ml; American Reagent, Inc. Shirley, NY, USA) was administered through a cannula into the corpora cavernosa. The ICP during tumescence was recorded as "ICP after papaverine" 5 minutes after the injection. After the ICP decreased below 40 mmHg saline was infused through another cannula, increasing infusion rate by 0.05 ml/min every 10 seconds, until the ICP reached 100 mmHg ("infusion rate"), then the infusion rate was adjusted to maintain a steady ICP level just above 100 mmHg ("maintenance rate"). The "drop rate" was determined by recording the fall in ICP within the next 1 minute after the infusion was stopped.

Electrical field stimulation of the cavernosal nerve (EFS). EFS was performed preceding cavernosometry as previously described **(2-5).** Briefly, under penthotal anesthesia (50 mg/kg IP; Hospira, INC., Lake Forest, IL, USA), the cavernosal nerve was exposed and hooked by a bipolar platinum electrode. Systemic arterial and intracavernosal pressure measurements were obtained by simultaneous femoral artery and cavernosal catheterization, respectively. EFS was applied at increasing voltages up to 10 V and a frequency of 15 Hz for 60 seconds, separated by 1-min intervals, with a Lab-Trax-4/24T data acquisition device with build in stimulator (WPI Inc. Sarasota, FL, USA). Arterial and intracavernosal pressures were simultaneously recorded, and values were expressed in mmHg. The ratio between the maximal intracavernosal pressure **(MIP)** and the mean arterial pressure **(MAP)** at the peak of erectile response was calculated, to normalize for variations in systemic arterial blood pressure.

Hormonal assays

Terminal blood was collected and serum was prepared, snap frozen, and stored at -80 °C until shipping on dry ice from NCTR to Gonzalez-Cadavid's laboratory at LABioMed at Harbor-UCLA Medical Center.Testosterone was assayed by applying a validated LC-MS/MS method **(2, 3, 6).**

Determinations in tissue sections

After cavernosometry, animals were sacrificed and aliquots of the skin-denuded penile shafts were fixed overnight in 10% neutral-buffered formalin, washed, and stored in alcohol (70%) at 4°C until shipping at room temperature from NCTR to our laboratory at LABioMed at Harbor-UCLA Medical Center and processed for paraffin embedded tissue sections (6-8 μm). Adjacent tissue sections were used for **(2-5, 6-8)** Masson trichrome staining for collagen (blue) and smooth muscle (SM) cells (red)

Aliquots of the penile shaft were alternatively embedded in Optimal Cutting Temperature compound (OCT) and used for obtaining frozen tissue sections that were subjected to Oil Red O staining for detecting fat droplets **(2,3,5,8).** The OCT-embedded samples were snap frozen on dry ice and stored at -80 °C until being shipped on dry ice from NCTR to our laboratory at LABioMed at Harbor-UCLA Medical Center.

Quantitative image analysis **(QIA)** was performed by computerized densitometry using the ImagePro 5.1.1 program (Media Cybernetics, Silver Spring, MD), coupled to an Olympus BHS microscope equipped with a Spot RT color digital camera **(2-5,6-8)**. For Masson and, Oil Red O staining, 40X magnification pictures were taken comprising the whole cross-section of the penile shaft. For all determinations, only the corpora cavernosa and the tunica albuginea were analyzed in a computerized grid and expressed as % of positive area vs. total area. In all cases, the total penile cross section was analyzed per tissue section, with at least 3 matched sections per animal and 8 animals per group.

Determinations in fresh tissue

Western blots **(2-5, 6-8).** Penile tissue homogenates (about 50 mg fresh tissue flash-frozen in liquid nitrogen, shipped on dry ice from NCTR to to Gonzalez-Cadavid's laboratory at LABioMed at Harbor-UCLA Medical Center, and stored at -80 °C until use) were obtained using Bullet Blender Storm 24 (Next Advance, Inc, NY) using one scoop of SSP14B (1.4 mm) beads and 4 SSB32 beads (3.2 mm) in boiling lysis buffer consisting in 1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris pH 7.4 and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1mM phenyl methyl sulfonyl fluoride), cutting the tissue into small pieces, adding the beads and the lysis buffer, and then running the blender for 5 min at speed 8 for 3 times and centrifuging at 16,000 g for 5 min. The supernatant proteins (20-30 μg) were subjected to western blot analyses by 7-10 % Tris-HCl polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, Hercules, CA) in running buffer (Tris/Glycine/SDS).

Proteins were transferred overnight at 4°C to nitrocellulose membranes in transfer buffer (Tris/glycine/methanol) and the next day, the non-specific binding was blocked by immersing the membranes into 5% non-fat dried milk, 0.1% (v/v) Tween 20 in PBS for 1 hour at room temperature. After several washes with washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 1 hour at room temperature. The monoclonal antibodies used were as follows: a) calponin 1 (Calp 1) mouse monoclonal (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), 1:500; b) nNOS rabbit monoclonal (Abcam, Cambridge, MA), 1:750; c) α-smooth muscle actin (αSMA), mouse monoclonal (Sigma), 1:1,000; d) vasoendothelial growth factor (VEGF) mouse monoclonal (Santa Cruz Biotechnology), 1:500; e) eNOS (Calbiochem, La Jolla, CA), 1:1000 ; f) nNOS polyclonal (BD Bioscience, San Jose, CA), 1:500; g) monoclonal anti-mouse macrophage NOS2A IgG (1:500; BD Pharmingen, Transduction Laboratories, San Jose, CA); h) BDNF (Abcam, Cambridge, MA), 1:1000; and h) as a reference housekeeping protein, mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal (Millipore, Billerica, MA), 1:5000.

The washed membranes were incubated for 1 hour at room temperature with 1/3,000 monoclonal antibody, followed by a secondary antibody linked to horseradish peroxidase. After several washes, the immunoreactive bands were visualized using the ECL plus western blotting chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The densitometric analyses of the bands were performed with Image J (NIH, Bethesda, MD). A running control, (one identical sample) was run throughout all gels for each antibody to standardize for variations in exposures and staining intensities. Negative controls were performed omitting the primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for GAPDH, upon reprobing.

Collagen estimation. For collagen estimation in fresh tissue, as previously described, the tissue was homogenized in saline, hydrolysed with 2 M NaOH for 30 min at 120 °C, followed by the estimation of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent, against a hydroxyproline standard curve and measuring at 550 nm [5]. Values were expressed as µg of collagen per mg of tissue.

Smooth muscle cell cultures from the rat penis. Fresh penile shaft was excised from 2 rats from each dose group, and skin and fascia were denuded **(9)**, stored in DMEM-20% fetal calf serum, stored at 4 °C, and then shipped overnight in wet ice pads to Gonzalez-Cadavid's laboratory at LABioMed at Harbor-UCLA Medical Center. Smooth muscle cultures were obtained by our procedure as previously described **(9)** and cultures were expanded to the third or fourth passage, checking purity by smooth muscle marker αSMA staining. These cultures were used for RNA isolation.

Gene global transcriptional profiles (signatures) (gene-GTS) **(2,3,10-12).** For the multiple mRNA profiles, RNA was isolated from smooth muscle cell cultures, and then maintained in RNA later, using RNeasy Plus Micro kit (Qiagen) with quality determined by the Agilent 2100 Bioanalyzer. Assays were performed in a duplicate set of penile tissue RNAs by the UCLA DNA microarray core, applying the Affymetrix Rat Gene array for 29,215 sequences. Only genes that

were up- or down-regulated, compared as ratios to the control group, by at least 2-fold were considered unless specifically detailed.

Statistical analysis

Values are expressed as the mean ± SEM. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by single factor ANOVA, followed by *post hoc* comparisons with the Tukey test. Differences among groups were considered statistically significant at $P < 0.05$.

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