Supporting Information

Cocoa extract exerts sex-specific effects in an aggressive hyperglycemia model: A pilot study

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MATERIALS AND METHODS

Cocoa extract production and characterization [1–5].
Folin-Ciocalteu colorimetric assay. Cocoa extract (*n=3*) was diluted with 40% EtOH to a final concentration of 0.2 mg/mL. A standard curve of gallic acid was prepared at 0.0-1.0 mg/mL. Each sample and standard was diluted 10X with ddH_2O, followed by addition of 2N Folin-Ciocalteu reagent (1:2.5 sample solution:Folin). Sodium carbonate solution (7.5%, v/v) was added to all samples and standards. Samples and standards were incubated for 2 h at room temperature and then read at 765 nm. Total polyphenol concentration is expressed as mg Gallic Acid Equivalents (GAE).

4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay. DMAC solution was prepared by combining stock HCl with EtOH (1:10 HCL:EtOH) and chilling at 4°C for 15 min. DMAC powder was added to the chilled solution at 0.001 g/mL. Cocoa extracts were diluted with EtOH to a final concentration of 100 ppm. A standard curve of procyanidin B2 (PCB2) was prepared at 0-100 ppm. DMAC solution was added to each sample and standard (1:5 sample:DMAC), mixed thoroughly, and read at 640 nm. Total flavanol concentration is expressed as mg PCB2 equivalents.

Thiolysis. Cocoa extract was diluted with MeOH to 0.5 mg/mL and then mixed (50 µL) with 50 µL HCl (3.3%, water) and 100 µL benzyl mercaptan (5%, MeOH). Samples were placed in a 90°C water bath for 5 min and then cooled on ice for 5 min. Unthiolyzed controls were prepared with cocoa extract and MeOH without heating in the water bath. Each thiolyzed sample (100 µL) was combined with 900 µL of 0.1% formic acid in water and 0.1% formic acid in ACN (95:5 v/v). Samples were analyzed on a Waters Acquity H-Class separations module with an Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm) at 40°C. Binary gradient elution was performed using 0.1% formic acid in water (Phase A) and 0.1% formic acid in ACN (Phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution was as followed: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7-10.5). (−)-electrospray ionization (ESI) together with tandem mass spectrometry (MS/MS) was used to analyze UPLC effluent on a Waters Acquity triple quadrupole (TQD) MS. (−) mode electrospray ionization (ESI) was performed with capillary, cone,

and extractor voltages of −4.24 kV, 30.0 V, and 3.0 V respectively. Source temperature was 150°C and desolvation temperature was 400°C. Cone gas flows at a rate of 75 L/h and desolvation gas at 900 L/h. Argon (0.25 mL/min) was used as the collision gas in MS/MS. Multi-reaction monitoring (MRM) with a mass span of 0.2 Da was performed on parent ions and collision-induced dissociation (CID) on daughter ions. Inter-channel delays and interscan time was 1.0 s each. Additional calculations are done to account for the native monomers and are reported as DP of total flavanols. mDP oligomers and polymers and mDP of total flavanols are calculated as follows:

$$mDP\ (O+P) = \frac{\text{net number of monomers} + \text{net number of thiolytic derivatives}}{\text{net number of monomers}}$$

$$mDP\ (total\ flavanols) = \frac{\text{total monomers} + \text{net number of thiolytic derivatives}}{\text{total monomers}}$$

UPLC-MS/MS. A Waters Acquity H-class separation module equipped with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm, 40°C) and VanGuard HHS T3 precolumn (1.8 µm). Samples were maintained at 10°C. Binary gradient elution was performed with 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution was carried out as followed: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.75 min), 95% A (8.85-10 min). An injection volume of 10 µL was used for all samples and standards. MS/MS analysis of column effluent was performed by (−)-ESI on a Waters Acquity TQD mass spectrometer equipped with a Z-spray electrospray interface. Ionization settings are as follows: −4.25 kV ESI capillary voltage, 150°C source temperature, 400°C desolvation temperature. N_2 was used for cone and desolvation gasses with flow rates of 75 L/h and 900 L/h, respectively. Ar was used as a collision gas. A standard curve of (−)-epicatechin, catechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 (CinA2) was prepared and flavanol concentrations (procyanidin tetramer – decamer) are expressed as CinA2 equivalents.

TABLES AND FIGURES

Supplementary Table 1. Mouse genotype results

Sample ID[a]	Detected *ob/ob* mutation
Male	
WT (A)	--
WT (B)	--
WT ©	--
ob/ob (A)[b]	NA
ob/ob (B)	++
ob/ob (C)	++
ob/ob + *c* (A)	++
ob/ob + *c* (B)	++
ob/ob + *c* (C)	--
Female	
WT (A)	--
WT (B)	--
WT ©	--
ob/ob (A)	++
ob/ob (B)	++
ob/ob (C)	++
ob/ob + *c* (A)	++
ob/ob + *c* (B)	++
ob/ob + *c* (C)	++

[a]A, B and C designate individual animals within each group

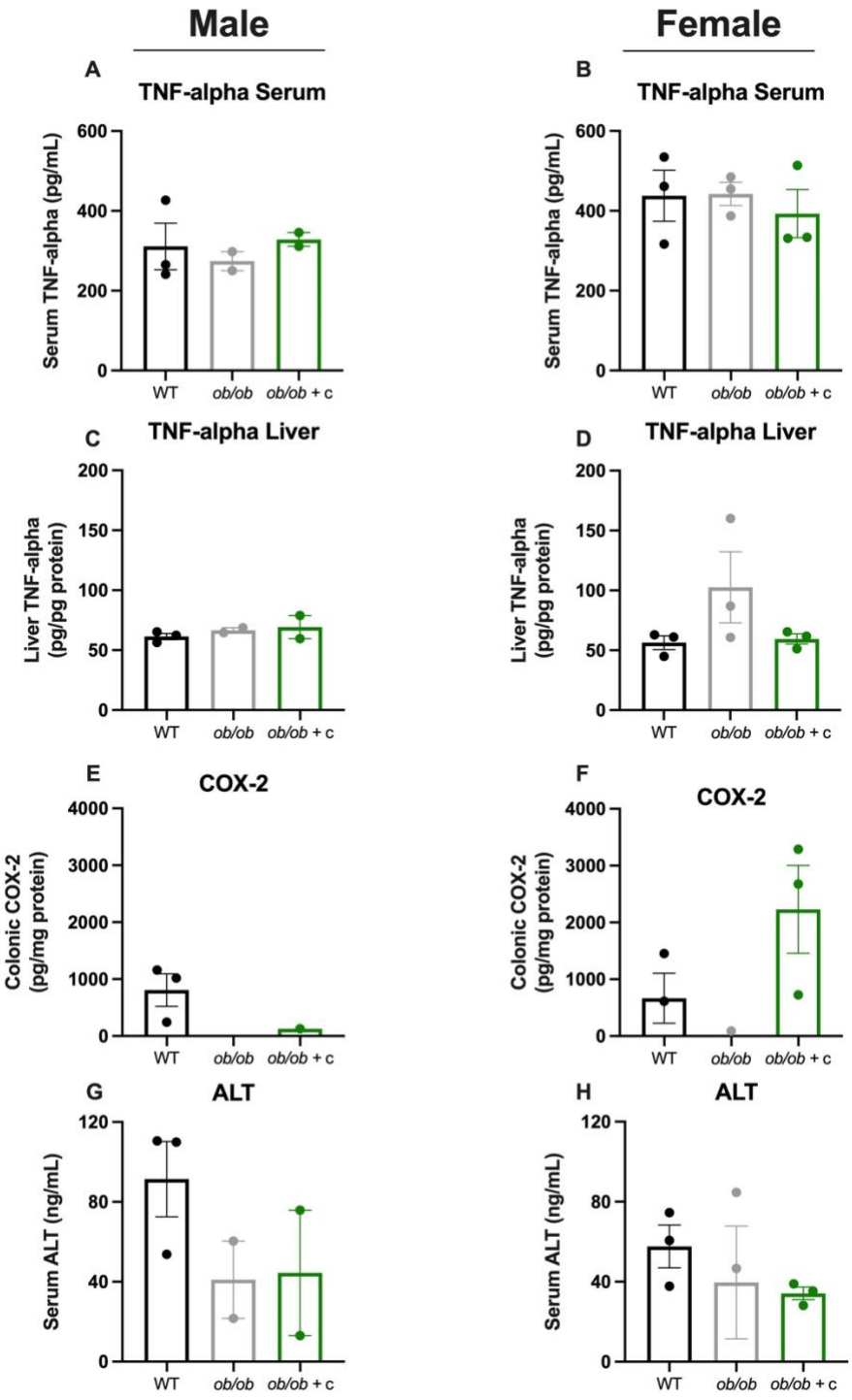
[b]unable to genotype, mouse died at week 10 prior to euthanasia

Supplementary Table 2: Cocoa extract characterization

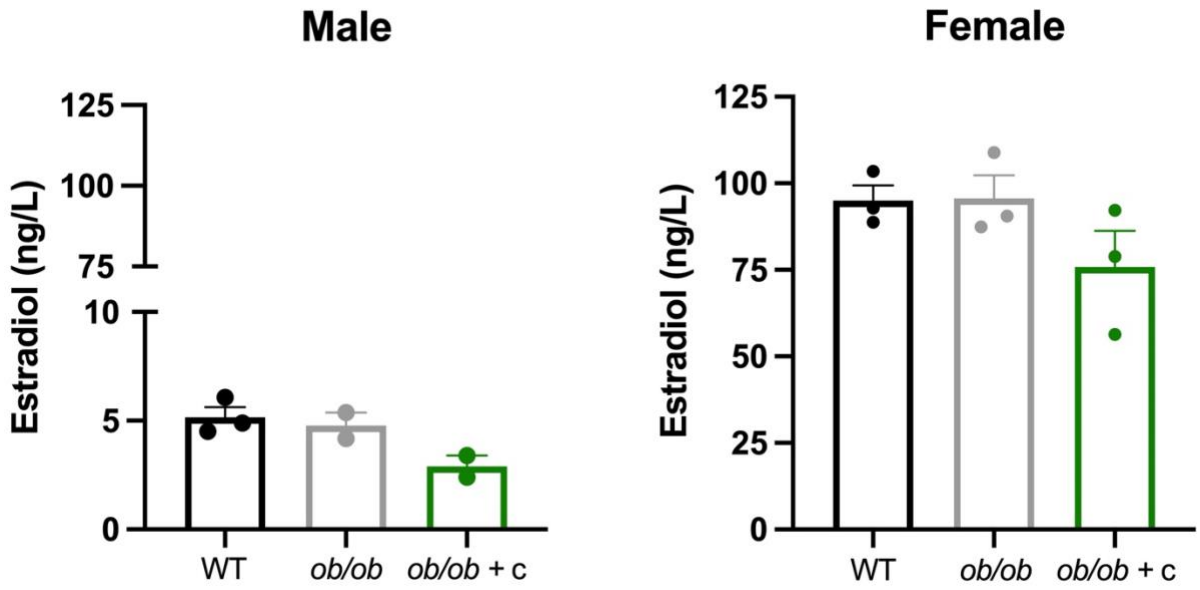
Measure	Value
Total polyphenols (Folin) (mg GAE/mg extract ± SEM[a])	0.2536 ± 0.008
Total flavanols (DMAC) (mg PCB2/mg extract ± SEM[b])	0.1996 ± 0.013
Mean degree of polymerization (thiolysis) (mDP ± SEM)[c]	Including monomers: 2.144 ± 0.024 Excluding monomers: 2.547 ± 0.048
Procyanidin characterization (LC-MS/MS) (mg/g extract ± SEM)	
Catechin	8.5589 ± 0.095
Epicatechin	18.9425 ± 0.360
Dimer	5.6865 ± 0.163
Trimer	6.7480 ± 0.231
Tetramer	3.9345 ± 0.043
Pentamer	3.6162 ± 0.078
Hexamer	1.6119 ± 0.107
Heptamer	1.2715 ± 0.1424
Octamer	0.6641 ± 0.027
Nonamer	0.3757 ± 0.041
Decamer	0.1193 ± 0.019

[a]Gallic acid equivalents
[b]Procyanidin B2 equivalents
[c]Mean degree of polymerization (monomer residues per flavan-3-ol molecule)

Supplementary Figure 1. *Cocoa extract intake for both male and female mice. Male and female treatments without cocoa extract supplementation are not shown.*



Supplementary Figure 2. *Serum TNF-α (A-B), hepatic TNF-α (C-D), colonic COX-2 (E-F), and serum ALT (G-H) levels following 10 weeks of treatment. Values are presented as mean ± SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different (P<0.05). Values not shown were not detectable or not within the quantifiable range (negative).*



Supplementary Figure 3. *Serum estradiol levels in male and female mice following 10 weeks of treatment. Values are presented as mean ± SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different (P<0.05). Bars displaying no superscript letter have no significant differences (P<0.05).*

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