Cocoa Extract Exerts Sex-Specific Effects in an Aggressive Hyperglycemia Model: A Pilot Study

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Abstract

Type 2 diabetes (T2D) is characterized by hyperglycemia and insulin resistance. Cocoa may slow T2D development and progression. This study employed male and female BTBR.Cg-*Lep*$^{ob/ob}$/WiscJ and wild type (WT) controls to assess the potential for cocoa to ameliorate progressive T2D and compare responses between sexes. Mice received diet without (WT, *ob/ob*) or with cocoa extract (*ob/ob* + c) for 10 weeks. Glucose and insulin tolerance tests (GTT/ITT) were conducted at weeks 1, 5 and 2, 6, respectively. Cocoa provided mild non-significant protection against weight gain vs. *ob/ob* control in males but not females. Male *ob/ob* + c had increasing fasting glucose at weeks 1 and 5 GTTs, with significantly higher levels of fasting glucose than *ob/ob* control at week 5. This was not seen in females. Cocoa protected against elevated 4-hour fasting glucose in week 2, but not week 6, ITTs. Cocoa partly suppressed hyperinsulinemia in males but significantly amplified it in females and protected against loss of beta cell area in females only. The mechanisms of these sex-specific effects remain to be elucidated. This study informs additional experiments with larger sample sizes and demonstrates that sex differences must be considered when designing dietary interventions for T2D.

Keywords: BTBR, *ob/ob*, type-2 diabetes, flavanol, insulin, beta cell

1. Introduction

Diabetes is a health crisis, imposing great public health and socio-economic burdens globally. The International Diabetes Federation reported in 2019 that ~463 million adults worldwide were living with diabetes [1]. It is estimated that 90% of these type 2 (T2D), with another 374 million people at risk of developing T2D by 2045 [1]. T2D is often preceded by inflammation and chronic metabolic stress as well as beta cell dysfunction [2]. While beta cell dysfunction is traditionally viewed as a late-stage event in T2D, growing data suggest that it may be an early event [3–5]. Mismanagement of T2D results in complications including cardiovascular disease, kidney failure, neuropathy, and retinopathy. It is critical to develop effective strategies for T2D prevention and management. Obesity is one of the most prominent risk factors for T2D, as it can result in insulin resistance [6,7]. During insulin resistance, perceived insulin demand is heightened, resulting in increased stress on beta cells- ultimately increasing beta cell mass and disrupting the balance of proliferation and apoptosis [8]. This combination of hyperinsulinemia and dysregulated beta cell physiology is, in large part, the precursor to beta cell exhaustion and failure and ultimately hypoinsulinemia in late-stage T2D.

Flavanols have drawn interest for their potential role in T2D prevention and management [9–11]. Cocoa (*Theobroma cacao*) is one of the most concentrated dietary sources of flavanols [12–14]. Numerous preclinical studies have suggested a potential anti-diabetic role for cocoa flavanols [15]. Most of these studies focus on obesity, hyperglycemia, and insulin resistance with few examining the impact of cocoa flavanols on beta cell function. Pancreatic beta cells secrete insulin and are responsible for maintenance of glucose homeostasis, along with glucagon-secreting alpha cells. Physiological conditions accompanying T2D, such as chronic oxidative stress and inflammation, eventually induce beta cell failure and death and accelerate disease progression.[6] Promising studies suggest that flavanols protect beta cells *in vitro* [8,16–19] and *in vivo* [20–22], but this has not been investigated as thoroughly as effects in adipose tissue and skeletal muscle. We previously demonstrated that monomeric flavanols enhance beta cell function by increasing mitochondrial respiration [23]. Human studies of cocoa in the context of obesity and hyperglycemia have had mixed results [24–28]. To effectively harness the potential benefits of cocoa in humans, more information from preclinical models is needed regarding the specific contexts (disease state, genetics, and sex) in which cocoa may be effective.

Rodent T2D models include insulin resistance and beta cell failure phenotypes. Popular monogenic models include Lep$^{ob/ob}$ ("*ob/ob*"), Lepr$^{db/db}$ ("*db/db*"), and Zucker diabetic fatty rats. These models are deficient in leptin signaling and exist in a state of perceived starvation, consuming excess food but suppressing energy expenditure [29]. The *db/db* and ZDF models develop hyperglycemia around 8-10 weeks of age whereas *ob/ob* are hyperglycemic by 4 weeks [30]. Environmental models include high fat feeding and/or streptozotocin (STZ) administration. High fat feeding mirrors human disease more accurately than genetic models but involves slow development of beta cell damage, while STZ rapidly induces hyperglycemia by damaging pancreatic beta cells. In this study, we aimed to employ an aggressive T2D model with the potential to reach beta cell damage. To achieve this, the *ob/ob* mutation on the novel BTBR (black and tan, brachyuric) background (BTBR.Cg-*Lep^{ob}*/WiscJ) was used with wild type (WT) controls. Although data are limited, a few groups have compared this model with *ob/ob* mice on the C57BL/6J (B6) background genome [29,31–34]. BTBR *ob/ob* mice progress more rapidly to late-stage T2D and experience a loss of specific microRNAs (miRNAs) in pancreatic islet cells, potentially resulting in an inability to increase beta cell replication in response to obesity that

happens on the B6 background [35]. BTBR *ob/ob* mice have significantly higher food intake and gain significantly more weight than B6 *ob/ob*, and display glycosuria [29].

Our objectives were to perform a pilot study to 1) determine the potential for cocoa to ameliorate progressive T2D, and 2) compare sex differences in the response of T2D mice to cocoa supplementation. We hypothesized that cocoa supplementation would protect against progressive T2D, but that efficacy would differ by sex.

2. Materials and Methods

2.1 Chemical standards and reagents. LC-MS grade acetonitrile and methanol were obtained from Thermo-Fisher Scientific (Waltham, MA). Glacial acetic acid and acetone were obtained from VWR (Radnor, PA). Authentic standards of (−)-epicatechin, (±)-catechin, and procyanidin B2 were obtained from ChromaDex (Irving, CA). Folin-Ciocalteu reagent and 4-dimethylaminocinnamaldehyde (DMAC) were obtained from Sigma-Aldrich (St. Louis, MO). Solvents were ACS grade or higher.

2.2 Cocoa flavanol-rich extract production and characterization. A flavanol-rich cocoa extract (CE) was prepared as described previously [36]. Full methodological details can be found in Supplementary Information. CE was analyzed by the Folin-Ciocalteu and 4-dimethylaminocinnamaldehyde (DMAC) methods as described previously [36,37] to approximate total polyphenols and total procyanidins, respectively. CE was thiolyzed with benzyl mercaptan and the resulting products quantified by UPLC-MS/MS as described previously [36,38] to determine flavanol mean degree of polymerization (mDP). CE was also analyzed by UPLC-MS/MS as previously described [39] to measure monomeric flavanols through decameric procyanidins. Methodological details for characterization procedures are found in Supplementary Material.

2.3 Animals. Approval for this study was obtained from the Animal Care and Use Committee at the David H. Murdock Research Institute (#20-011). Four-week-old BTBR.Cg-*Lep^{ob}*/WiscJ mice (stock # 004824) were obtained from Jackson (Bar Harbor, ME): *N*=12 males and 12 females homozygous for the *Lep^{ob}* mutation (*ob/ob*), and *N*=6 male and 6 female wild-type (WT) controls. Mice were acclimatized to the facility for 4 d before randomization to experimental diets. Mice were housed under standard laboratory conditions (12 h light/dark cycle, 30-70% relative humidity, 20-26°C, 3 mice/cage) on aspen bedding. Animals were allowed access to food and water *ad libitum* except where specified.

2.4 Diets. Mice were fed the 10% fat, 7% sucrose control diet from the Diet-Induced Obesity series (D12450J, Research Diets, New Brunswick, NJ) alone or supplemented with 0.8% CE by weight (Supplementary Table 1). This dose was selected based on studies[40,41] demonstrating that 8% cocoa powder in the diet blunts diet-induced obesity without altering food intake in WT mice. Based on an estimated extraction yield of 10% from cocoa powder and food intake of ~0.1 kg diet/kg body weight/day in WT mice, this dose was designed to provide ~800 mg CE/kg body weight/day (equivalent to ~8000 mg cocoa powder/kg body weight/day) to mice eating normal amounts of food (as we had not previously worked with *ob/ob* or BTBR mice). Based on body surface area conversion [42], this dose corresponds to 65 mg CE/kg body weight/day (~650 mg cocoa powder/kg body weight/day) in adult humans. For a 60 kg individual, this would equate to 3900 mg CE or 39 g cocoa powder. This equates to roughly 8 doses of cocoa powder (5 g/dose).

The treatment groups for each sex were as follows: WT, *ob/ob*, *ob/ob* + cocoa extract (*ob/*ob + c) (*n*=3/sex/group). Diet treatments were started on the first day of week 1.

2.5 Body weight and food intake. Body weight and food intake (measured by difference between food provided and food remaining) were measured weekly.

2.6 Glucose and insulin. Insulin tolerance tests (ITTs) were performed at weeks 2 and 6. Mice were fasted for 4 h, weighed, and provided insulin (Humulin R, Cardinal Health, Dublin, OH) i.p. at 0.65 U/kg body mass in saline (USP-grade, Hospira, Inc., Lake Forest, IL). Blood was collected at 0, 15, 30, 45, and 60 min and glucose measured using OneTouch Ultra Blue glucose test strips (LifeScan, Inc., Milpitas, CA) and a glucometer. The upper limit of the glucometer was 600 mg/dL; values above that were noted, and 600 mg/dL used for calculations. AUC was calculated for week 2 and 6 ITT.

2.7 Euthanasia and tissue collection

Upon study completion, animals were fasted 12 h and euthanized by CO_2, followed by cervical dislocation, per AVMA guidelines. Blood was collected via cardiac puncture and samples collected from the colon, cecum, small intestine, pancreas, and liver. Samples were stored in RIPA buffer (Thermo Fisher, Waltham, MA) for protein analyses. Pancreas and liver samples were preserved in cassettes immersed initially in 10% neutral buffered formalin solution (Thermo) overnight and then stored in 70% ethanol. Blood was collected in MiniCollect Serum Tubes (Greiner Bio-One, Monroe, NC) and clotted at room temperature for 30 min. Serum was prepared by centrifuging (2000 × *g*, 10 min). Samples were stored at −80°C.

2.8 Blood biomarkers. Fasting blood insulin was quantified using a mouse ultrasensitive insulin ELISA kit with the 0-12.8 ng/mL "wide" standard curve range option (90080, Crystal Chem, Elk Grove Village, IL). Fasting blood triglycerides were quantified by a Triglyceride Quantification Colorimetric/Fluorometric Kit (MAK266-1KT, Sigma, St. Louis, MO).

2.9 Histology. Histology of collected pancreata was conducted per established methods and full methodological details can be found in Supplementary information.

2.10 Animal genotyping. Formalin fixed liver tissue was used to genotype each animal to confirm the presence or absence of the homozygous *ob/ob* mutation. Samples were sent to Transnetyx (Cordova, TN) and results reported for all samples collected.

2.11 Statistical analysis. Data were analyzed using GraphPad Prism v9.1.2 (La Jolla, CA, USA). Outliers were removed by Dixon's q-test at $\alpha = 0.05$. When values were missing due to removal of outliers or missing sample, a mixed-effects model was used. When initial one-way or two-way ANOVA indicated a significant overall treatment effect ($P<0.05$), individual means were compared using Tukey's or Sidak's post-hoc tests to control for multiple comparisons ($\alpha = 0.05$).

3. Results

3.1Genotyping. Genotyping is presented in Supplementary Table 2. We identified and excluded one male (*ob/ob* + c) that did not carry the *ob* mutation on either allele.

3.2 Cocoa. Composition measures of cocoa extract are shown in Supplementary Table 3. The yield of CE from powder was 18%, higher than the 10% used to calculate diet formulations. The estimated total polyphenol and total flavanol content of the extract were 0.25 ± 0.008 mg GAE/mg CE and 0.200 ± 0.013 mg PCB2 equivalents/mg CE, respectively. The mDP of all flavanols was 2.14 ± 0.024 mDP. The mDP of flavanols other than monomeric flavanols was 2.55 ± 0.048. These values agree with our previous analyses of cocoa extract [38,43].

3.3 Body weights and food intake. Weight gain is presented in Figure 1. Absolute weights and food intake are presented in Supplementary Figure 1. WT males and females experienced similar weight gain, approximately 12 g total (~30% increase of initial body weight) over 10 wks. Interestingly, homozygotes (*ob/ob*) fed the control diet exhibited significant variation in weight gain in both sexes, with weight gains of ~2-30 g (~0-50%). It is unclear whether this variation is typical on the BTBR background, but it could be partially due to weight loss (or, in this case, lack of weight gain despite *ob/ob*-induced hyperphagia) often exhibited in severe diabetes [44–46]. WT male and female had greater % weight gain, compared to *ob/ob* controls but this is likely due to the higher initial weights of *ob/ob* mice (Supplementary Figure 1 A, C). While no statistically significant differences were observed (due to small sample size and large variability in the *ob/ob* on control diet), CE appeared to slightly reduce weight gain in males, with no such trend in females. The *ob/ob* mice for both sexes ate more food compared to WT controls (Supplementary Figure 1B, D), consistent with known hyperphagia in *ob/ob* mice. Importantly, inclusion of CE did not reduce food intake, indicating that observed changes in body composition, metabolism, or other markers in the CE treatment were not due to reduced caloric intake. This has been reported before for equivalent cocoa doses[40,41]. Based on food intake and body weights over the study, the average intake of was ~1400-1450 mg CE/kg/d or 7800-8100 mg cocoa powder/kg/d (based on a yield of 18%) (Supplementary Figure 2). This dose corresponds to previous work reporting ~5700 mg cocoa powder/kg/d, when utilizing C57BL/6J mice- a model that consumes lower quantities of food than that of the *ob/ob* utilized in this study [41].

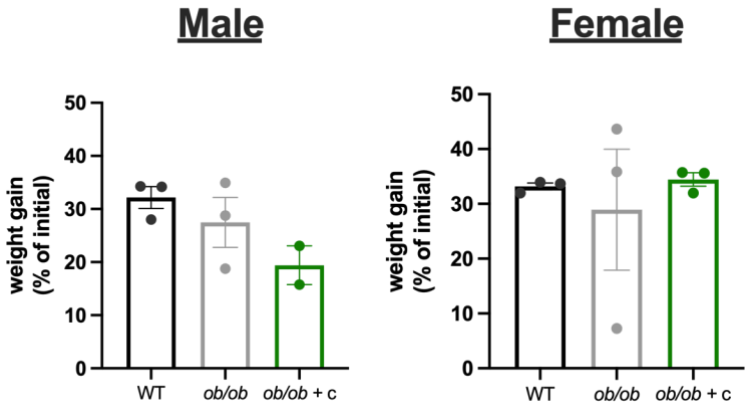

Figure 1. *Weight gain as % of initial. Values are mean ± SEM. Data were analyzed by 1-way ANOVA; due to lack of observed overall treatment effect for any graph, no post hoc tests were performed to compare treatment means.*

3.4 Insulin sensitivity. ITT results from weeks 2 and 6 are shown in Figure 2. For males, fasting glucose was elevated in *ob/ob* and *ob/ob* + c mice compared to WT controls at 2 and 6 weeks (Figure 2A). In females, distinct effects on fasting blood glucose were observed compared to males (Figure 2D). At 2 weeks, *ob/ob* females had significantly elevated fasting glucose

compared to WT controls, but supplementation with CE prevented this increase. At week 6, fasting blood glucose had significantly worsened for both *ob/ob* and *ob/ob* + c, and the protective effect of CE supplementation observed at 2 weeks was lost. The apparent lack of variability for the female *ob/ob* and *ob/ob* + c mice at week 6 is due to the large number of readings above the measurable scale of the glucometer

Glucose AUCs for week 2 and 6 ITTs are shown in Figures 2B, C, E, F. For males at week 2, *ob/ob* control mice had significantly worse AUCs compared to WT mice, and CE supplementation worsened AUC. AUCs worsened for both groups of *ob/ob* mice by week 6, and CE supplementation appeared to slightly but non-significantly worsen AUC. For females, CE supplementation blunted the elevation in AUC seen in *ob/ob* controls at week 2, but AUCs worsened in both groups by week 6 and the protective effects of CE were no longer observed (protective effects may have been present, but as the glucometer could not read >600 mg/dL, such protection was not detectable).

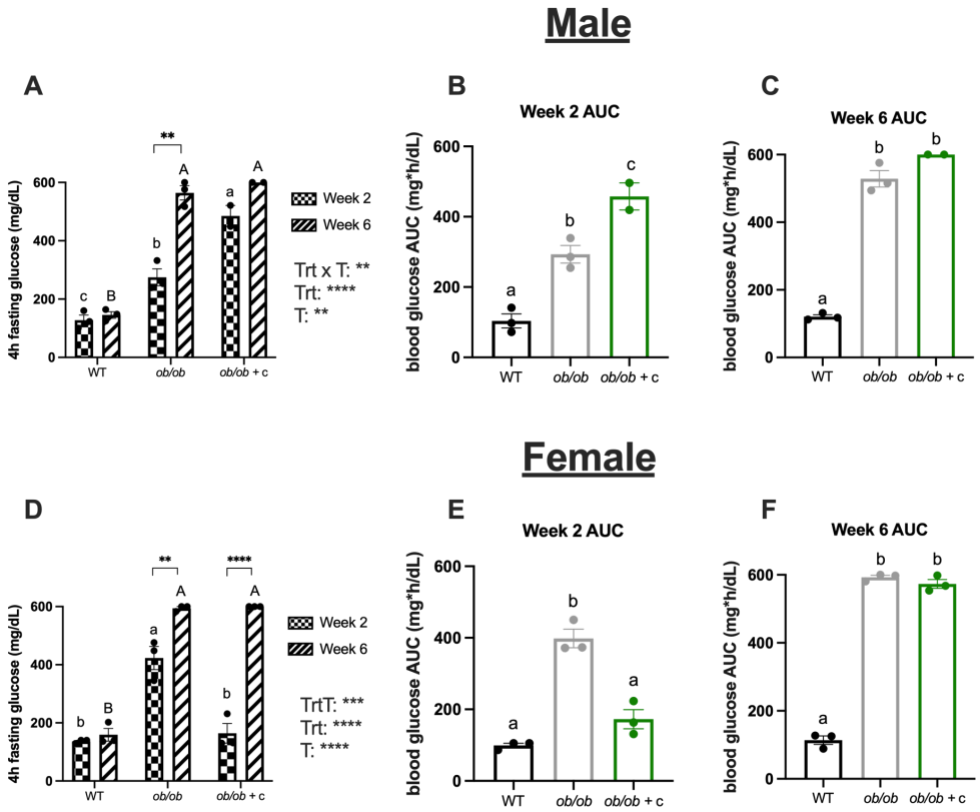

Figure 2. *Insulin tolerance test measures. Four hour fasting blood glucose at week 2 and 6 for males (A) and females (D). Blood glucose area under the curve for male (B,C) and female (E,F) mice. Values are mean ± SEM. Data were analyzed by 2-way ANOVA: treatment (Trt), time (T, 0-60 min or week 1 vs. 5), and significance of main effects/interactions are shown in inset boxes for fasting glucose graphs. If a significant main effect or interaction was detected, Sidak (time) or Tukey's (treatment) post hoc test for multiple comparisons were performed: *, **, *** and **** indicate P ≤ 0.05, P ≤ 0.01, P ≤ 0.001 and P ≤ 0.0001, respectively. Lower-case and upper-case superscript letters indicate significance for week 1 and week 5, respectively. Blood glucose area under the curve (AUC) for week 2 (G, H) and 6 ITT (I, J). 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).*

3.5 Insulin. Fasting blood insulin levels at euthanasia are shown in Figure 3. In males, fasting insulin was significantly elevated in *ob/ob* compared to WT controls. This was reversed by CE supplementation. The opposite was observed in females: fasting insulin was significantly elevated in *ob/ob* compared to WT controls, and CE supplementation further increased levels by > 2-fold. For some *ob/ob* + c females the absorbance values were ~2.5x higher than the maximal standard curve concentrations. Therefore, these should be treated with caution. Nevertheless, the extremely high insulin values suggest that, despite prolonged severe hyperglycemia, *ob/ob* + c females were still hyperinsulinemic and had not progressed to beta cell failure in terms of insulin secretion capacity.

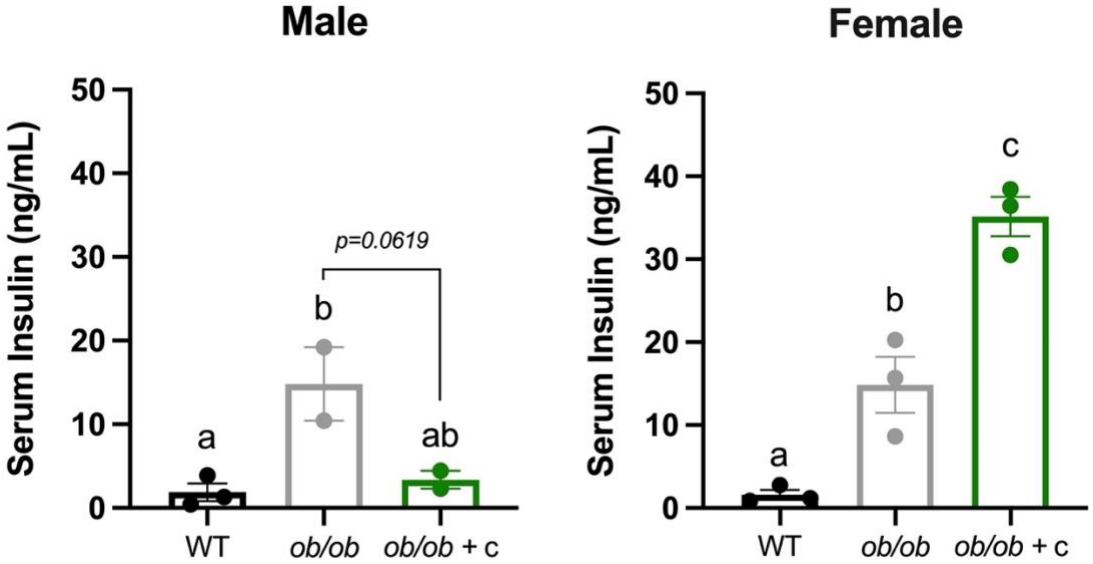

Figure 3. *Fasting blood insulin following 10 weeks of treatment for males (**A**) and females (**B**) Values are mean ± SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different (P<0.05).*

3.6 Serum triglycerides.

Serum triglyceride levels were measured and presented in Figure 4. Sex-specific effects of CE supplementation were observed. In males, levels were slightly but non-significantly elevated in *ob/ob* mice compared to WT controls, with no effect of CE supplementation. In females, serum triglycerides were slightly but non-significantly elevated in *ob/ob* mice compared to WT controls (as in males), but CE supplementation resulted in substantial (~15-fold) increases.

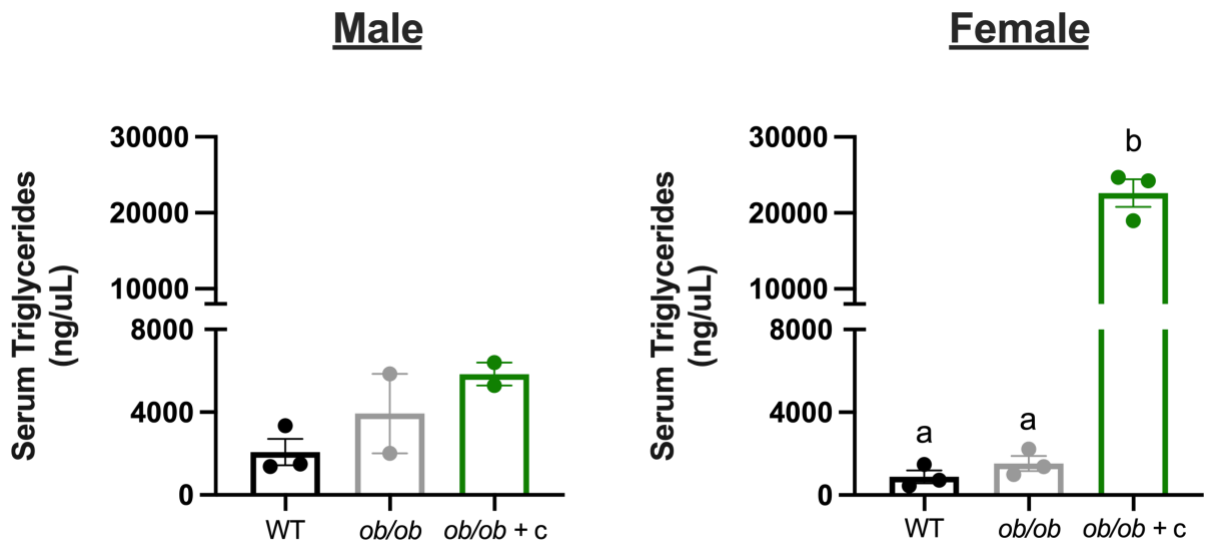

Figure 4. *Serum triglycerides following 10 weeks of treatment for males and females. Values are mean ± SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different (P<0.05).*

3.7 Pancreatic beta cell area

Total beta cell area was measured by immunohistochemistry (% insulin positive area) to determine if *ob/ob* mutation and/or CE feeding affected beta cell survival, illustrated in Figure 5. In males, although the overall treatment effect was not significant (p=0.073), WT had slightly greater beta cell area overall compared to both *ob/ob* and *ob/ob* + c treatments. In females, *ob/ob* controls had significantly reduced beta cell area compared to WT, but CE administration protected against beta cell loss.

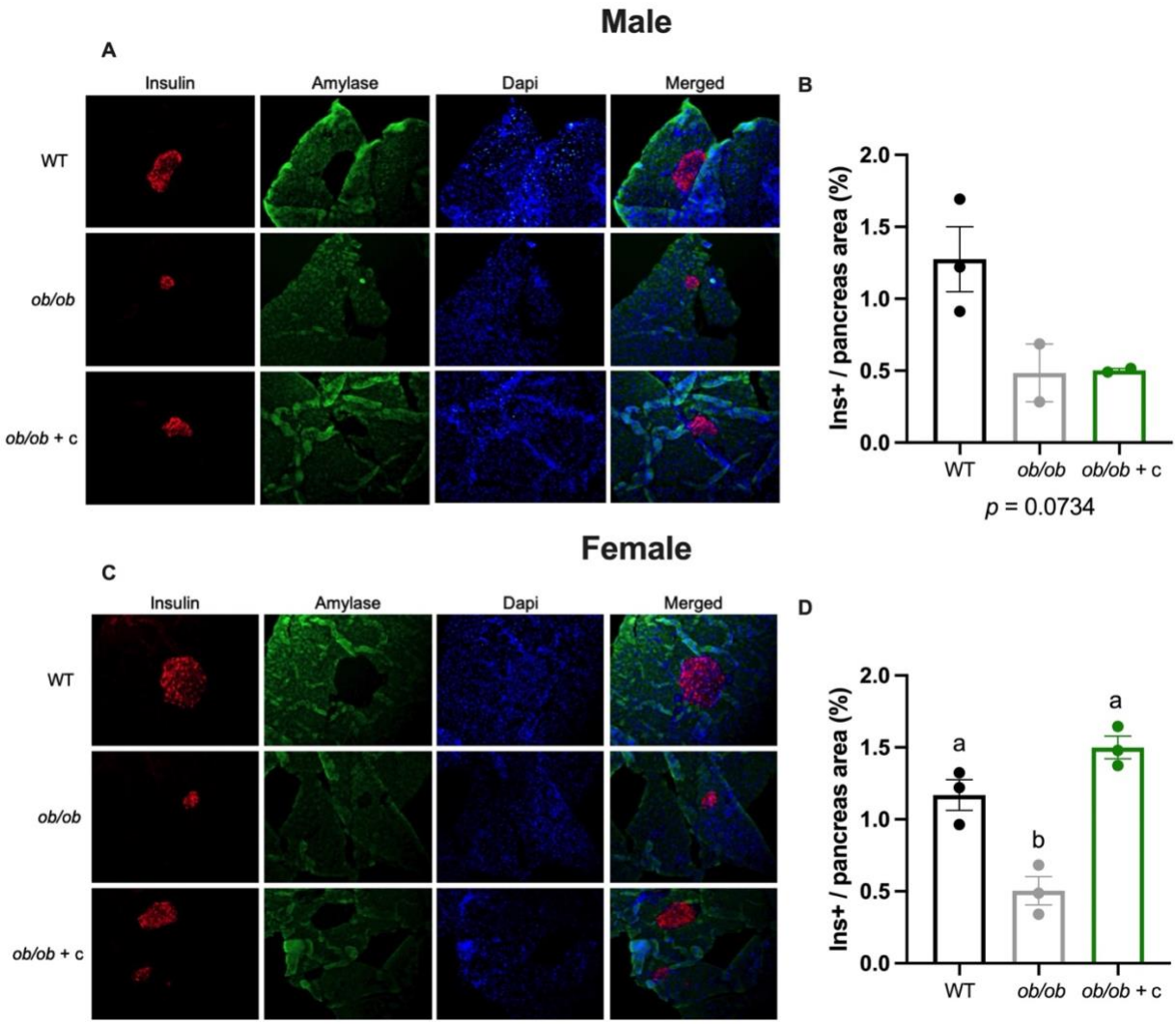

Figure 5. *Representative images for pancreata (males:* ***A****, females:* ***C****). Total beta-cell (% insulin positive) area following 10 weeks of treatment (males:* ***B****, females:* ***D****). Images were captured at 20x magnification and analyzed using cellSens and ImageJ software for five section slides per animal. Values are 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).*

3.8 Animal survival.

Due to rapid progression to severe hyperglycemia, the study was terminated early. At later stages of the study, veterinarian supervision was needed due to rapidly declining health of multiple animals. At week 10 (14 weeks old), one male mouse (*ob/ob* control) was found deceased in its cage, and 3 female mice (*ob/ob* + c) required euthanasia on humane grounds. Therefore, the study was terminated. This highlights the aggressive phenotype as well as the potentially deleterious nature of cocoa in females in this context. It is worth noting that tissue and blood were not obtainable for the male mouse found deceased and are thus not included in the data and was unable to collect genotypic identification (Supplementary Table 2).

4. Discussion

In this pilot study, we compared effects of cocoa on obesity-induced T2D in both male and female BTBR.Cg-*Lep*^{ob}/WiscJ mice. The *ob/ob* mutation on the BTBR background produces an aggressive model of hyperglycemia. To the best of our knowledge, this is the first study of any flavanol or flavanol-rich food in *ob/ob* T2D mice on the BTBR background, as well as one of the few studies to simultaneously compare the effects of cocoa on both sexes. Within the realm of cocoa and its effect on inflammation, obesity, and associated disease pathologies, very few investigators utilize male and female mice simultaneously [15,47]. Despite the small sample size, our results suggest significant differences between male and female *ob/ob* mice when fed a diet supplemented with flavanol-rich cocoa extract.

Sex differences emerged not only in established markers of T2D but also in physical observation and weight gain. Although not statistically significant due to small sample size, our data suggest that cocoa supplementation may have provided slight protection against obesity in male *ob/ob* + c mice, yet females of the same treatment displayed similar weight gain over the 10 weeks as *ob/ob* control (Figure 1). Large variability was seen within weights of both male and female *ob/ob* control mice, yet when cocoa supplementation was introduced, that variability was lost. We have previously reported that impacts of flavanol supplementation on body weight and glycemic control vary significantly between sexes in mice [48] but a larger sample size is needed to further understand this phenotypic variation as it has not been previously reported in BTBR *ob/ob* males or females [7,34,49,50].

The speed of T2D progression was notable in this study, as the cascade of hyperglycemia and hyperinsulinemia was accelerated with the *ob* mutation on the BTBR background. It has been previously reported that BTBR *ob/ob* mice are unable to maintain adequate insulin production, have impaired glucose tolerance, and are insulin resistant at 4-6 weeks of age, in contrast to B6 *ob/ob* mice which are capable of compensating for insulin resistance [31]. In our study, 4 h fasting blood glucose at 6 weeks of age (week 2 ITT) was between 275 – 425 mg/dL and at 10 weeks of age (week 6 ITT) was over 560 mg/dL for male and female *ob/ob* control (Figure 2A, D). Lan *et al* [7] reported similar fasting blood glucose levels for BTBR *ob/ob* mice and directly compared these mice to age-matched B6 *ob/ob* that display fasting blood glucose levels <250 mg/dL at 9 weeks of age.

Cocoa supplementation appeared to have acute effects early in the intervention. *Ob/ob* + c females exhibited significant protection against acute insulin resistance compared to *ob/ob* control (week 2), but this protection was lost by roughly the midpoint of the study. Due to the extremely aggressive nature of T2D progression in BTBR *ob/ob* mice, this benefit observed in females was not sustainable through nutritional prevention strategies. As mentioned above, BTBR *ob/ob* mice are reported to be insulin resistant by 6 weeks of age, furthering our hypothesis that cocoa does in fact provide protection against initial disease progression, specifically in female mice.

These data also suggest that cocoa effects potentially reverse based on sex as T2D progresses. Cocoa appeared to improve hyperglycemia-induced hyperinsulinemia in males but resulted in worsening of markers in females under fasting conditions (Figure 4). Whether this is due to changes in beta cell function and/or differences in insulin resistance is unknown, and further studies are needed to delineate the underlying mechanism(s). However, large (10-fold) differences in fasting insulin levels between males and females fed CE cannot be explained by differences in fasting blood glucose alone. Cocoa protected against beta cell loss in females (Figure 5) and together with fasting insulin expression, these results potentially indicate that in females, although CE did not provide relief against insulin resistance and hyperglycemia-induced

hyperinsulinemia, it protected beta cell health and function. On the other hand, CE reduced insulin resistance and hyperglycemia-induced hyperinsulinemia in males, but that protection was not carried across to the beta cells. We previously reported that cocoa monomers and cocoa flavanol microbial metabolites promote beta cell stability and enhance/stimulate beta cell function *in vitro* [23,51]. These effects appear to translate *in vivo* in female mice supplemented with CE, but it remains unknown as to the mechanisms behind these effects. If these effects are translatable to humans, cocoa may be useful at different stages of beta cell function and insulin resistance based on sex.

We also observed that cocoa did not appear to affect fasting serum triglycerides in males but caused large increases in females, having significantly higher levels than female *ob/ob* control (Figures 4). The mechanism for this difference, as well as its implications, are unknown as these differences were not reflected in body weights (Figure 1). The combination of elevated circulating insulin and serum triglycerides, particularly in female *ob/ob* + c mice, can potentially be explained by more severe hyperglycemia and hyperinsulinemia. The extreme worsening of hyperinsulinemia and apparent insulin resistance in females remain therefore unexplained. A recent study reported worsening glucose metabolism in a mice fed purified polyphenols diet-induced obese mouse model [52]. These data suggest that the potential anti-obesity and anti-diabetic benefits of cocoa may differ in mice based on T2D stage, biological sex, and/or hormonal status. Whether these sex-specific effects are seen in humans remains to be established. Future studies should also capture the metabolic response in the liver, skeletal muscle, and adipose tissue to better understand these relationships between sex, beta cell function, and glucose homeostatic perturbation.

This experiment was designed as a pilot, with the inherent limitation of small sample size (n=3). An additional limitation is the aggressive nature of this model. Nutritional intervention was not able to blunt long-term disease onset, however, these data provide insights into the potential effects of cocoa in the context of severe hyperglycemia. Despite these limitations, sex-specific effects of cocoa on hyperglycemia and related metabolic biomarkers were evident. Additionally, diet formulation was based on WT mice, therefore the dose of cocoa employed was elevated due to hyperphagia characteristic of the *ob/ob* mutation. Future research is needed using more translatable doses. There are also significant challenges in working with this model. First, the cost for both heterozygotes and homozygotes of the *ob* mutation on the BTBR background is high, and availability is also limited compared to mice on the B6 background. The lead times for delivery were also long or unknown and age availability was limited. Thus, the cost and availability of these mice inherently restrict the experiments that can be performed, consequently leading to the small sample size for this pilot study. However, given the preliminary findings, larger studies with more statistical power are warranted to further evaluate these findings.

The findings of this pilot study, along with recent studies from our group and others, highlight the need to factor in sex-specific disease biology and treatment effects when studying interventions in pre-clinical animal and human studies [48,53]. Too often, pre-clinical animal studies of a single strain and/or sex are utilized to evaluate the potential for a dietary bioactive to prevent or ameliorate chronic disease [54,55]. Studies should be performed to elucidate whether sex differences are primarily due to hormonal differences [56–58] as well as the role that genetic background plays in disease outcome. We previously reported that the anti-obesity and anti-diabetic activity of quercetin was highly dependent on genetic background in mice [48]. It is unknown whether the sex-specific effects of cocoa in the present study are limited to mice with the *ob/ob* mutation or the BTBR genetic background. These findings will need to be evaluated in

the context of multiple preclinical mouse models of T2D (DIO, *db/db*, HF + STZ, etc.). If the results are generalizable across strains, human work is warranted. If the results are specific to narrow genetic contexts, further studies can be performed to identify the genetic loci responsible for such effects. These results could then be applicable to genes with human orthologues for potential personalized nutrition approaches targeting humans with specific genetic characteristics [59]. Future studies are needed in both males and females, using multiple complementary preclinical models of obesity and hyperglycemia (diet-induced C57BL/6J, *ob/ob*, etc.) to further interrogate sex-specific effects of cocoa at various stages of hyperglycemia.

5. Conclusions

Cocoa supplementation appears to have sex-specific effects in the context of severe hyperglycemia. Cocoa appears to acutely prevent against and insulin resistance in females in the short term. Chronic supplementation appears to ameliorate obesity-induced hyperinsulinemia in males and worsen hyperlipidemia and obesity-induced hyperinsulinemia, yet preserve and enhance beta cell survival, in females. The underlying mechanisms of these sex differences, as well as their implications for humans, warrant further study. These pilot data inform experiments with larger sample sizes. Whether these sex-specific effects are observed in other preclinical models of T2D remains to be established. OVX and estrogen inhibitor/replacement studies are justified to determine the hormonal basis for such differences. If sex differences are apparent in subsequent studies, clinical studies will be warranted to establish whether these differences are relevant in humans. Sex differences may need to be taken into consideration when designing intervention strategies to prevent or ameliorate T2D.

Funding: This work was funded by the US Department of Agriculture by NIFA AFRI foundational grant 2020-67017-30846 (APN, JST, CDK, and MGF).

Acknowledgements. The authors wish to acknowledge Dr. Glicerio Ignacio, DVM (attending veterinarian) and Mr. Daniel Peralta (facility manager) of the David H. Murdock Research Institute for their invaluable contributions to animal care, husbandry, and phenotype monitoring, as well as Lauren Essenmacher for her contribution to animal husbandry and blood glucose testing and Lyric Ramsue for her assistance with ELISA assays.

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