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Opposing Effects of S-equol Supplementation on Metabolic and Behavioral Parameters in Mice Fed a High Fat Diet

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Abstract

Phytoestrogens, such as daidzein and genistein, may be used to treat various hormone-dependent disorders. Daidzein can be metabolized by intestinal microbes to S-equol. However, not all individuals possess bacteria producing this metabolite, resulting in categorization of equol vs. non-equol producers. Past human and rodent studies have suggested supplementation of this compound might yield beneficial metabolic and behavioral effects. We hypothesized that administration of S-equol to diet-induced obese male and female mice would mitigate potential diet-induced metabolic and comorbid neurobehavioral disorders. To test this possibility, we placed five-week-old C57 mice on a high fat diet (HFD) to mimic the diet currently consumed by many Western adults. Animals were randomly assigned: S-equol supplementation (10 mg/kg body weight) or vehicle control group. After four weeks on HFD with or without S-equol supplementation, metabolic and behavioral phenotyping was performed. While the initial hypothesis proposed S-equol treatment would improve metabolic and neurobehavioral outcomes, this supplementation instead exacerbated aspects of HFD-induced metabolic disease, as indicated by suppressed physical activity in treated individuals, reduced energy expenditure in treated males, and serum chemistry changes (hyperglycemia in treated individuals; hyperinsulinemia and hypoleptinemia in treated males). Conversely, S-equol individuals exhibited less anxiety-like and depressive-like behaviors, as evidenced by increased exploratory time in the elevated plus maze by treated males and increased time spent mobile in the tail suspension test for treated individuals. In summary, S-equol may be beneficial in mitigating depression and anxiety disorders in individuals, but for indeterminate reasons, supplementation may worsen facets of metabolic disorders in obese individuals.

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Keywords

daidzein; equol; phytoestrogen; gut microbiome; obesity; mood disorders; anxiety; depression; metabolic disorders; physical activity

1. Introduction

Isoflavones are phytoestrogens, or plant-based estrogenic molecules, abundant in soy, soy-based foods, and other legumes. Soy emerged as a food of importance with the observation that nations that consumed more soy were overall healthier. Soy isoflavones may reduce incidence of breast cancer [1], cardiometabolic disease [2–5], and improve cognitive function [6]. Some studies suggest that such protective effects, like that of soy in breast cancer prevention, are absent in non-Asian populations [7], although recent work may contradict this claim [8]. Notwithstanding, the variation in response to soy between Asian and Western individuals may suggest that the benefits of soy may not be inherent to the protein itself. Instead, the benefits may lie in differences of the consumer, such as the ability to produce an intermediary phytoestrogen-derived metabolite that confers protection.

These phytoestrogens are metabolized, in some cases, to more active forms by gut microbes. S-equol is one metabolite produced when select intestinal bacteria metabolize daidzein/daidzin [9]. Disparity exists in individual's ability to produce S-equol, with 60% of Asian individuals having the gut microbes needed for such conversion, but only 25% of Western individuals possessing S-equol-producing gut bacteria [10]. Those individuals not producing S-equol instead produce O-desmethylangolensin (ODMA); importantly, the ODMA-producer, but not S-equol phenotype, tends to be associated with obesity development [11]. Within Asian populations, the rate of equol producers is positively associated with the amount of soy intake [12].

While S-equol was first identified in humans in the 1980s [13], research examining potential therapeutic benefits of this metabolite is still in its relative infancy [14]. To date, S-equol has been attributed to inducing several health benefits, such as reduced prostate cancer incidence in men [15] and alleviation of menopausal symptoms in women [16]. Importantly, S-equol has also been associated with positive metabolic and neurobehavioral outcomes [17–24]. While not all rodent studies agree on potential cognitive benefits of S-equol [25], reports in humans show equol producers have overall better cognitive performance and improved emotional responses [23, 24].

Previous studies suggest that S-equol supplementation might be a useful adjuvant in treating metabolic disorders in obese individuals. Additionally, many individuals with obesity experience comorbidity with depression, anxiety, and other mood disorders, giving rise to the term “metabolic-mood syndrome” [26–29]. Thus, S-equol might also be useful in combating such associated conditions.

We therefore hypothesized that administration of S-equol to diet-induced obese (DiO) male and female mice would mitigate potential diet-induced metabolic and neurobehavioral disorders, as previous reports have suggested beneficial effects in humans and rodents

supplemented with this compound [17–24]. The current studies also sought to clarify potential discordant results in terms of whether S-equol supplementation provides beneficial behavioral effects. The main research objectives were to place adult male and female C57BL6J mice on a Western HFD designed to replicate the diet and metabolic state of many obese individuals and cause DiO. A DiO approach was used as it is considered one of the best methods to examine obesity and other metabolic disorders in non-genetically altered rodent models [30–32]. To test whether S-equol supplementation would be beneficial in alleviating metabolic and behavioral disorders associated with obesity, male and female mice were randomly assigned then to receive either S-equol supplementation or vehicle control for eight weeks. Another aim of this study was to examine for potential sex differences in response to S-equol treatment, necessitating both males and females to be examined. After one month on the HFD with or without S-equol supplementation, metabolic and behavioral indices were performed in all male and female mice.

2. Methods and materials

2.1. Animals and treatments

Five-month-old C57BL6J mice (14 males and 14 females) were shipped from Jackson Labs to the University of Missouri. Mice were allowed to acclimate to their new environment for one week, during which time they were fed the AIN93G diet (Envigo, Madison, WI). After the one week habituation period, males and females were switched over from the AIN93G to a HFD diet and provided a distinguishing tattoo mark (based on cage assignment). At this time, they were also randomly assigned to one of two groups: 1) HFD + daily oral administration of vehicle alone (2.5% dimethyl sulfoxide [DMSO]/0.5% carboxymethyl cellulose [CMC]), the control group (CTL) or 2) HFD + daily oral administration of 10 mg S-equol /kg body weight in 2.5% DMSO/0.5% CMC, the S-equol group. Each group consisted of seven males and seven females. The dose of S-equol was chosen based on past studies suggesting that similar doses show beneficial metabolic and behavioral effects [18, 33]. All mice are considered natural S-equol producers [9], and thus, we did not measure their internal equol production before randomly assigning them to one of these two treatment groups. All animals were maintained on a HFD to challenge them metabolically and recapitulate the obesogenic diet many Westerners are currently consuming. This HFD included no soy derivatives to ensure that the mice had minimal endogenous production of S-equol and only received it exogenously, if in the S-equol group. We, and others, have previously used this diet to induce DiO in various mouse models [34–37]. The ingredient composition of this diet is listed in Table 1. Animals of the same sex and receiving the same treatment were group-housed in polystyrene cages, provided aspen bedding, and given *ad libitum* access to the HFD and water (administered in glass water bottles). All experiments were approved by the University of Missouri Animal Care and Use Committee (Protocol # 8693). Studies were conducted in accordance with the National Institute of Health guidelines for the proper handling of laboratory animals. Sentinel animals in the rooms are routinely screened for major rodent pathogens, and none have been found to date. Animals were weighed weekly on an Ohaus SC-4010 Scout II Portable Electronic Balance, 400g, 120 V scale (Ohaus, Paisippany, NJ) for the entire duration of the study.

2.2. Metabolic assessments

2.2.1. Promethion indirect calorimetry unit—After one month on HFD $\pm$ S-equal supplementation, each mouse was placed in an individual Promethion indirect calorimetry unit (Sable Systems International, Las Vegas, NV) for 3 full days, as detailed previously [34, 38]. Data were divided into 12-hour light and 12-hour dark cycles. Parameters that were measured include energy expenditure, RQ value of oxygen consumption vs. CO₂ production, number of beam breaks on various axes (X – vertical, Y – horizontal, and Z – rearing), food, and water intake. Beam break quantification is indicative of total movement within the cage. The first day of data was not analyzed, as this is considered the habituation period. Each cage included a weighing chamber, a full water bottle, food chamber for *ad libitum* feeding of the HFD, and a thin layer of aspen shavings. While in the chamber, mice were provided their daily dose of CTL vehicle or S-equal on a mini Nabisco wafer to ensure measurements of metabolic readings were not disrupted.

2.2.2. EchoMRI—Following indirect calorimetric testing, mice were placed in the EchoMRI 1100 (EchoMRI LLC, Houston, TX) to measure body composition, as described previously [34, 38]. Parameters were measured in a rapid (approximately 30 seconds) and non-invasive manner, and included total muscle and fat, free water, and total water mass. Tubes were disinfected with 70% ethanol between testing each mouse.

2.2.3 Glucose tolerance tests (GTT)—Two days after behavioral testing, discussed below, GTT were conducted. All mice were fasted five hours prior to baseline glucose measurement, weighed, and subsequently placed in clean single housing units with *ad libitum* access to water. One hour prior to baseline testing, mice were allowed to habituate to the testing room, and cages were placed on low-heat generating heating pads (SnuggleSafe, UK) to encourage blood flow to the tail. The tail of each mouse was disinfected with isopropyl alcohol and lidocaine was applied five minutes prior to baseline testing. At the indicated time, tails were slightly nicked with disinfected surgical scissors, and blood extruded from the tail vein. Using an AlphaTRAK glucometer and AlphaTRAK 2 blood glucose test strips (Zoetis, Chicago Heights, IL), baseline blood glucose concentrations were assayed for each mouse. Glucose was then drawn up into 1 cc tuberculin syringes and mice were intraperitoneally injected with 2 g glucose/kg body weight. Tail vein blood was then collected in the same manner as the initial time and blood glucose concentrations measured and recorded at 15, 30, 45, 60, and 120 minutes post-glucose administration [39, 40].

2.2.4. Serum chemistry and hormone profile measurements—Two days after GTT were performed, mice were fasted at 17:00 hours. The following day, mice were humanely euthanized with CO₂ inhalation followed by cervical dislocation, consistent with guidelines for the Panel on Euthanasia of the American Veterinary Medical Association, and cardiac blood was collected and immediately placed on ice in 1.5 mL microcentrifuge tubes. The blood was centrifuged for 15 minutes at 7500X g in a Hettich Zentrifugen centrifuge (Hettich Lab Technology, Beverly, MA). The serum fraction was then collected and stored at -80°C until metabolite and hormone analyses were performed. Serum glucose was measured by using a commercially-available hexokinase glucose-6-phosphate assay (Beckman-Coulter, Brea, CA) on an automated clinical chemistry analyzer (Beckman-

Coulter AU680, Brea, CA). Two levels of serum quality control (Randox Laboratories, Kearneysville, WV) for the chemistry analyzer were utilized. Plasma insulin (Crystal Chem, Downers, Grove, IL. Catalog # 90080), adiponectin (Crystal Chem, Catalog # 80569), corticosterone (Crystal Chem, Catalog # 80556), and leptin (Crystal Chem, Catalog # 90030) concentrations were analyzed according to the manufacturer's instructions for each of these ELISA kits, but without any serum dilution, as detailed previously [34, 38].

2.3. Behavioral assessments

2.3.1. Elevated plus maze (EPM)—The EPM was used to examine anxiogenic and exploratory behaviors, as detailed previously [34, 41–43]. The EPM is arranged in a plus configuration and includes two opposite open arms (30 cm), a central platform region (5 × 5 cm), and two opposite closed arms (30 cm). Each animal was placed in the center of the maze and permitted to explore it for 300 seconds. Each trial was recorded with a Canon Vixia HF HD hand held camcorder (Canon, Melville, NY). Between trials, the maze was disinfected with 70% ethanol, which also removed olfactory cues from previous mice tested. The video trials were then analyzed with the Observer Version 11.5 software (Noldus Technologies, Leesburg, VA). Parameters measured include number of entries into the open and closed arms, duration of time spent in the open and closed arms and center, and non-anxious or exploratory behavior, measured by head-dipping and rearing.

2.3.2. Tail suspension test (TST)—The TST measures depressive-like behaviors, based on the amount of time spent immobile. The test was performed as described previously [44]. Using non-adhesive painter's tape, mice were suspended by their tails 20 cm above the floor for 6 minutes. Trials were recorded with a Canon Vixia HF HD hand held camcorder (Canon). The first two minutes of the trial were considered habituation period and not analyzed. The remaining four minutes of each trial were analyzed with the Observer Version 11.5 software (Noldus Technologies). Time spent immobile, as evidenced by complete cessation of movement, vs. mobile, was determined.

2.4 Statistical analyses

Sample size was determined based on previous mouse studies testing effects of equol on metabolic and behavioral parameters [18, 19, 45, 46] and SAS Power and Sample Size application (PSS, SAS analysis, Cary, NC). Data from the Promethion Indirect Calorimetry Unit was first run through ExpeData analyses. In addition, all behavioral assessments listed above were first recorded with a camera, with the manners noted above, timed using a stop watch, and recorded. All dependent variables were analyzed for normality using the Wilk-Shapiro test (SAS, v9.4, Cary, NC). Serum adiponectin and corticosterone concentrations were logarithmically and square root-transformed, respectively, to approach a normal distribution for data analysis. Data for the dependent variables of EchoMRI body muscle, fat, free and total water mass, serum insulin and leptin, EPM data, time spent mobile and immobile in the TST, and food intake were analyzed by using the general linear model (GLM) procedure of Statistical Analysis Systems (SAS). Sources of variation considered were treatment, sex, and treatment × sex interaction. Data for dependent variables determined by Promethion indirect calorimetry unit were also analyzed by GLM procedure of SAS (V9.4) with treatment, mouse sex, light cycle (light and dark) and interactions

between them as source of variance. Repeated body weight and blood glucose concentration measurements obtained with GTT were analyzed as a repeated measures, as outlined by Littell et al. [47]. The ANOVA model contained the effects of treatment, sex, and the interaction of treatment X sex. The individual served as the experimental unit. These data were analyzed by using the PROC MIXED procedure in SAS (Ver. 9.4). Differences in body weight and glucose between treatment groups over time were determined by Fisher's protected least-significant difference (LSD) to control for multiple testing. A p value of 0.05 was considered significant. All data are presented as means $\pm$ standard error of the means (SEM).

3. Results

3.1. Body weight and food intake

Over the course of the studies, S-equol treatment alone and in interaction with sex did not affect overall body weight compared to controls counterparts (Fig. 1, $p > 0.05$). Food intake did not differ between the two treatment groups (Fig. 2, $p = 0.88$).

3.2. Promethion indirect calorimetry unit assessments

3.2.1 Average energy expenditure and respiratory quotient (RQ)—For average energy expenditure, a two-way interaction between S-equol treatment and sex existed ($p = 0.0006$). CTL males expended more energy when compared to S-equol males (0.51 ± 0.01 vs. 0.46 ± 0.01 kcal/hour, respectively, $p < 0.0001$; Fig. 3A). However, no differences were detected in females. For average RQ value, an interaction existed between treatment and sex ($p < 0.0001$). S-equol treated females had a higher RQ value than control females, suggesting that the latter were burning more fats (0.84 ± 0.01 vs. 0.74 ± 0.01 , respectively, $p < 0.0001$; Fig. 3B). No differences were detected between male groups.

3.2.2 Activity Level Assessments—Total number of Y beam breaks was affected by treatment ($p = 0.01$). S-equol individuals broke the beam less often than controls (11975.94 ± 669.87 vs. 13478.88 ± 741.55 episodes, respectively, $p = 0.01$; Fig. 4A). Total meters traveled was affected by treatment ($p = 0.006$). When considering both males and females combined, S-equol treated individuals traveled less than controls (147.19 ± 12.88 vs. 168.28 ± 13.45 meters, $p = 0.006$; Fig. 4B). Total percentage of time spent walking and remaining still or sleeping were also affected by treatment ($p < 0.0001$ and $p = 0.0003$, respectively). S-equol individuals walked less percentage of the time while in these cages than control counterparts (27.14 ± 1.88 vs. 31.81 ± 2.02 %, respectively, $p < 0.0001$; Fig. 5A). In contrast, S-equol individuals spent a greater percentage of time remaining still vs. CTL group (71.15 ± 2.03 vs. 66.72 ± 2.19 %, respectively, $p = 0.0003$; Fig. 5B). S-equol individuals spent greater percentage of time sleeping relative to controls (65.60 ± 2.35 vs. 60.57 ± 2.51 %, respectively, $p = 0.0003$; Fig. 5C).

3.3 EchoMRI

None of the parameters measured with EchoMRI differed based on treatment. These included lean weight, fat weight, free water, and total water.

3.4 Glucose tolerance tests (GTT), serum chemistry, and metabolic hormones

Treatment did not affect overall GTT nor individuals time-point measurements (Fig. 6, $p > 0.05$). After animals were fasted and euthanized, glucose serum levels were affected by treatment ($p < 0.0001$). S-equol animals had elevated glucose concentrations when compared to controls (376.58 ± 21.86 vs. 174.93 ± 22.39 mg/dL, respectively, $p < 0.0001$; Fig. 7). A two-way interaction existed between treatment and sex for serum insulin concentrations ($p = 0.02$). S-equol males had elevated insulin concentrations vs. CTL males (1.16 ± 0.18 vs. 0.61 ± 0.09 ng/mL, respectively, $p = 0.0084$; Fig. 8A). No differences were detected between female groups. For serum leptin concentrations, a two-way interaction existed between treatment and sex ($p = 0.03$). S-equol males had reduced leptin concentrations compared to CTL males (45.65 ± 14.69 vs. 113.85 ± 27.88 ng/mL, respectively, $p = 0.0467$; Fig. 8B). No differences were detected between female groups.

3.5. Behavioral parameters

3.5.1. Elevated plus maze (EPM)—Overall exploratory time (defined as time spent engaging in head-dipping and rearing behaviors) in the EPM showed a two-way interaction existed between treatment and sex ($p = 0.015$). S-equol males spent more time engaging in such exploratory behaviors compared to CTL males (20.38 ± 3.85 vs. 9.06 ± 2.95 seconds, respectively, $p = 0.01$; Fig. 9A). No differences were detected between female groups. Time spent rearing alone also showed a two-way interaction between treatment and sex ($p = 0.01$). S-equol males reared more than CTL males (12.02 ± 2.84 vs. 4.82 ± 1.89 seconds, respectively, $p = 0.02$; Fig. 9B). No differences were detected between female groups.

3.5.2. Tail suspension test (TST)—Time spent mobile in the TST was affected by treatment ($p = 0.02$). S-equol individuals spent more time mobile than CTL individuals (176.4 ± 10.31 vs. 142.57 ± 7.98 seconds, respectively, $p = 0.0195$; Fig. 10), suggestive of decreased depressive-like behaviors in the former group.

4. Discussion

The current studies sought to test whether S-equol supplementation helped to combat metabolic and behavioral disorders, especially anxiety- and depressive-like traits, in a diet-induced (DiO) mouse model for human obesity. Based on past rodent and human studies, the prediction at the outset was that both types of disorders would be alleviated in mice consuming a HFD [17–24, 48, 49]. However, conflicting results were obtained in the current work.

Metabolic and voluntary physical activity in a home cage setting, i.e. within the Promethion indirect calorimetry, suggest that mice, especially males, supplemented with S-equol showed disruptions in these categories relative to CTL mice also on the HFD. This was evident by reduced energy expenditure and burning of fats vs. carbohydrates, and increased serum glucose, even though treated males had higher insulin concentrations, but reduced leptin concentrations. Conversely, mice fed a HFD provisioned with S-equol showed improved behavioral patterns. Increased exploratory behavior in the EPM suggests that they were less anxious. Increased mobility in the TST indicates S-equol treated males and females had less

depressive-like tendencies. It is not clear why this supplementation approach helped mitigate anxiogenic and depressive-like behaviors in the DiO mouse model while potentially exacerbating metabolic parameters and voluntary physical activity in a home cage setting. Different outcomes may have been obtained in mice maintained on a standard chow diet.

It is not clear why S-equol treatment improved behavioral outcomes but worsened metabolic parameters, especially as comorbidity with behavioral and metabolic disorders often exist. One possibility is that S-equol stimulates different estrogen receptors (ESR) in metabolic organs compared to various brain regions, including those such as the amygdala that regulate emotive behaviors. Equol tends to bind with higher affinity to ESR2 (also called ER β) than ESR1 (ER α) [14, 50]. However, equol isomers can act as well through ESR1. Equol might also induce anti-androgen effects by binding and sequestering 5 α -dihydrotestosterone (DHT), preventing this hormone from binding and activating androgen receptors (AR) [51]. Conversely, a study with male rats did not detect anti-androgen effects in the brain, prostate gland, and male reproductive tract, but this compound suppressed ESR1 and ESR2 expression in the hypothalamus [52]. Taken together, past studies suggest that S-equol can interact and affect both ESRs and might also induce anti-androgenic effects in at least some tissues. Depending on the balance of steroid receptors that are targeted within a given tissue could affect the ultimate phenotype; thereby possibly explaining opposing S-equol effects on metabolic vs. behavioral parameters.

Intestinal bacteria can metabolize daidzein to S-equol with mice possessing several gut microorganisms capable of this conversion with rodents therefore considered natural S-equol producers [9]. In contrast, differences exist across human populations in ability to produce this compound. 60% of Asian individuals possess bacteria able to do this conversion, while only 25% of Western individuals contain S-equol producing gut bacteria [10]. Non-equol-producing individuals instead tend to be ODMA producers, which is associated with a greater incidence of obesity [11]. Supplementation of recent menopausal women with equol and resveratrol (a natural phenol found in several plants, including grapes, blueberries, and raspberries) resulted in improved menopausal signs, including reductions in heart discomfort, vaginal dryness, sexual problems, and sleep disorders [53]. It remains to be determined though whether S-equol alone or in combination with resveratrol improves metabolic and neurobehavioral disorders.

A strong correlation exists between obesity and mood disorders in males and females showing depressive symptoms and other mood disorders [26–29]. In addition, a 2013–2016 study from the Center of Disease Control and Prevention, found that Asian adults report lower depression when compared to Hispanic, non-Hispanic black, or non-Hispanic white adults [54]. Our current results suggest that S-equol supplementation might be useful in treating depression in obese individuals, especially those who do not naturally produce S-equol. However, the current mice data suggests that such adjuvant treatment should be used with caution, as it could also worsen aspects of metabolic syndrome, although further work is needed in this area. Potential conflicting results in the current mouse model and past human studies might also be explained by the fact that all mice are natural S-equol producers [9]. Treating mice with supplemental S-equol presumably only raises levels of this

metabolite. However, there could be a dosage range in which S-equol is protective against metabolic and behavioral disorders, but beyond which may exacerbate metabolic parameters.

To recapitulate humans who do not naturally produce this metabolite, future studies might consider testing germ-free (GF) mice, transferred with fecal samples from non-equol-producing individuals. Such “humanized” GF mice could then be provided exogenous S-equol and metabolic and behavioral parameters assessed. If the metabolite results in beneficial metabolic and behavioral outcomes in these mice, it would provide robust evidence that obese and depressed individuals who do not naturally produce S-equol would benefit from supplementation of S-equol.

Thus, the limitations of the current study are that the effects of S-equol were tested in DiO mice that already have gut bacteria capable of producing S-equol. To reduce endogenous S-equol production the HFD was designed to be phytoestrogen-free. Even so, the findings might not fully translatable to human populations, especially non-equol producers. While the current studies have begun to examine the effects of S-equol on metabolic state and mood-related disorders, direct effects on adipose tissue and other metabolic organs have not been considered. Additionally, future studies should examine how S-equol affects other behaviors, and molecular changes in specific brain regions should be examined. Such experiments might also help elucidate the underpinning reasons why S-equol results in beneficial behavioral but detrimental metabolic effects in DiO mice, e.g., whether it is due to differential binding and activation of ESR1 or ESR2. The current studies also only considered a single dose of S-equol and mice were only provided this compound for a relatively short time frame. Further studies are needed to establish dose response curves and the long-term effects of S-equol supplementation.

In conclusion, our current findings with conventional mice fed a HFD show that S-equol treatment ameliorates anxiogenic and depressive-like behaviors, while possibly intensifying HFD-induced metabolic disorders. Why this is the case remains to be determined with additional and longer-term supplemental studies in conventional mice. Based on the current findings, we partially accept the initial research hypothesis in that this supplement helped curb depressive-like behaviors in the mice, but the predicted beneficial metabolic effects were not observed. Rather, this compound seemed to enhance metabolic disruptions in DiO mice. S-equol provisioning to GF mice that have been transplanted with human gut microbiota from non-S-equol producers may result in both metabolic and behavioral benefits in such future studies. If such is the case, this would have translational impact to non-S-equol producing human populations.

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Abbreviations

AR	androgen receptor
CMC	carboxymethyl cellulose
CTL	control group
DHT	5 α -dihydrotestosterone
DiO	diet-induced
DMSO	dimethyl sulfoxide
EPM	Elevated Plus Maze
ESR	estrogen receptor
GF	germ-free
GLM	general linear modeling
GTT	Glucose Tolerance Tests
HFD	high fat diet
LSD	least significant difference
ODMA	O-desmethylangolensin
RQ	Respiratory Quotient
SEM	standard error of the means
TST	Tail Suspension Test
VRSP	Veterinary Research Scholars Program

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Fig. 1. Measurement of body weight gain for S-equol treated and control male and female mice. S-equol treated did not affect body weight gain in male or female mice compared to CTL counterparts ($p > 0.05$). Predictably, males weighed more than females over the course of the studies ($p < 0.05$). Data represent means $\pm$ SEM.

Fig. 2. Food intake over a 24 hour period.

There was no difference in food intake over a 24 hour period between S-equol vs. CTL group. Data represent means $\pm$ SEM. $p=0.8$.

Fig. 3. Effects of S-equol treatment on average energy expenditure and respiratory quotient (RQ).

A. Comparison of energy expenditure in male and female mice treated with S-equol compared to control counterparts. **B.** Comparison of RQ in male and female mice treated with S-equol vs. control counterparts. Data represent means $\pm$ SEM. * $p=0.0006$; ** $p<0.0001$.

Fig. 4. Effects of S-equol treatment on voluntary physical activity.

A. Comparison of the number of Y beam breaks in mice treated with S-equol vs. CTL. **B.** Effects of S-equol on total distance traveled while in Promethion Indirect Calorimetry Unit. Data represent means $\pm$ SEM. * $p=0.01$; ** $p=0.006$.

Fig. 5. Activity within the Promethion indirect calorimetry unit.

A. Comparison of percentage of time spent walking by S-equol vs. CTL individuals. **B.** Comparison of percentage of time spent remaining still by S-equol vs. CTL individuals. **C.** Comparison of percentage of time spent sleeping by S-equol vs. CTL individuals. Data represent means $\pm$ SEM. *p 0.0001; **p=0.0003.

Fig. 6. Serum glucose tolerance curves. Blood glucose comparisons were not significantly different based on S-equol treatment at any of the time point measurements ($p > 0.05$). Data represent means $\pm$ SEM.

Fasting Serum Glucose Concentrations

Fig. 7. Fasted serum glucose measurements.

Comparison of fasting serum glucose concentrations in S-equal treated vs. CTL mice. Data represent means $\pm$ SEM. *p 0.0001.

Fig. 8. Serum insulin and leptin concentrations.

A. Comparison of serum insulin concentrations in male and female S-equol treated vs. CTL counterparts. **B.** Comparison of serum leptin concentrations in male and female S-equol treated individuals compared to CTL counterparts. Data represent means $\pm$ SEM. * $p=0.02$; ** $p=0.05$.

Fig. 9. Behavioral differences in the elevated plus maze (EPM) testing.

A. Comparison of total exploratory time, as defined by time spent engaging in rearing and head-dipping behaviors, in EPM for male and female S-equol treated individuals compared to CTL counterparts. **B.** Comparison of number rearing episodes for male and female S-equol treated individuals vs. CTL counterparts. Data represent means $\pm$ SEM. * $p=0.01$; ** $p=0.02$.

Fig. 10. Total time spent mobile in tail suspension test (TST).
Comparison of total time spent mobile for S-equal treated individuals vs. CTL counterparts.
Data represent means $\pm$ SEM. * $p=0.02$.

Table 1.

Ingredient and macronutrient composition of the high fat diet (HFD).

	HFD (7% CO, 15% Lard) TD.130957
Ingredient	g/Kg
Casein	240
L-Cystine	3.6
Corn Starch	93.626
Maltodextrin	132
Sucrose	200
Corn Oil	70.0
Lard	150
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	42
Calcium Phosphate, dibasic	3.6
Calcium Carbonate	1.2
Ferric Citrate	0.24
Vitamin Mix, AIN-93-VX (94047)	12
Choline Chloride (85% Choline)	1.62
TBHQ, antioxidant	0.014
Red Food Color	0.1
Total (g)	1000
Macronutrient	% by wt
Protein	21.2
CHO	43.1
Fat	22.2
Kcal/g	4.6
	% kcal
Protein	18.6
CHO	37.7
Fat	43.8