

Xanthohumol derivatives improve insulin sensitivity in obese mice

By

Joshua Hay

An Undergraduate Thesis Submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Baccalaureate of Science in BioResource Research,
Toxicology Option

May 27, 2015

Xanthohumol derivatives improve insulin sensitivity in obese mice

Abstract

Metabolic syndrome, characterized by a collection of risk factors including abdominal obesity, insulin insensitivity, elevated plasma triglycerides, hypertension and low plasma high-density lipoproteins (HDLs), is a major contributing factor to the development of cardiovascular diseases and type II diabetes (T2D). Xanthohumol (XN), a flavonoid isolated from *Humulus lupulus* cones, has been shown to reduce some markers of metabolic syndrome by a proposed mechanism of mitochondrial uncoupling (Legette et al, 2012 and Kirkwood, et al, 2013). Two XN derivatives, dihydroxanthohumol (DXN) and tetrahydroxanthohumol (TXN) appear to have a more profound effect on mitochondrial uncoupling *in vitro*, but their effect *in vivo* is unknown. Male C57BL/6J mice were fed a high fat diet (60% kcal fat, 20% kcal protein and 20% kcal carbohydrate) supplemented with XN, DXN or TXN at a dose of 30 mg/kg body weight/day for 14 weeks. Insulin resistance was measured via glucose tolerance assays (GTT) during weeks 4 and 11, where plasma glucose levels were measured at 15, 30, 60 and 120 minutes following an intraperitoneal glucose injection of 2 g/kg body weight. Body weights were measured weekly, and plasma glucose, insulin, triglycerides, and cholesterol were examined following blood collection at sacrifice after 14 weeks on test diets. All mice in XN, DXN and TXN treatment groups showed improved insulin sensitivity demonstrated by smaller increases and more rapid recovery of blood glucose level compared to control (vehicle-diet) mice in the GTT. Only TXN mice showed significantly reduced fasting plasma glucose and insulin levels compared to the control, and none of the three compounds reduced plasma triglycerides or cholesterol levels.

Mice given TXN showed significantly reduced weight gain compared to control mice ($p < 0.05$), though mice given DXN also displayed a reduction in weight gain to a lesser extent. These results suggest that XN, DXN and TXN have a protective effect against metabolic syndrome by improving insulin sensitivity and glucose metabolism.

Introduction

Cardiovascular disease remains the number one cause of death worldwide, in spite of educational outreach and new medical procedures (7). According to the National Heart, Lung, and Blood Institute, cardiovascular diseases were responsible for more than 811,000 deaths in 2008 alone (7). This also resulted in an estimated medical care cost of 179.3 billion U.S. dollars (7). Cardiovascular disease has many underlying causes, though by far the most important factor in developed countries is obesity. In the United States, overweight and obese adults now make up fully two-thirds of the population (12). Though this proportion has somewhat stabilized over the past decade, little to no evidence indicates any decrease in prevalence. It is commonly known that a balanced diet and regular exercise are keys to a healthy cardiovascular system, though the availability of this knowledge has done little to diminish the energy imbalance that is responsible for weight gain and obesity. This information highlights the need for new strategies to combat the threat posed by obesity and related medical conditions.

The risk of cardiovascular disease is strongly correlated to a series of risk factors, collectively referred to as metabolic syndrome (7). Metabolic syndrome includes several common medical conditions, which include hypertension, excess abdominal fat, high blood sugar, high plasma triglycerides, and low plasma levels of high density lipoproteins (HDLs) (7). Any combination of

three or more of these conditions greatly multiplies cardiovascular disease risk. Currently, approximately 34 percent of US adults are affected by metabolic syndrome (7). Metabolic syndrome is almost completely preventable by changes in activity level and improved nutritional status; however, its high prevalence indicates that current preventative measures are not having a significant enough impact to eliminate the condition.

While lifestyle changes have proven effective at reversing the progression of metabolic syndrome, medicinal treatments have remained frustratingly elusive. However, new possibilities have been presented with the discovery of xanthohumol's (XN) beneficial health effects (16). XN is the most abundant of the flavonoids found in the cones of *Humulus lupulus*, or hops (16). Much interest has been generated regarding the various health benefits of XN, including improved cognitive flexibility in mice and its potential as a chemotherapeutic agent (17, 18). Most importantly for this study, however, is XN's ability to reduce weight gain (19).

The first demonstration of XN's promise involved a rat model of obesity (19). Zucker rats were fed a high fat diet (60% of Kcal) to induce rapid weight gain and dysfunctional lipid metabolism. Three treatment groups received low, medium and high dose XN supplements in their diet, and the study lasted 6 weeks. The most compelling difference was seen in male rats in the high dose group, which averaged 14% less weight gain when compared to rats on control diets (Figure 1). Follow up research has provided mounting evidence to support the hypothesis that the reduction in weight is at least partially due to mild mitochondrial uncoupling, though other studies have also indicated an interaction between XN and the farnesoid X receptor (FXR), which could explain some of the improved fasting blood sugar levels seen in treated mice (20).

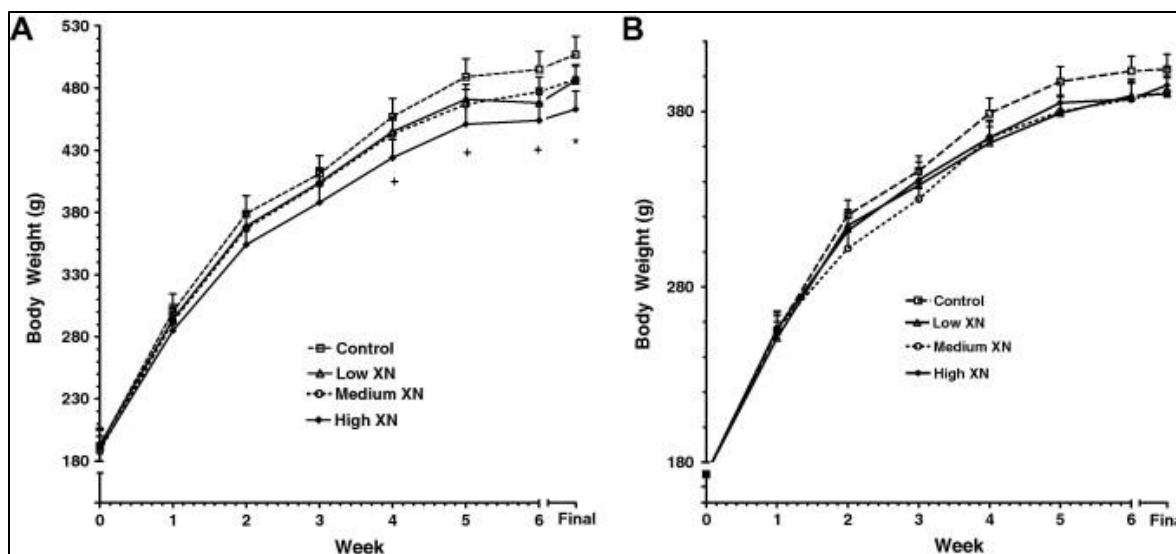


Figure 1. Reduction in weight gain of Zucker rats fed a high fat diet supplemented with xanthohumol. While females (Graph B) did not show a significant reduction in weight with treatment, males (Graph A) in the high dose group experienced an average of 14% reduction in weight gain (19).

Mitochondrial uncoupling is a process that alters the normal functioning of the electron transport chain and ATP synthase in the mitochondria (10, 11). An uncoupling agent will allow protons to reenter the mitochondrial matrix and bypass ATP synthase, partially dissipating the electrochemical gradient established by the electron transport chain (11) (Figure 2). Additional reducing equivalents such as NADH and FADH₂ are then needed to reestablish the proton gradient, resulting in higher energy expenditure and additional heat generation as the activity of electron transport chain proteins is increased and additional oxygen is reduced to water (11). Changes in cellular oxidative phosphorylation rates can therefore be estimated by the rate of oxygen consumption over time in a closed system.

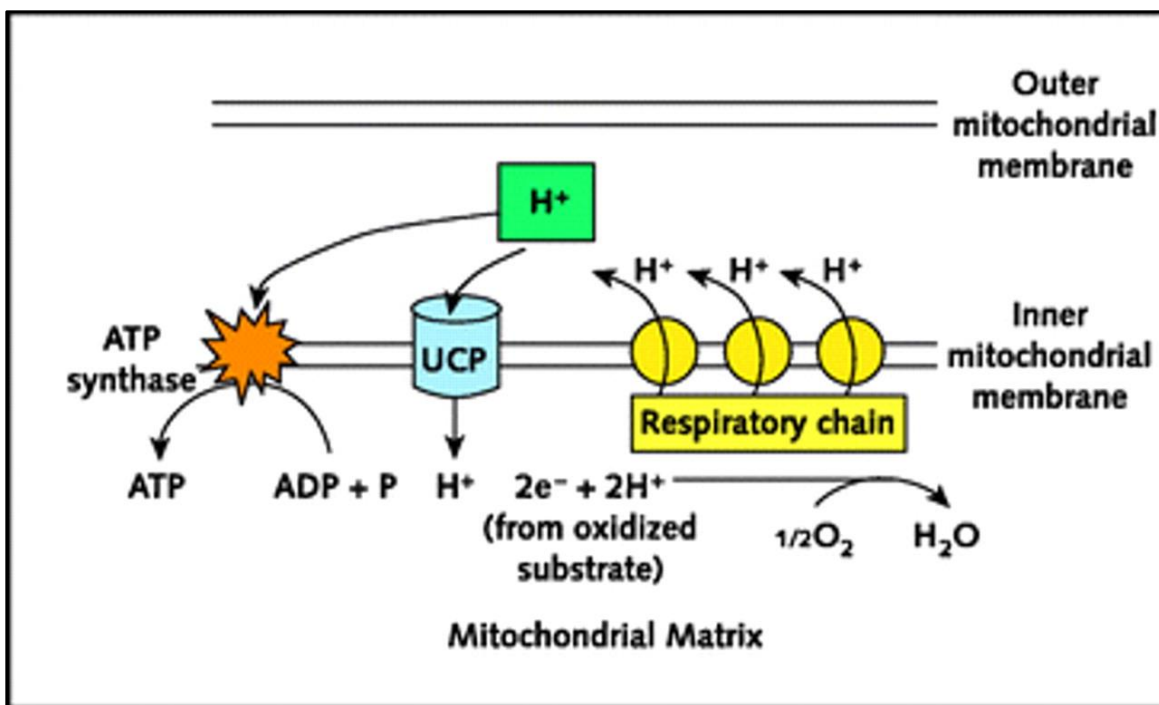


Figure 2. Mitochondrial uncoupling schematic. Uncoupling agents allow protons in the mitochondrial intermembrane space to reenter the matrix, thereby partially dissipating the proton gradient established by the electron transport chain. Oxidative phosphorylation via ATP synthase is decreased, while additional energy is expended by increased reduction of NADH and FADH₂. Image courtesy of *Annals of Internal Medicine*.

Nozawa, et al., provided evidence to show that XN is also a ligand for the FXR receptor (20). The FXR is a nuclear receptor and transcriptional activator, having a wide range of effects in many cell types. The FXR has an important role in glucose and lipid metabolism, and studies have shown that FXR null mice have poor insulin sensitivity and glucose metabolism (4). Further, additional evidence indicates that poor or improper FXR function may be linked to obesity and fatty liver disease (4). While primarily activated by bile acids, it appears that XN is able to

modulate FXR activity, possibly explaining some of the improved glucose sensitivity seen in mouse and rat studies (20).

To investigate XN's mechanism of action, cellular oxygen consumption was monitored in C2C12 mouse myocytes, a cellular model selected for high levels of oxidative phosphorylation (2). Cells were monitored using a Seahorse Biosciences XF analyzer, allowing test substances to be injected into the cell medium at specified times. An ATP synthase inhibitor was first injected to reduce oxygen consumption via oxidative phosphorylation to consistent background, or baseline levels. Once a baseline was achieved, XN was injected into the medium to reestablish the electron transport chain function. XN was able to increase oxygen consumption, providing evidence to show that it acts as a protonophore, allowing protons to translocate from the inter-membrane space of the mitochondrion to the matrix (2).

Despite the promising results of these investigations, XN presents one possible side-effect. Due to the presence of an α,β -unsaturated ketone in its chemical structure, XN can spontaneously form a stable isomer known as isoxanthohumol (IX) (8). IX can be further metabolized, and through several steps can be transformed into a compound known as 8-prenylnaringenin (8-PN). 8-PN is one of the most potent phytoestrogens currently known, and can interact with both α and β estrogen receptors (8). Because of this ability, XN supplementation could be coupled with upregulation of estrogenic pathways. Current research indicates that phytoestrogens have both beneficial and potentially disruptive effects, which would be amplified if large quantities of XN were being administered continuously and over a long period of time. To avoid this, two XN derivatives that do not have the same unsaturated ketone in

their structures, dihydroxanthohumol (DXN) and tetrahydroxanthohumol (TXN), are under investigation (figure 3). Cell culture analysis of DXN and TXN revealed that they also have effects on cellular oxygen consumption, indicating that they are also likely mitochondrial uncouplers (de Montgolfier, 2014). Uncoupling effects of DXN and TXN appear to be even more dramatic than that of XN at the same concentration (5 μ M), with DXN and TXN inducing 20% and 50% increases in oxygen consumption, respectively, compared to approximately 10% seen in XN (de Montgolfier, 2014). TXN's uncoupling effect is so robust in fact that the question of toxicity and dose response will need to be examined further.

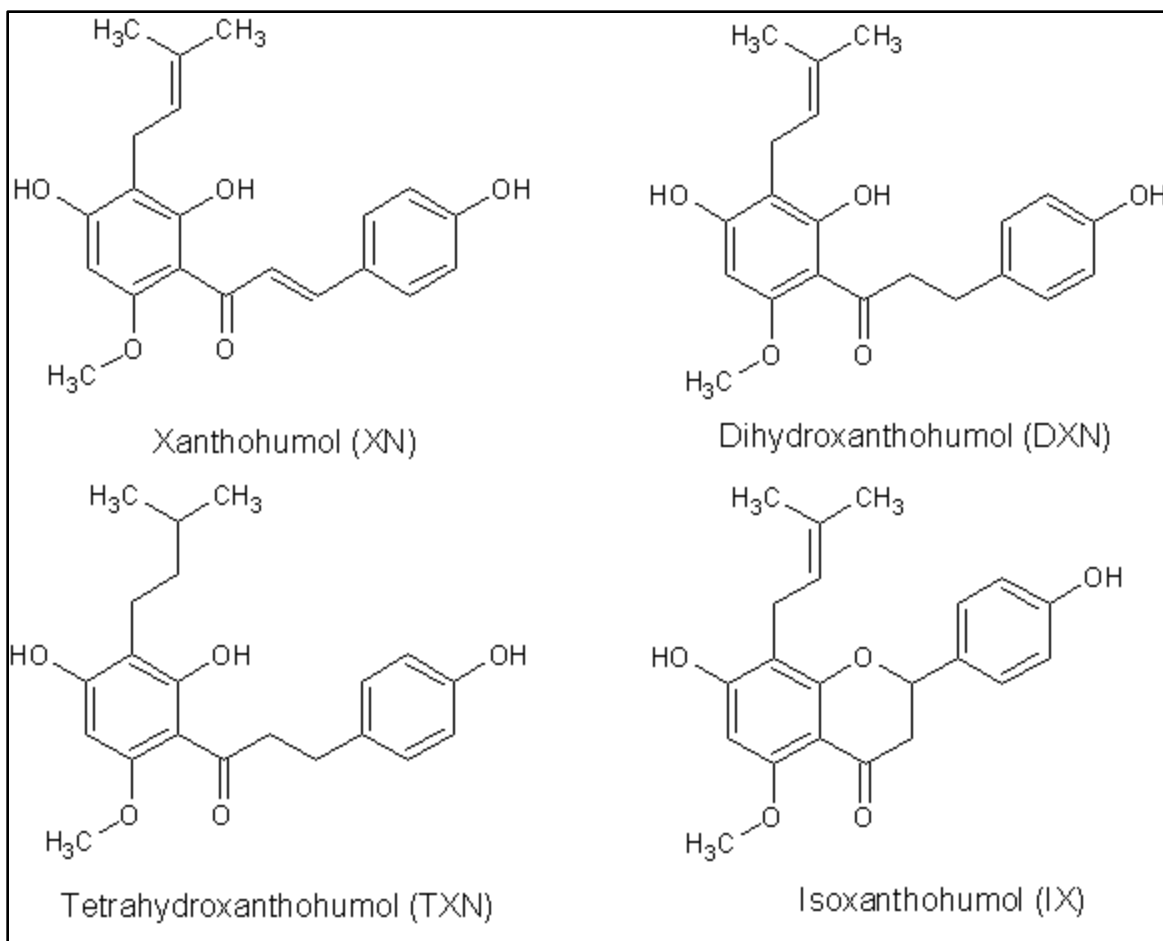


Figure 3. XN, DXN, TXN chemical structures. The α,β -unsaturated ketone found within XN's structure has been identified as the component enabling spontaneous isomerization to IX. DXN and TXN do not share this structural feature, reducing the possibility of phytoestrogen formation while retaining its mild uncoupling ability.

Mitochondrial uncoupling compounds have been used in the past to help induce weight loss, the most prominent of these compounds was 2,4-dinitrophenol (DNP). DNP is a very potent uncoupling agent, and induced rapid weight loss for many people (1). Responses to DNP vary widely, however, and some individuals experienced acute toxicity, primarily severe hyperthermia and tachycardia, and has resulted in at least 62 deaths (1). The unpredictability

and small margin of safety for DNP prompted the Food and Drug Administration (FDA) to ban its medicinal use. As this case exemplifies, any mitochondrial uncoupling agent must have a more predictable dose response and therapeutic index if it is to be administered to humans. The milder uncoupling effects of XN, DXN and TXN help to limit their potency, increasing their safety and enhancing their potential as supplements.

Now that compelling cellular metabolic data demonstrates the efficacy of DXN and TXN, the next step toward understanding their effects are experiments *in vivo*. This study involves supplementing a high fat mouse diet (60% kcal) with low doses of XN, DXN or TXN and comparing weight gain among mice in each group to one another and to a group of control mice receiving vehicle diet only. We expected that mice in the TXN group would experience the least weight gain, and those in the XN and DXN groups would have only a moderate decrease in weight gain, all compared to mice receiving the same diet without XN.

Because XN, DXN, and TXN have such similar structures, it was unclear why there are such dramatic differences in metabolic effect between XN and its two derivatives. Differences in each compound's activity or cellular uptake could provide possible explanations. For this reason, cellular uptake of each compound was compared using C2C12 mouse myocytes, and the first part of this experiment addresses this question.

The second part of this experiment examined some of the metabolic effects of XN, DXN and TXN in mice on a high fat diet. In accordance with results from previous feeding experiments, it was expected that mice receiving a supplemented diet would experience reduced weight gain. Further, mice receiving DXN or TXN would experience the least weight gain among the four

groups. We expected that less weight gain would also correlate with improved fasting blood sugar levels and insulin sensitivity.

Materials and Methods

Cell culture

Cellular uptake investigations were performed using C2C12 mouse myocytes. Cells were cultured in 75 cm² flasks using Dulbecco's Modified Eagle Medium (DMEM) obtained from Sigma-Aldrich (St. Louis, MO), which was supplemented with 10% Fetal Bovine serum (FBS) and 1% penicillin/streptomycin solution (PS). Cultures were maintained in an incubator at 37° C and 5% CO₂.

Cellular Uptake of XN, DXN and TXN

Uptake comparisons were conducted by seeding three 12-well plates with 1 mL of C2C12 cells in suspension with a density of 100,000 cells/mL, which were then incubated until confluent. Four treatment groups were compared, including XN, DXN, TXN and a no compound control. Each compound was tested in quadruplicate in the presence of cells and in medium not exposed to cells. Medium not exposed to cells was examined for compound concentration to help account for protein binding in medium. Confluent cells were subsequently treated with fresh DMEM containing 1% FBS, 1% PS, as well as 5 µM XN, DXN, or TXN, and incubated at 37° C and 5% CO₂ for one hour. Following incubation, cell medium was collected and the cells were washed once using Hanks Balanced Salt Solution (HBSS). HBSS was then aspirated, 0.5 mL of cell extraction buffer (50% methanol:ethanol, v:v) was added to each well and the plates were

covered with a plastic adhesive plate cover. The plates were placed in a freezer and maintained at -80° C for 24 hours. After freezing, the plates were removed from the freezer and the cells were removed from the wells using a cell scraper and collected. An additional 0.5 mL of extraction buffer was added to each well as a rinse, which was collected and added to the cell extracts. Samples were centrifuged for 5 minutes at 13,000 g, and the supernatant was placed in liquid chromatography tandem mass spectrometry (LC-MS/MS) vials for analysis. Cell medium samples were prepared by 1:4 dilution with acetonitrile (ACN) and subsequent centrifugation for 5 minutes at 13,000 g. Supernatants were transferred to LC-MS/MS vials. A volume of 20 µL of 2 µM 2,4'-dihydroxychalcone (DHC) in ethanol (EtOH) was added to all LC-MS/MS samples as an internal standard.

Mass Spectrometry

All cell culture samples were analyzed by LC-MS/MS using an Applied Biosystems API 4000 Q-trap triple quadrupole mass spectrometer (Carlsbad, CA). Analytes were separated by high performance liquid chromatography (HPLC) using a Phenomenex pentafluorophenyl (PFP) Luna 5 µm column (Torrance, CA). Once concentrations could be determined, mass balances of XN, DXN or TXN were calculated based on the average total volume of the sample, approximately 0.995 mL for cell medium, and 0.75 mL for cellular extracts.

Mouse Feeding and Maintenance

A total number of 48 C57BL6/J mice, with 12 mice belonging to each of 4 treatment groups. Duration of the feeding was 14 weeks. Mouse feed contained high fat content (60% of kCal), and was supplemented with XN, DXN, or TXN in the treatment groups. XN, DXN and TXN

content within the mouse diet were designed to deliver a dose of 30 mg/kg/day body weight. Food intake was measured three times per week by weighing the food pellet before placement in the cage, and subsequently weighing uneaten food at the time of feed changing. Mice were unrestricted in dietary intake, being allowed to eat as much food as desired. Mice were weighed once per week. Groups included low dose (30 mg/kg) XN, DXN, TXN and no supplement control. Mice were maintained in accordance with the Oregon State University Institutional Animal Care and Use Committee (IACUC) regulations. One mouse was maintained on a standard AIN-93M chow diet to be used as a healthy reference during glucose tolerance assays.

Mouse Glucose Tolerance Testing

Glucose tolerance tests were conducted during week 4 and week 11 of the feeding trial. Five mice from each treatment group were used in each glucose tolerance assay. Mice were fasted for 6 hours prior to baseline blood glucose testing. Blood was collected from the mice by tail puncture, and glucose measurements were taken using a One Touch UltraMini glucometer (LifeScan, Inc., Milpitas, CA). Mice were weighed and then given an intraperitoneal (IP) injection of glucose equal to 2 g/kg body weight. Glucose readings were taken at 0 minutes (prior to glucose injection), 15 minutes, 30 minutes, 1 hour, and 2 hours.

Plasma Metabolites

Plasma was collected at sacrifice (14 weeks) to analyze for plasma glucose, triglycerides, cholesterol and insulin. Plasma glucose was assayed using the Wako Autokit Glucose kit (Wako Chemicals USA, Inc., Richmond, VA). A 96-well plate (Corning Costar® assay plate, 9017) was

used to combine 5 μL of plasma with 195 μL buffer solution (60 mM Phosphate buffer (pH 7.1), 5.3 mM Phenol) containing color reagent (0.13 U/mL Mutarotase, 9.0 U/mL Glucose oxidase, 0.65 U/mL Peroxidase, 0.50 mM 4-aminoantipyrine, 2.7 U/mL Ascorbate oxidase). Samples were allowed to incubate for 5 minutes, and then the absorbance of each well was measured at a wavelength of 505 nm using a SpectraMax 190 spectrophotometer (Molecular devices, Sunnyvale, CA). Glucose was calculated using a 6-point calibration curve containing concentrations of 0, 25, 50, 100, 200 and 400 mg/dL.

Plasma triglycerides were measured using the Infinity™ Triglycerides Reagent kit (Fisher Diagnostics, Middletown, VA). 5 μL from each sample of plasma was combined with 195 μL of Infinity Triglycerides Reagent (53 mM phosphate buffer (pH 7) containing 2.5 mM ATP, 2.5 mM Mg acetate, 0.8 mM 4-aminoantipyrine, 1.0 mM 3, 5-dichloro-2-hydroxybenzene sulfonate (DHBS), 3000 U/L glycerolphosphate oxidase (GPO), 100 U/L glycerol kinase, 2000 U/L lipoprotein kinase, 300 U/L peroxidase (horseradish)) in a separate well of a 96-well plate (Corning Costar® assay plate, 9017), and allowed to incubate for 5 minutes at room temperature. Absorbance at 500 nm was then measured on a SpectraMax 190 spectrophotometer. Triglyceride levels were calculated from a 6-point calibration curve.

Plasma cholesterol was measured using the Infinity™ Cholesterol Liquid Stable Reagent assay kit (Fisher Diagnostics). Five μL of plasma from each sample was combined with 195 μL of Infinity™ Cholesterol Liquid Stable Reagent (50 mM phosphate buffer (pH 6.7) containing 200 U/L cholesterol oxidase, 500 U/L cholesterol esterase, 300 U/L peroxidase (horseradish), 0.25 mM 4-aminoantipyrine, 10 mM hydroxybenzoic acid) and incubated for 5 minutes at room

temperature. Cholesterol absorbance was then measured using a SpectraMax 190 spectrophotometer at 500 nm, and concentrations were calculated using a 6-point calibration curve.

Plasma insulin levels were assayed using the ALPCO 80-INSMS-E01 mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (ALPCO diagnostics, Salem, NH). Ten μL of plasma from each sample was loaded into a 96-well plate (provided in kit) and combined with 75 μL of provided conjugate buffer. The plate was placed on a shaker and incubated for 2 hours at room temperature while shaking at 800 rpm. The buffer was then discarded and the wells were washed 5 times with 100 μL of provided wash buffer. One hundred μL of 3,3',5,5'-tetramethylbenzidine (TMB) was then added to each well and incubated at room temperature on a plate shaker (800 rpm) for 15 minutes. After incubation, 100 μL of stop solution was added to each well, and the plate was analyzed on a SpectraMax 190 spectrophotometer at 450 nm. Sample insulin concentrations were calculated using a 6-point calibration curve.

Statistical analysis

Cellular uptake data was analyzed and evaluated using Microsoft Excel, and significance was determined using one-way ANOVA plus post-hoc. Data from the mouse feeding experiment was statistically evaluated using GraphPad statistical software. Differences between mouse treatment groups were determined via one-way ANOVA.

Results

Cellular uptake

Mass spectrometry analysis of cell contents showed uptake of XN to be approximately 6.6%, with DXN and TXN uptake at 7.8% and 6.9%, respectively (Figure 4). Differences in uptake were not statistically significant.

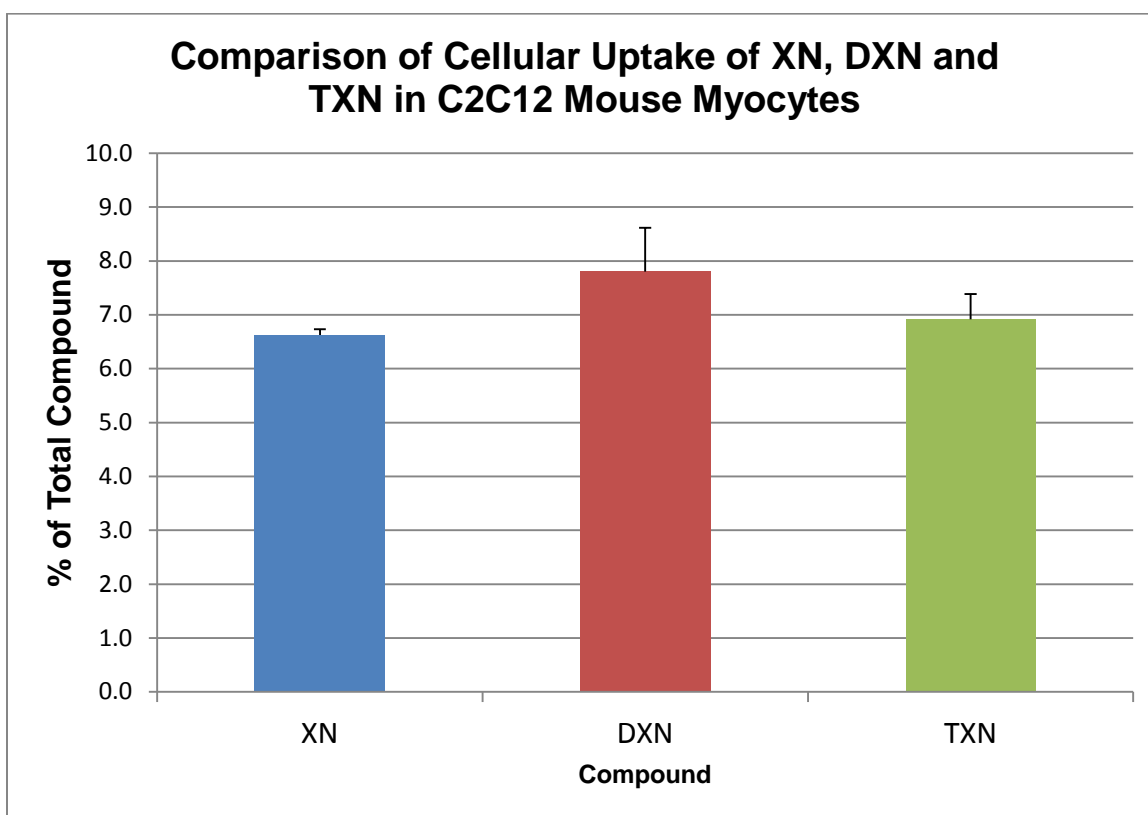


Figure 4. Cellular uptake of XN, DXN, and TXN in C2C12 cells. Percentages are of total amount of compound applied to cells. Differences were small, and not statistically significant (n=12). Error bars indicate standard error of the mean (SEM).

Mouse Feeding Experiment

Weight gain

Over the course of 12 weeks, mouse weights were recorded once per week and the average weights were calculated for each group. The TXN treated mice appeared to have the most dramatic reduction in weight gain compared to the control, followed DXN, then XN (Figure 5).

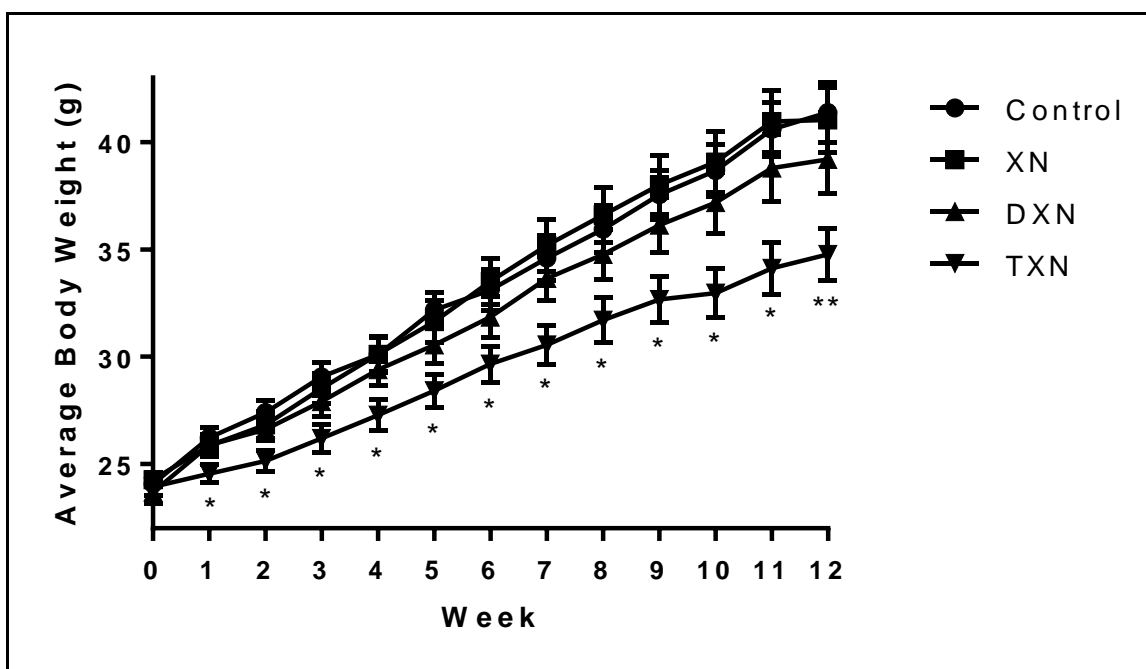


Figure 5. Average mouse weights over 12 weeks. While approximate dose was the same, the TXN mice gained the least weight on average, followed by DXN and XN, compared to the control. Values from each week were analyzed using one-way ANOVA, comparing treated mice to controls. Statistically significant values are marked with a * ($p < 0.05$), extremely significant values are marked with ** ($p < 0.01$)

Insulin Sensitivity

During the 4 week glucose tolerance test, average fasting blood glucose levels for all groups except TXN were partially elevated (normal range = 148 – 210 mg/dL). Peak glucose levels were achieved by 15 minutes in the XN and TXN groups, with the average for XN at 508.8 mg/dL and 407.2 mg/dL for the TXN group. The control and DXN groups reached peak glucose levels at 30 minutes, with average blood glucose for control mice >600 mg/dL and 423.0 mg/dL for the DXN group (Table 1). Glucose levels in all groups returned to fasting levels by 2 hours. High levels of variation occurred among DXN treated mice, though all treated mice demonstrated improved glucose recovery as indicated by less dramatic increases in plasma glucose. The fastest average recovery time was seen in TXN treated mice, though all groups had returned to their fasting glucose levels within 2 hours (Figure 6).

Table 1. Mean plasma glucose levels at time points 0, 15, 30, 60 and 120 minutes.

Glucose Tolerance Assay Mean Plasma Glucose Levels - Week 4 (mg/dL)					
Group	0 Min	15 min	30 min	60 min	120 min
Control	281.8 (± 20.5)	485.2 (± 45.8)	596.6 (± 3.4)	499.8 (± 48.9)	222.4 (± 15.6)
XN	283.4 (± 4.6)	508.8 (± 28.3)	456.0 (± 41.5)*	389.6 (± 29.8)*	167.0 (± 3.8)*
DXN	297.6 (± 6.6)	396.4 (± 55.9)	423.0 (± 76.3)*	342.8 (± 70.4)	176.2 (± 40.0)
TXN	193.6 (± 17.9)	407.2 (± 38.1)	394.6 (± 50.1)*	235.8 (± 21.9)*	149.0 (± 15.3)*

Values showing statistically significant deviation from the control are marked with * ($p < 0.05$).

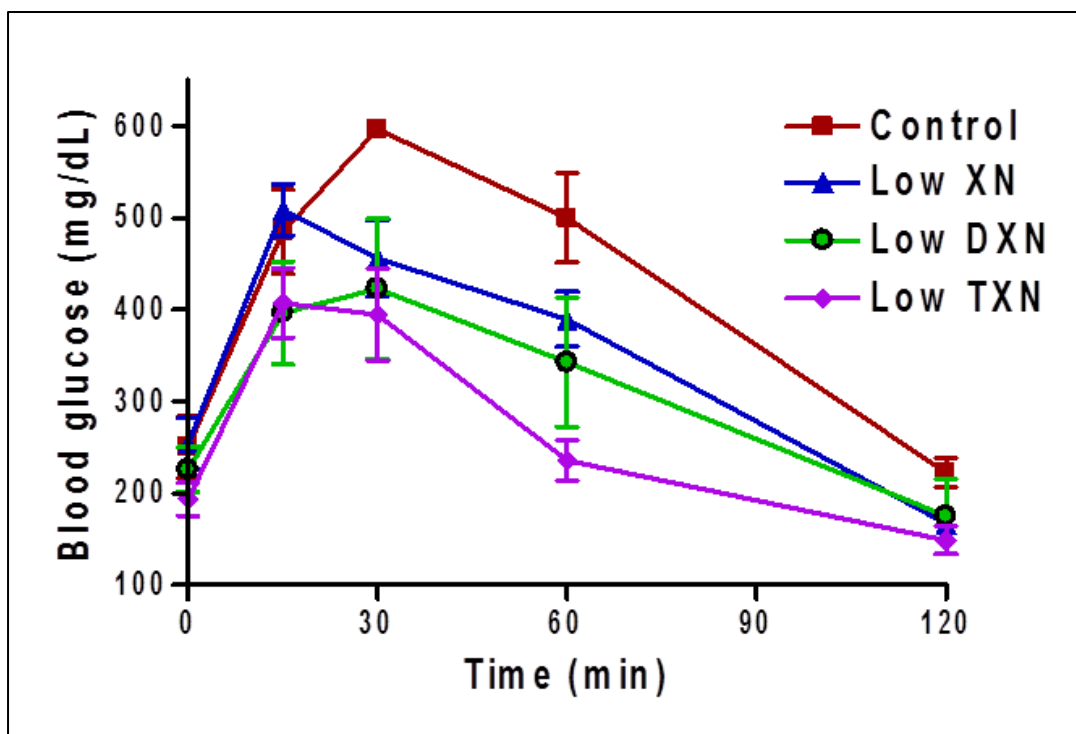


Figure 6. Week 4 plasma glucose levels in treated mice over a period of 2 hours following a 2 g/kg glucose injection. Peak glucose levels were achieved at 15 minutes for XN and TXN, and 30 minutes for DXN and the control mice. The highest average increase in plasma glucose was seen in the control group at >600 mg/dL, while the XN, DXN and TXN groups reached peaks of 508.8, 407.2, and 423.0 mg/dL, respectively.

The week 11 glucose tolerance test showed a much more extended glucose recovery length, as well as higher glucose increases following injection. At two hours post glucose injection, average plasma glucose for all groups except TXN remained above fasting levels. Peak glucose levels occurred in XN at 15 minutes at a concentration of 465.0 mg/dL, while DXN and TXN peaked at 30 minutes, at levels of 508.0 and 513.6 mg/dL, respectively (Figure 7). By 2 hours post-injection, average plasma glucose was highest among control mice at 422.2 mg/dL, while XN, DXN and TXN mice had averages of 373.4, 298.8, 266.8 mg/dL, respectively (Table 2).

Table 2. Mean plasma glucose levels measured during the week 11 glucose tolerance assay.

Glucose Tolerance Assay Mean Plasma Glucose Levels - Week 11 (mg/dL)					
Group	0 Min	15 min	30 min	60 min	120 min
Control	325.6 (\pm 14.8)	550.6 (\pm 30.4)	573.6 (\pm 22.6)	576.8 (\pm 14.4)	422.2 (\pm 36.1)
XN	193.2 (\pm 21.9)	465.0 (\pm 30.0)*	452.0 (\pm 41.6)*	463.8 (\pm 52.2)	373.4 (\pm 58.1)
DXN	249.8 (\pm 22.1)	474.4 (\pm 46.0)	508.0 (\pm 41.2)	494.8 (\pm 38.9)	298.8 (\pm 45.5)*
TXN	245.0 (\pm 17.8)	408.0 (\pm 29.8)*	513.6 (\pm 37.6)	450.8 (\pm 5.0)*	266.8 (\pm 32.1)*

Values showing statistically significant deviation from control are marked with * ($p < 0.05$).

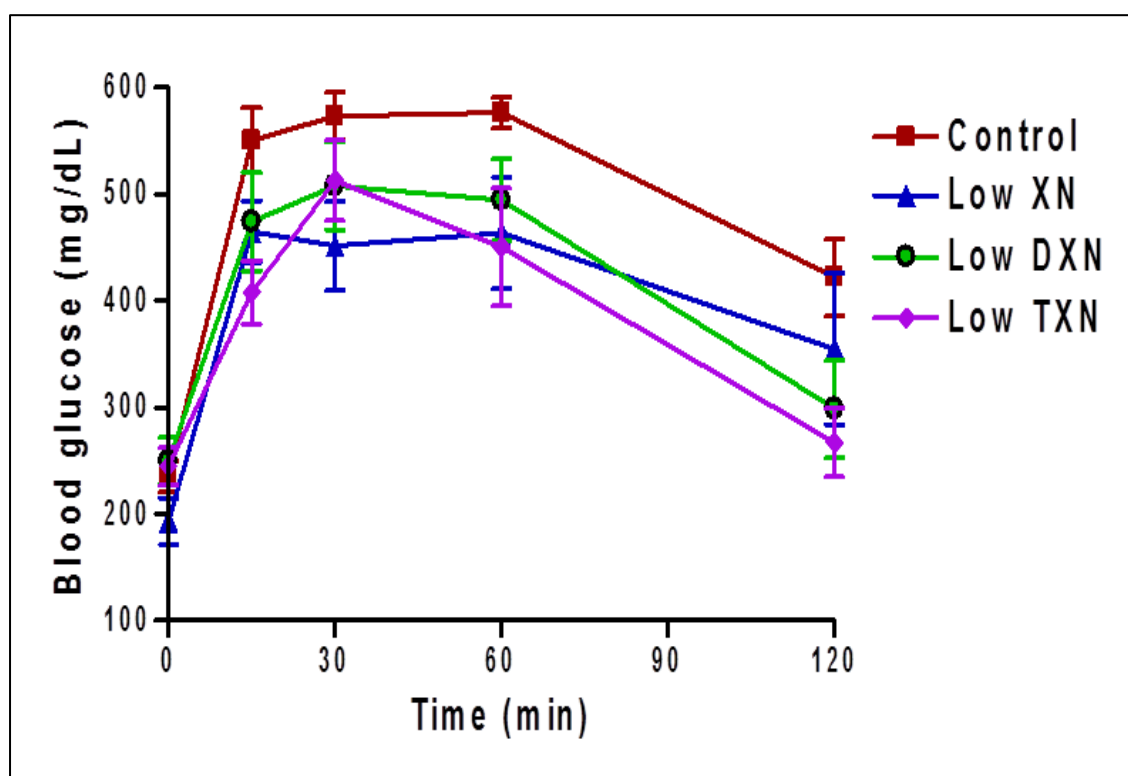


Figure 7. Week 11 glucose tolerance test blood glucose levels over a 2 hour period following a 2g/kg glucose IP injection. All mice showed an extended plasma glucose recovery time, as well as larger glucose increases after injection. In contrast to testing at 4 weeks, few mice had recovered to fasting plasma glucose levels within 2 hours.

Plasma Metabolic Markers

Mean fasting plasma glucose at time of sacrifice was 158.2 ± 29.6 mg/dL for control mice, and 169.1 ± 28.7 , 150.5 ± 45.0 and 116.1 ± 24.1 mg/dL for XN, DXN and TXN, respectively (Figure 8).

Average plasma triglycerides detected were 87.5 ± 6.6 , 80.7 ± 4.2 , 84.5 ± 4.9 and 76.4 ± 7.8 mg/dL for the control, XN, DXN and TXN groups, respectively. Plasma cholesterol averages were 173.9 ± 31.9 mg/dL for the control, and 196.2 ± 28.3 , 167.2 ± 33.3 and 160.7 ± 30.7 mg/dL for XN, DXN and TXN respectively. Insulin levels averaged 1.01 ± 0.44 ng/mL for control mice, and 1.19 ± 0.68 , 1.04 ± 0.77 and 0.500 ± 0.24 ng/mL for XN, DXN and TXN, respectively.

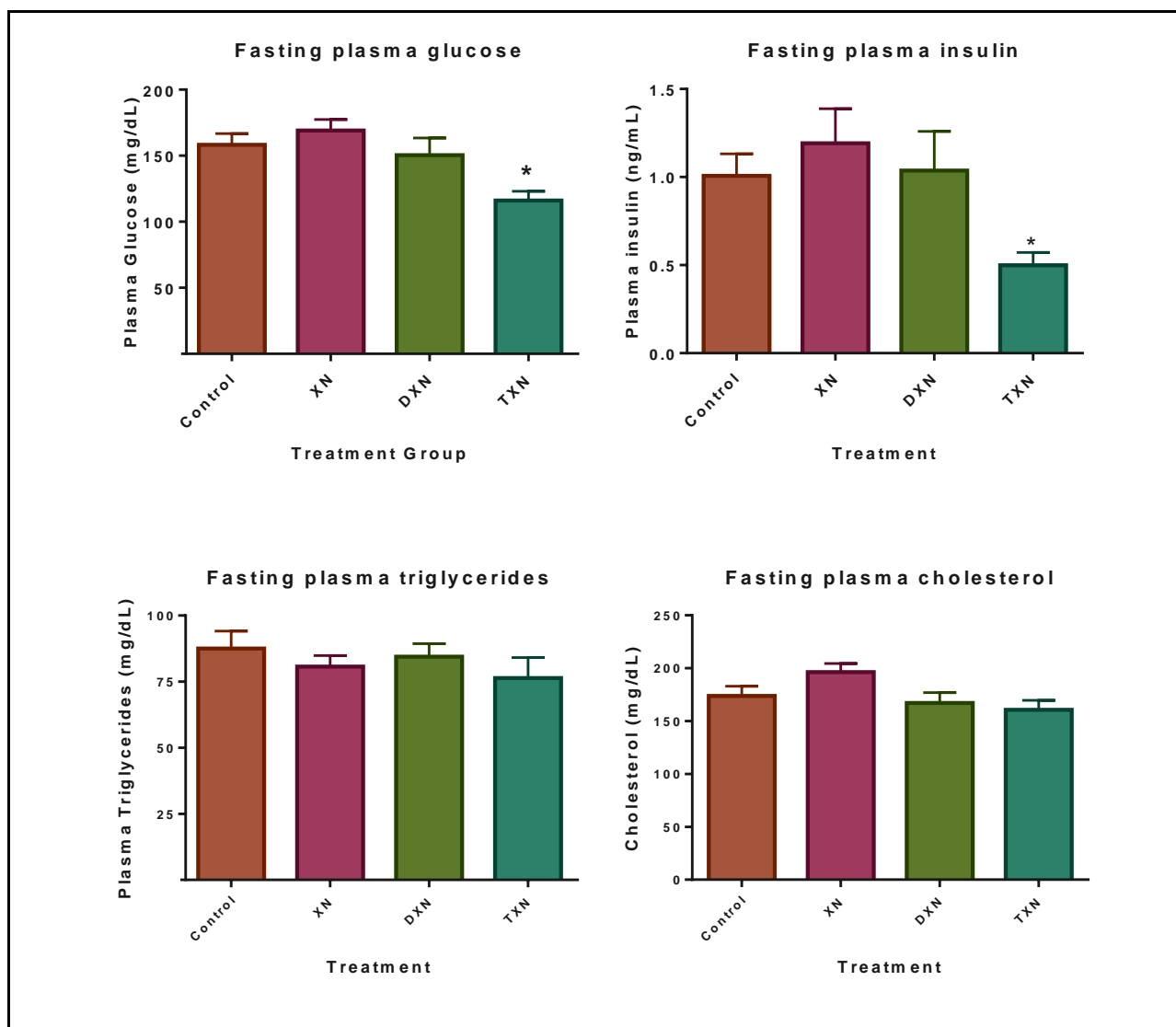


Figure 8. Fasting plasma glucose, insulin, triglycerides and cholesterol, measured from plasma collected at time of sacrifice following a 6 hour fast. TXN mice showed significantly lower ($p < 0.05$) glucose and insulin levels compared to control. No differences between treatment groups were observed in cholesterol or triglycerides.

Discussion

Xanthohumol (XN) and its derivatives dihydroxanthohumol (DXN) and tetrahydroxanthohumol (TXN) all show efficacy in improved insulin sensitivity as measured by the intraperitoneal

glucose tolerance test, and TXN shows efficacy at reducing fasting plasma glucose and insulin levels. Corresponding reductions in weight gain also contributed to reducing the impact of metabolic syndrome. However, the dose used during this experiment was not sufficient to wholly prevent the development of diabetic-like symptoms or a pre-diabetic condition. While at 4 weeks mice receiving XN, DXN or TXN showed glucose tolerance roughly comparable to mice on a healthy diet, by 11 weeks all mice demonstrated prolonged glucose recovery times and experienced much larger increases in plasma glucose following glucose administration.

Metabolic markers such as glucose, insulin and triglycerides did not differ significantly from the controls, except in the case of TXN. A high degree of variability hampered statistical analysis of several of these parameters, and some of this variation could have resulted from mice coming from different litters. Whether or not a higher dose would be more beneficial with respect to disease prevention is unclear. Further investigations will need to include multiple dose-level comparisons.

Though not as yet comprehensive, the data provides evidence of a protective effect against metabolic syndrome, primarily by reduction in weight gain and enhanced insulin sensitivity. TXN appeared to have the most protective effect of the three treatments, though more information is needed regarding dose response. While diet and exercise remain the most important factors to reducing cardiovascular disease and diabetes risk, XN and its metabolites could prove to be effective preventative agents or assistive treatments to help those with metabolic disorder make healthy lifestyle changes.

References

1. Grundlingh, J., Dargan, P. I., El-Zanfaly, M., and Wood, D. M. (2011) 2,4-Dinitrophenol (DNP): A Weight Loss Agent with Significant Acute Toxicity and Risk of Death. *J. Med. Toxicol.* **7**, 205–212
2. Kirkwood, J. S., Legette, L. L., Miranda, C. L., Jiang, Y., and Stevens, J. F. (2013) A Metabolomics-driven Elucidation of the Anti-obesity Mechanisms of Xanthohumol. *J. Biol. Chem.* **288**, 19000–19013
3. Després, J.-P., and Lemieux, I. (2006) Abdominal obesity and metabolic syndrome. *Nature.* **444**, 881–887
4. Ma, K., Saha, P. K., Chan, L., and Moore, D. D. (2006) Farnesoid X receptor is essential for normal glucose homeostasis. *J Clin Invest.* **116**, 1102–1109
5. Stevens, J. F., Taylor, A. W., Clawson, J. E., and Deinzer, M. L. (1999) Fate of Xanthohumol and Related Prenylflavonoids from Hops to Beer. *J. Agric. Food Chem.* **47**, 2421–2428
6. Ježek, P., Engstová, H., Žáčková, M., Vercesi, A. E., Costa, A. D. T., Arruda, P., and Garlid, K. D. (1998) Fatty acid cycling mechanism and mitochondrial uncoupling proteins. *Biochimica et Biophysica Acta (BBA) - Bioenergetics.* **1365**, 319–327
7. Lloyd-Jones, D., Adams, R. J., Brown, T. M., Carnethon, M., Dai, S., Simone, G. D., Ferguson, T. B., Ford, E., Furie, K., Gillespie, C., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, P. M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M. M., Meigs, J., Mozaffarian, D., Mussolino, M., Nichol, G., Roger, V. L., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Thom, T., Wasserthiel-Smoller, S., Wong, N. D., Wylie-Rosett, J., and Subcommittee, on behalf of the A. H. A. S. C. and S. S. (2010) Heart Disease and Stroke Statistics—2010 Update A Report From the American Heart Association. *Circulation.* **121**, e46–e215
8. Legette, L., Karnpracha, C., Reed, R. L., Choi, J., Bobe, G., Christensen, J. M., Rodriguez-Proteau, R., Purnell, J. Q., and Stevens, J. F. (2014) Human pharmacokinetics of xanthohumol, an antihyperglycemic flavonoid from hops. *Mol. Nutr. Food Res.* **58**, 248–255
9. Simon, E. W. (1953) Mechanisms of Dinitrophenol Toxicity. *Biological Reviews.* **28**, 453–478
10. Ricquier, D., and Bouillaud, F. (2000) Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *J Physiol.* **529**, 3–10
11. Echtay, K. S. (2007) Mitochondrial uncoupling proteins—What is their physiological role? *Free Radical Biology and Medicine.* **43**, 1351–1371
12. Ogden CL, Carroll MD, Kit BK, and Flegal KM (2014) Prevalence of childhood and adult obesity in the united states, 2011-2012. *JAMA.* **311**, 806–814
13. Brand, M. D., and Esteves, T. C. (2005) Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metabolism.* **2**, 85–93
14. Wunderlich, S., Wurzbacher, M., Back, W. (2013) Roasting of malt and xanthohumol enrichment in beer - Springer 10.1007/s00217-013-1970-5
15. Eckel, R. H., Alberti, K., Grundy, S. M., and Zimmet, P. Z. (2010) The metabolic syndrome. *The Lancet.* **375**, 181–183
16. Stevens, J. F., and Page, J. E. (2004) Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry.* **65**, 1317–1330

17. Venè, R., Benelli, R., Minghelli, S., Astigiano, S., Tosetti, F., and Ferrari, N. (2012) Xanthohumol Impairs Human Prostate Cancer Cell Growth and Invasion and Diminishes the Incidence and Progression of Advanced Tumors in TRAMP Mice. *Mol Med.* **18**, 1292–1302
18. Zamzow, D. R., Elias, V., Legette, L. L., Choi, J., Stevens, J. F., and Magnusson, K. R. (2014) Xanthohumol improved cognitive flexibility in young mice. *Behavioural Brain Research.* **275**, 1–10
19. Legette, L. L., Moreno Luna, A. Y., Reed, R. L., Miranda, C. L., Bobe, G., Proteau, R. R., and Stevens, J. F. (2013) Xanthohumol lowers body weight and fasting plasma glucose in obese male Zucker fa/fa rats. *Phytochemistry.* **91**, 236–241
20. Nozawa, H. (2005) Xanthohumol, the chalcone from beer hops (*Humulus lupulus* L.), is the ligand for farnesoid X receptor and ameliorates lipid and glucose metabolism in KK-Ay mice. *Biochemical and Biophysical Research Communications.* **336**, 754–761