Xanthohumol lowers body weight and fasting plasma glucose in obese male Zucker fa/fa rats

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Abstract

Obesity contributes to increased risk for several chronic diseases including cardiovascular disease and type 2 diabetes. Xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus*), was tested for efficacy on biomarkers of metabolic syndrome in 4 week old Zucker fa/fa rats, a rodent model of obesity. Rats received daily oral doses of xanthohumol at 0, 1.86, 5.64, and 16.9 mg/kg BW for 6 weeks. All rats were maintained on a high fat (60% kcal) AIN-93G diet for 3 weeks to induce severe obesity followed by a normal AIN-93G (15% kcal fat) diet for the last 3 weeks of the study. Weekly food intake and body weight were recorded. Plasma cholesterol, glucose, insulin, triglyceride, and monocyte chemoattractant protein -1 (MCP-1) levels were assessed using commercial assay kits. Plasma and liver tissue levels of XN and its metabolites were determined by liquid-chromatography tandem mass spectrometry. Plasma and liver tissue levels of xanthohumol were similar between low and medium dose groups and significantly ($p<0.05$) elevated in the highest dose group. There was a dose-dependent effect on body weight and plasma glucose levels. The highest dose group (n=6) had significantly lower plasma glucose levels compared to the control group (n=6) in male but not female rats. There was also a significant decrease in body weight for male rats in the highest dose group (16.9 mg / kg BW) compared to rats that received no xanthohumol, which was also not seen for female rats. Plasma cholesterol, insulin, triglycerides, and MCP-1 as well as food intake were not affected by treatment. The findings suggest that xanthohumol has beneficial effects on markers of metabolic syndrome.

Keywords: xanthohumol, *Humulus lupulus*, hops, metabolic syndrome, obesity, rats, type 2 diabetes

1. Introduction

Metabolic syndrome is a condition defined by clinical diagnosis of three or more of the following conditions: abdominal obesity, atherogenic dyslipidemia, insulin resistance and/or impaired glucose tolerance, hypertension, pro-inflammatory state, and prothrombotic state (Grundy et al. 2004) . The 2003-2006 National Health and Nutrition Examination Survey (NHANES) revealed that over 34% of US adults have metabolic syndrome and are at increased risk for cardiovascular disease and type 2 diabetes (Ervin 2009). Direct health care costs arising from obesity and/or related disorders are estimated to be 7-10% of all US health care expenditures annually (Trogdon et al. 2011).

Dietary bioactives may act as preventive and/or mitigating agents in the progression of several chronic diseases (Ferrari 2004; Kris-Etherton et al. 2002; Milner 2004). In particular, xanthohumol (XN), the principal prenylated flavonoid from hops (Stevens et al. 2000), has been shown to have broad biological activity and may have several health benefits (Stevens and Page 2004). Studies have demonstrated that XN may exert beneficial health effects as an antioxidant (Miranda et al. 2000), anti-inflammatory (Lupinacci et al. 2009; Peluso et al. 2010), anti-microbial (Gerhauser 2005), cancer chemopreventive agent (Colgate et al. 2007; Miranda et al. 1999), and an immune system modulator (Xuan et al. 2010). With growing interest in health promoting abilities of XN, several researchers have examined aspects of XN metabolism. *In vitro* and *in vivo* animal studies have revealed key steps of XN metabolism which are depicted in Fig 1 and have been described previously (Legette et al. 2012).

Emerging evidence on the role of XN in cholesterol regulation has led to speculation that XN may reduce risk factors associated with metabolic syndrome including hypercholesterolemia and dyslipidemia. Nozawa identified XN as an agonist of the farnesoid X receptor (FXR), which

is involved in cholesterol and bile acid metabolism (Nozawa 2005). Nozawa also observed that feeding XN for four weeks led to lower plasma glucose and triglyceride levels in KK-A^y mice, a model for obesity and type 2 diabetes (Nozawa 2005). Additional *in vitro* work has shown that XN may have anti-obesity effects through its actions on adipocytes. XN inhibited differentiation, reduced proliferation and increased apoptosis in 3T3-L1 (murine preadipocytes) cells (Mendes et al. 2008; Rayalam et al. 2009; Yang et al. 2007).

Dietary flavonoids may have potential as therapeutic agents for metabolic syndrome by influencing factors associated with obesity and type 2 diabetes (Xia and Weng 2010). Most of the promising results of XN effects on lipid metabolism were observed *in vitro* (Mendes et al. 2008; Rayalam et al. 2009; Yang et al. 2007) with only one *in vivo* study assessing the effects of feeding XN via the diet (Nozawa 2005). The aim of the present study was to determine the effect of oral administration of XN at various doses on biomarkers of metabolic syndrome utilizing an obese rodent model. To our knowledge, this is the first investigation to determine relationships between circulating plasma levels, tissue levels, and effects of XN on biomarkers of metabolic syndrome following chronic administration.

2. Results and Discussion

2.1 Study Design

Disadvantages of administering a potential therapeutic agent in the diet include variation in dose, potential negative effect of the test agent on food intake, and effect of the diet on bioavailability of the test agent, all of which may result in difficulties in accurately assessing its biological activities *in vivo*. Based on available safety data on oral doses of xanthohumol in rodents (Vanhoecke et al. 2005), we selected a dose range equivalent to daily oral doses of 20, 60, and 180 mg in humans. Findings from our recent pharmacokinetic study of XN (Legette et al.

2012) predict that the selected doses lead to steady-state plasma levels of XN and metabolites in the high nanomolar to low micromolar concentration range. Using allometric scaling and an interspecies scaling factor (Food and Drug Administration 2005) for an individual weighing ca. 64 kg (141 lb), this results in equivalent doses of 1.86, 5.64 and 16.9 mg/kg BW for rats (here referred to as low, medium and high doses, respectively). In this study, test meals were utilized to ensure accurate administration of defined XN doses. A test meal is a defined dose incorporated in a compatible matrix for oral consumption. In addition to using test meals, we examined the effect of XN on metabolic syndrome with the use of a genetic model of obesity, the Zucker obese fa/fa (ZOF) rat. ZOF rats have an autosomal recessive mutation that prohibits the production of leptin, a regulatory hormone involved in appetite control and energy metabolism. This mutation in ZOF rats leads to hypercholesterolemia, hyperinsulinemia, and obesity (Zucker and Zucker 1962).

2.2 Body weight and Food intake

A dose-dependent effect of XN treatment on body weight was observed in male rats (Fig. 2A). During the last three weeks of the study, a significant trend ($0.05<p<0.10$) emerged, with male rats on high XN having a lower body weight compared to rats receiving no XN. The difference in body weight between treatments only became significant ($p<0.05$) at sacrifice indicating that longer treatment duration may be needed to fully evaluate health effects of XN. Although lower body weight was observed in female rats with XN treatment (Fig. 2B), it was not significant suggesting that higher treatment dose of XN may be needed to induce significant change in body weight of female rats. Differences in body weight cannot be attributed to changes in food intake, which was not affected by treatment (data not shown). Similar to our findings, others have

shown that soy isoflavonoid consumption decreases body weight (Blake et al. 2011; Guo et al. 2009).

2.3 Xanthohumol analysis

Plasma levels of XN and its metabolites for low, medium, and high dose groups are shown in Table 1. There were no detectable plasma levels of XN or its metabolites (isoxanthohumol, IX; 8-prenylnaringenin, 8PN; 6-prenylnaringenin, 6PN) in control animals. As observed previously (Legette et al. 2012), the only major metabolites in plasma after XN treatment were IX and 8PN and they were largely present in their conjugated forms. Steady-state plasma XN levels observed in this study correspond to predicted values from earlier research. (Legette et al. 2012). As seen in Table 2, projected steady-state plasma levels calculated from the single-dose pharmacokinetics (PK) study are in good agreement with the observed experimental steady-state concentrations measured in this chronic treatment study.

Liver tissue concentrations of XN, IX and 8PN are displayed in Table 3 and follow the same trends observed with plasma levels including the presence of only IX and 8PN as XN metabolites. Plasma and liver concentrations of XN and its metabolites were higher in female rats compared to males across all treatment groups. This is attributed to the fact that XN formulations were based on average body weight of treatment groups including both male and female rats. Since females tended to weigh less than males, they received slightly higher doses at each XN level**.** As expected, both male and female rats consuming high XN had significantly higher plasma and liver concentrations of XN than all other dose groups. There were no detectable liver tissue levels of 8PN for animals receiving low XN dose. There was also no detectable liver concentration of IX in male rats on the low dose whereas female rats had small

amounts of IX in liver. Although plasma and liver tissue levels were higher for rats receiving medium XN compared to those on low XN, the differences were not significant, probably due to considerable variation across animals within treatment groups. This suggests that perhaps a larger population size is needed in subsequent studies to thoroughly evaluate the effects of low and medium doses of XN. Additional investigations are also needed to determine which XN metabolite is contributing to health effects of XN supplementation. Perhaps the biological effects observed at high XN dose are due to increasing levels of 8PN, which has been shown to have greater estrogenic activity (Bovee et al. 2004; Coldham and Sauer 2001; Milligan et al. 2002; Milligan et al. 1999; Milligan et al. 2000) than most flavonoids including its parent compound, XN.

2.4 Plasma biochemical markers

Plasma glucose levels were significantly ($p<0.05$) decreased in the high dose group compared to control but not in the other dose groups. Nozawa and others also observed a decrease in plasma glucose levels after four weeks of feeding XN (1000 mg/kg BW) to obese mice (Nozawa 2005). Our findings showed that XN exerted an effect on glucose metabolism at a much lower dose (16.9 mg/kg BW). The difference in dose effect could be due to form and/or mode of XN administration in the studies. Our study used a XN formulation composed of a self-emulsifying mixture which allows for high absorption as detailed previously (Legette et al. 2012) whereas Nozawa provided XN in a basal diet. The effect of the highest XN dose on plasma glucose, as with body weight, was seen in male (Fig. 3A) but not in female (Fig. 3B) rats.

The gender differences we found in response to flavonoid treatment have also been observed for other flavonoids (Blair et al. 2002; Camper-Kirby et al. 2001; Guo et al. 2005).

Blair and others demonstrated that dietary consumption of soy and soy isoflavones for 16 weeks affects LDL plasma concentrations in male but not female hamsters (Blair et al. 2002). Additional research is needed to determine what factors are influencing differential responses. Possible causes include treatment dose and/or treatment duration. Previous study findings also illustrate an effect of chronic flavonoid consumption (16-20 weeks) on plasma cholesterol in obese animals (Ali et al. 2004; Blair et al. 2002). Guo *et al.* (2009) examined the effects of administration of daidzein, a soy isoflavone, via oral gavage for 4 weeks at doses of 25, 50 and 100 mg/kg BW in male obese mice and found no effect on plasma triglyceride levels but did observed a decrease in plasma cholesterol and free fatty acid concentrations. However, in our study plasma cholesterol, insulin and triglyceride levels were not affected by treatment (data not shown), which may be attributed to a variety of factors including base diet, sample size, and treatment duration. Our high fat AIN-93G base diet contained 60% kcal fat diet whereas Guo's diet (Guo et al. 2009) only had 45% kcal fat to induce obesity in male mice. Additionally, a small sample size (n=6/group), large variation, short exposure period (6 weeks), and relatively low doses of XN used in the treatment could have impaired our ability to detect an effect of XN on plasma biochemical markers.

Plasma MCP-1 levels, a biomarker of inflammation, were not altered by XN treatment (data not shown) which was unexpected since XN has been shown to inhibit MCP-1 formation in LPS-activated monocytes (Peluso et al. 2010) and RAW 264.7 mouse macrophages (Lupinacci et al. 2009) in culture.

2.5 Dose/plasma-effect relationships

The significantly higher steady-state XN plasma and liver tissue levels in the high-dose animals compared to the medium and low dose groups parallel the effects of treatment on body weight and plasma glucose in the high-dose males compared to the control group. This finding suggests that the low and medium doses fail to generate high enough plasma and especially tissue levels required to exert effects on the endpoints used in this study.

3. Concluding Remarks

Our exploratory study on chronic XN exposure showed that the highest XN dose (16.9 mg /kg BW) exerted beneficial effects on body weight and glucose metabolism in obese male rats. This suggests that XN holds promise as a therapeutic agent for treating obesity and dysregulation of glucose metabolism, conditions associated with metabolic syndrome. Our findings indicate that chronic administration of XN in a self-emulsifying matrix produces effects at doses low enough to be feasible as a supplement (16.9 mg/kg BW). The potential of XN as an effective preventive for metabolic syndrome warrants further investigation. Additional research is also needed to explore the mechanisms of action of XN and its metabolites, including factors related to pharmacologic and pharmacokinetic effects.

4. Experimental

4.1 General Information

Animals: Four-week old male and female Zucker fa/fa rats were purchased from Harlan (Livermore, CA, USA). Animals were housed in individual cages in temperature and humidity controlled rooms with a 12:12 on-off light cycle. All procedures were approved by and in

accordance with ethical standards of Oregon State University's Institutional Animal Use and Care Committee (Protocol # 3689).

Diet: All rats were maintained on a high fat (60% kcal) AIN-93G diet for 3 weeks to induce severe obesity followed by a normal AIN-93G (15% kcal fat) diet for the last 3 weeks of the study. Weekly food intake and body weight were recorded.

Treatment Groups: After a 2 day acclimation period, 48 animals (24 males and 24 females) were divided into four treatment groups (n=6/gender group): control (0 mg XN / kg BW), low (1.86 mg XN / kg BW), medium (5.64 mg XN / kg BW), and high (16.9 mg XN /kg BW). Animals were selected to ensure similar body weight average across treatment groups and received daily doses of XN for six weeks before undergoing sacrifice. Animals were euthanized with an overdose of CO_2. The sacrifice period occurred over 4 days with a total of 12 animals being euthanized daily. Three animals were randomly selected from each treatment group every day of the sacrifice period.

Statistics: Data were analyzed using Statistical Analysis Software (SAS) (Version 9.1, SAS Institute, Cary, NC). All values are expressed as mean±SD. Treatment effects on body weight were assessed using repeated measure analysis over time (PROC MIXED procedure). Effects on plasma endpoints were determined by one-way ANOVA (PROC GLM procedure) and post-hoc multiple comparison testing using LSD test. Significance was set at $p<0.05$.

4.2 Source Material

Animals received daily XN doses via a test meal. XN powder (> 99% pure by HPLC-UV and NMR; Anheuser Busch, St. Louis, MO, USA) was dissolved in a self-emulsifying isotropic

mixture (oleic acid, propylene glycol, and Tween 80; 0.9:1:1 by weight) to create various dosing solutions (0, 1.86, 5.64, and 16.9 mg / kg BW). XN solutions were added to a modified AIN-93G powdered diet (corn oil replacing soybean oil) and homogeneously mixed with mortar and pestle to result in a 3 g test meal for each dose. Test meals were administered after withholding food for 3 h to ensure complete XN consumption within 30 min. Test meals were made weekly for each treatment group based on average body weight of male and female rats. All test meals used for XN treatment were analyzed and found on average to be within 10% of their nominal doses.

4.3 Xanthohumol Analysis

Plasma levels of XN, IX, 8PN, and 6PN were determined using procedures and conditions reported previously (Legette et al. 2012). In brief, samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after sample preparation with and without enzymatic hydrolysis to determine amount of free and conjugated XN, IX, 8PN, and 6PN present in plasma. Tissue extracts were prepared in duplicate and on ice by homogenizing 0.500 g of liver tissue in 5 ml of extraction solvent (90% methanol: 10% H_2O) using an Omni Tissue Homogenizer (Omni TH, Omni International, Marietta, GA, USA) for 30 sec. Tissue homogenates underwent sonication for 1 min (Model F60 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA) prior to being centrifuged at 4000g for 15 min (Allegra X15R centrifuge, Beckman Coulter, Brea, CA, USA). The resulting supernatant was removed and stored as tissue extract. After obtaining tissue extracts, sample preparation and analysis were conducted as described for plasma samples. Test meals were analyzed by HPLC. Triplicate samples (400 mg) of test meals were extracted twice with 10 ml methanol using vortexing (20 sec) and sonication

(5 min). The extracts were centrifuged for 5 min at setting = 6 using a clinical centrifuge (International Model CL, International Equipment Co., Needham, MA, USA) and the resulting supernatants were centrifuged for 5 min at 13000g. The two centrifuged extracts were analyzed separately using an HPLC system (Waters Corp., Milford, MA) consisting of a model 600 pump/controller, a model 717 Plus autosampler, and a 2996 photodiode array detector. Analytes were separated on a 4 × 250 mm Luna 5 μm C18 reversed phase column (Phenomenex, Torrance, CA), eluted with a linear gradient of 40 to 100 % solvent B (acetonitrile) in solvent A (H_2O containing 0.1 % trifluoroacetic acid) in 15 min at a flow rate of 1 ml/min. XN was detected at 368 nm and quantified using the external standard method.

4.4 Biochemical Analysis

Animals underwent overnight fasting before sacrifice. Blood was collected via a cardio-puncture and centrifuged for 10 min at setting=7 using an International clinical centrifuge. Plasma was stored at –80ºC until analysis. Commercial assay kits were used to determine plasma cholesterol (Infinity™ Cholesterol Liquid Stable Reagent, Thermo Scientific, Middletown, VA, USA), glucose (Autokit Glucose, Wako Chemicals, Richmond, VA, USA), insulin (Alpco Insulin RAT ELISA, Alpco Diagnostics, Salem, NH, USA) , triglyceride (Infinity™ Triglycerides Liquid Stable Reagent, Thermo Scientific, Middletown, VA, USA), and MCP-1 (Alpco MCP-1 rat ELISA, Alpco Diagnostics, Salem, NH, USA) levels.

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Tables

Table 1. Mean plasma concentrations[1] of xanthohumol (XN) and its metabolites (isoxanthohumol, IX, and 8-prenylnarigenin, 8PN) of male (n=4-6/group) and female (n=5-6/group) obese Zucker fa/fa rats who received a daily supplementation of xanthohumol (XN) at four dose levels (0, 1.86, 5.64, and 16.9 mg / kg BW) for 6 weeks.

Treatment		**Control (0 mg / kg BW)**	**Low XN (1.86 mg / kg BW)**	**Medium XN (5.69 mg / kg BW)**	**High XN (16.9 mg / kg BW**
XN	Male rats	ND	50±14[a]	107±46[a]	389±153[b]
(nM)	Female rats	ND	75±28[a]	240±83[a]	555±228[b]
IX	Male rats	ND	10±3[a]	32±16[a]	100±24[b]
(nM)	Female rats	ND	21±8[a]	56±11[a]	181±50[b]
8PN	Male rats	ND	35±5[a]	82±17[a]	364±82[b]
(nM)	Female rats	ND	40±7[a]	111±31[a+]	211±52[b]
Total	Male rats	ND	95±21[a]	221±68[a]	853±178[b]
(nM)	Female rats	ND	136±37[a]	407±125[b]	947±322[b]

All values are expressed as means± SE. ND indicates levels were not detectable. Different letters denote statistical differences ($p<0.05$) and $^{+}$ denotes a significant trend ($0.05 < p < 0.10$) from low XN group.[1] Plasma concentrations of XN and metabolites include both free and conjugated forms as determined by LC-MS/MS following sample preparation with enzymatic hydrolysis.

Table 2. Mean plasma total concentrations (nM) of xanthohumol (XN) and its metabolites (isoxanthohumol, IX, and 8-prenylnarigenin, 8PN) of male rats from a single dose pharmacokinetic and a chronic supplementation study with the following doses of XN: (0, 1.86, 5.64, and 16.9 mg / kg BW)

Studies	**Low XN (1.86 mg / kg BW)**	**Medium XN (5.69 mg / kg BW)**	**High XN (16.9 mg / kg BW**
Single Dose Pharmacokinetic (PK) Study[1] Maximum Concentration, C_{max}	191 ± 20	434 ± 18	1510 ± 70
Projected Steady State Levels from PK Study[1]	94	129	213
Chronic Supplementation Study	50 ± 14	107 ± 46	389 ± 153

All values are expressed as mean±SE. [1]Results from a single XN dose pharmacokinetics study discussed in detail elsewhere (Legette et al. 2012).

Table 3. Mean liver tissue concentrations[1] of xanthohumol (XN) and its metabolites (isoxanthohumol, IX, and 8-prenylnarigenin, 8PN) of male (n=4-6/group) and female (n=5-6/group) obese Zucker fa/fa rats who received a daily supplementation of xanthohumol (XN) at four dose levels (0, 1.86, 5.64, and 16.9 mg / kg BW) for 6 weeks.

Treatment		**Control (0 mg / kg BW)**	**Low XN (1.86 mg / kg BW)**	**Medium XN (5.69 mg / kg BW)**	**High XN (16.9 mg / kg BW**
XN (nmol/g tissue)	Male rats	ND	0.15±0.04[a]	0.35±0.07[a]	1.1±0.35[b]
	Female rats	ND	0.35±0.06[a]	0.97±0.20[a]	1.9±0.50[b]
IX (nmol/g tissue)	Male rats	ND	ND	0.06±0.01	0.21±0.03
	Female rats	ND	0.04±0.00[a]	0.06±0.02[a]	0.39±0.11[b]
8PN (nmol/g tissue)	Male rats	ND	ND	0.05±0.01	0.22±0.04
	Female rats	ND	ND	0.06±0.01	0.11±0.03
Total (nmol/g tissue	Male rats	ND	0.15±0.04[a]	0.46±0.08[a]	1.5±0.38[b]
	Female rats	ND	0.39±0.05[a]	1.1±0.20[a]	2.4±0.53[b]

All values are expressed as mean±SE. ND indicates levels were not detectable. Different letters denote statistical differences ($p<0.05$) from low XN group.[1] Tissue concentrations of XN and

metabolites include both free and conjugated forms as determined by LC-MS/MS following sample preparation with enzymatic hydrolysis.

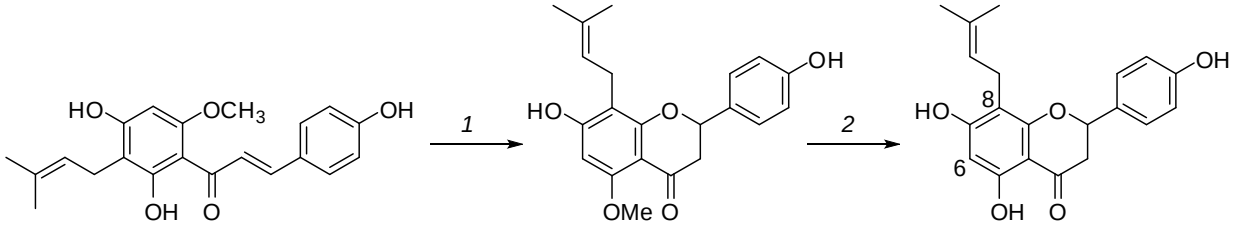
Figures and legends

Figure 1. Metabolic conversion of xanthohumol (XN) into isoxanthohumol (IX) and 8-prenylnaringenin (8PN). Step 1, non-enzymatic cyclization; Step 2, cytochrome P450-mediated demethylation. The plasma metabolite profile showed no support for the alternative metabolic pathway from XN to 6-prenylnaringenin by sequential demethylation and cyclization.

Figure 2. Mean body weight levels of A) male and B) female obese Zucker fa/fa rats (n=6 /group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg / kg BW) of xanthohumol (XN) for six weeks. $^{+}$ and * denotes a statistical trend ($0.05 < p < 0.10$) and difference ($p < 0.05$) from the control group, respectively. All values are expressed as mean±SE.

Figure 3. Mean plasma glucose levels of A) male and B) female obese Zucker fa/fa rats (n=4-6/group) receiving various daily doses (0, 1.86, 5.64, 16.9 mg / kg BW) of xanthohumol (XN) for six weeks. * denotes statistical difference from control group. All values are expressed as mean±SE.

Figure 1



xanthohumol (XN) isoxanthohumol (IX) 8-prenylnaringenin (8PN)

Figure 2A

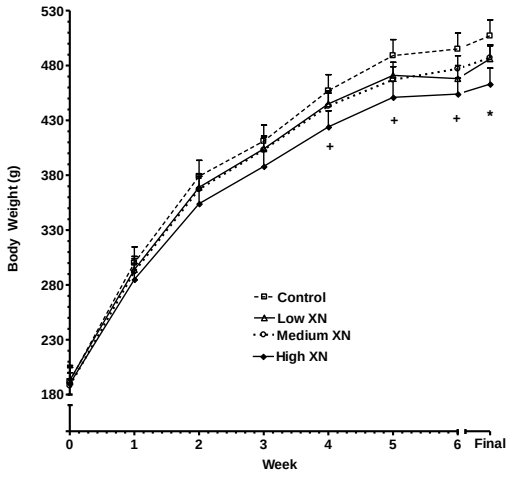


Figure 3A

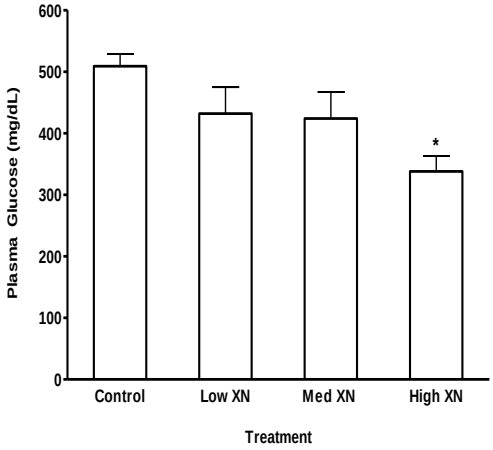


Fig ure 2B

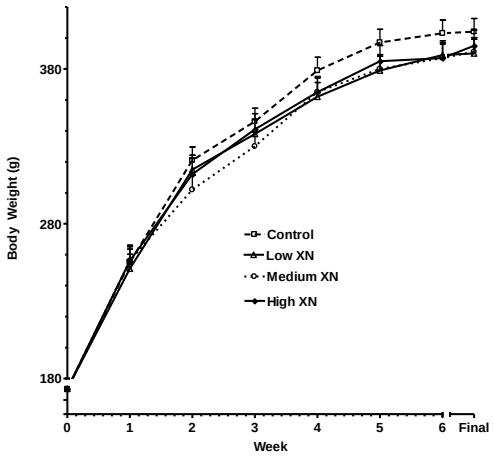


Figure3B