

**Investigating the potential anti-diabetic effect of sulforaphane**

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**Keywords: Sulforaphane, Obesity, T2D, Insulin Sensitivity**

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## **Investigating the potential anti-diabetic effect of sulforaphane**

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### **ABSTRACT**

Type 2 diabetes (T2D) is a major public health issue worldwide and it currently affects nearly 26 million people in the United States. It is estimated that one third of Americans will have diabetes by 2050. T2D is a result of chronic insulin resistance and loss of  $\beta$ -cell mass and function. Both in experimental animals and people, obesity is a leading pathogenic factor for developing insulin resistance, which is always associated with the impairment in energy metabolism, causing increased intracellular fat content in skeletal muscle, liver, fat, as well as pancreatic islets. Constant insulin resistance will progress to T2D when  $\beta$ -cells are unable to secrete adequate amount of insulin to compensate for decreased insulin sensitivity. In the present study, I investigated whether sulforaphane, a natural compound derived from cruciferous vegetables, can prevent high-fat (HF) diet-induced obesity and diabetes in C57BL/6 mice. Dietary intake of sulforaphane (250 mg/kg diet) prevented hyperglycemia and increased insulin sensitivity in HF diet-induced obese mice. Mice treated with sulforaphane had significant lower serum insulin levels ( $1.93 \pm 0.11 \mu\text{g/dl}$ ) as compared to those without treatment ( $3.09 \pm 0.27 \mu\text{g/dl}$ ,  $P < 0.05$ ). In second study, administration of sulforaphane (40 mg/kg body weight daily via gavage) in obese mice enhanced body weight loss and improved insulin sensitivity. Moreover, sulforaphane increased pyruvate oxidation by 28.85% ( $P < 0.05$ ) and enhanced fatty acid oxidation efficiency by 2.2 fold ( $P < 0.05$ ) in primary human muscle

cells. These results suggest that sulforaphane may be a naturally occurring insulin-sensitizing agent that is capable of preventing T2D.

Keywords: Sulforaphane, Obesity, T2D, Insulin Sensitivity

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## **Introduction**

Diabetes mellitus is one of the top causes of death and disabilities worldwide. More than 371 million children and adults worldwide have diabetes in 2012 [1] and are at high risk of disability and life-threatening complications, such as stroke, cardiovascular disease and heart attack. It was estimated that there were 4.8 million deaths from diabetes in 2012 and more than \$471 billion had been spent on the healthcare for diabetes [1].

Diabetes is defined as a fasting plasma glucose value higher than 7.0 mmol/L, or 126 mg/dl.

Diabetes is a chronic condition, which occurs when the body is not able to properly use insulin or do not produce adequate amount of insulin. There are three main categories of diabetes, type 1 diabetes (T1D), type 2 diabetes (T2D), and gestational diabetes mellitus (GDM). T1D is an autoimmune disease that is caused by T-cell-mediated destruction of pancreatic  $\beta$ -cells. As a result, there is not enough insulin in the body and glucose cannot be effectively utilized, thereby circulating in the blood that causes damages to vascular system and subsequently other tissues and organs. T2D, previously known as non-insulin-dependent diabetes, or adult-onset diabetes, is a result of chronic insulin resistance and loss of  $\beta$ -cell mass and function. GDM is named for women who are first diagnosed with diabetes when they are pregnant [2].

It is well established that obesity is a major contributing factor to the development of insulin resistance and thereby T2D [3]. Data from epidemiologic studies showed that weight control in overweight or obese people can improve insulin sensitivity and reduce the risk of T2D [4, 5]. Thus, investigating a therapeutic strategy to improve insulin resistance can be an effective method to treat obesity and prevent diabetes.

According to the National Center for Complementary and Alternative Medicine, Americans are increasingly using complementary and alternative medicine (CAM) during the past two decades. CAM is a variety of medical and health care practices and products, which are not currently considered medicine by public. The 2007 National Health Interview Survey conducted an inclusive survey on Americans who used CAM. The report revealed that approximately 4 out of 10 adults used CAM in 2007, with the most widely used CAM being natural products [6].

Some observational studies demonstrated that high consumption of cruciferous vegetables is associated with lower risks of numerous diseases [7-9]. On this basis, scientists have speculated that sulforaphane may be the potential active component responsible for the observed numerous health benefits, as it exhibits beneficial properties to prevent and/or treat these diseases both *in vivo* and *in vitro* studies. However, it is unknown whether dietary intake of sulforaphane provides a beneficial effect on T2D. In this thesis, I investigated the anti-obese and anti-diabetic potential of this compound *in vivo* and *in vitro*.

## Review of Literature

### Sulforaphane

Sulforaphane (Fig. 1) is a compound belonging to the isothiocyanate group. Cruciferous vegetables, such as broccoli, Brussels sprouts, cabbages, kale and radish, contain a variety of bioactive components such as selenium [10], flavonoid quercetin [11], and vitamin C [12]. One group of critical bioactive compounds that has been most studied in cruciferous vegetables is glucosinolates [13]. Glucosinolates are not active until they are hydrolyzed by myrosinase into isothiocyanates [14]. Myrosinase will be released from cell wall of cruciferous vegetables when they are physically ruptured. Sulforaphane is the hydrolysis product of glucoraphanin, which is the most abundant glucosinolates compound in cruciferous vegetables [15]. Cruciferous vegetables are commonly served after cooking in human diet. Therefore the enzyme myrosinase is inactivated by heat. Studies have shown that measuring the amount of mercapturic acid derivative in urine can be the appropriate method to determine the uptake of sulforaphane [16]. When served as raw vegetables, the bioavailability of sulforaphane mercapturic acid excretion found in urine is significantly higher than cooked vegetables [17]. The same study also found that the amount of plasma sulforaphane conjugate parameter is 10 times higher when consumed freshly.

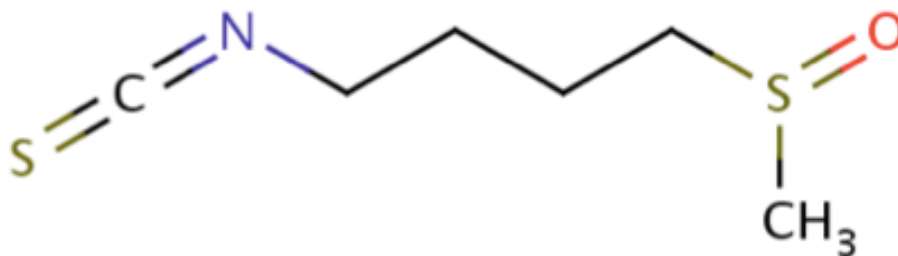


Fig. 1. Structure of sulforaphane

## **Sulforaphane and cancer**

There are numerous clinical studies demonstrating that large amounts of vegetables and fruits consumption can reduce the risk of chronic diseases [18, 19]. In epidemiological studies, it has been shown that dietary intake of cruciferous vegetables is associated with reduced risk of developing lung, kidney, prostate, bladder, colon and rectum cancer [20-25]. A meta-analysis of 87 case-control studies showed an inverse correlation between consumption of cruciferous vegetables and the risk of cancer. Specifically, consumption of cabbage, broccoli, cauliflower, and Brussels sprouts reduced cancer risk by 70, 56, 67, and 29%, respectively, for both genders [26]. These results suggest that the protective effect of cruciferous vegetables against cancer may be due to their relatively high content of glucosinolates. Indeed, data from a further study demonstrated that sulforaphane plays a major role in mediating the anti-cancer activity of consuming cruciferous vegetables [16].

The impact of dietary sulforaphane intake on cancer has been further examined in several studies. Results from clinical trials demonstrate that intake of more than two servings (1/2 cup counts as 1 serving [27]) of cruciferous vegetables per month can reduce the overall risk of cancer, especially in the early stages [28, 29]. There are epidemiological studies showing that dietary intake of sulforaphane-rich vegetables is associated with decreased risk of several types of cancers including colorectal cancer [29, 30], lung cancer [31], prostate cancer [28], breast cancer [28], and bladder cancer [32]. The relationship between the reduced risk factors of cancer and intake of sulforaphane-rich diet mainly depends on the dosage of sulforaphane. It was found that more than three servings of cruciferous vegetables per week reduced the risk of prostate cancer. The population-based, case-controlled study recruited 1,230 participants and found that consuming three or more servings of sulforaphane-rich diet per week can decrease the risk of prostate cancer by 59% compared with having one or less serving [23].



Sulforaphane in cruciferous vegetable has been proved to be the major constituent attributable to the anti-cancer effect. Sulforaphane diminished the occurrence and weight of tumor and retarded the mammary tumors progress in female Sprague-Dawley rats [33]. It was also found to moderate the benzo[a]pyrene-evoked fore-stomach tumor in mice [34]. Consistently, oral administration of sulforaphane at doses that can be obtained by dietary consumption of broccoli and other cruciferous vegetables reduces the formation of PC-3 human prostate cancer xenografts by more than 50% in nude mice [35].

### **Sulforaphane and cardiovascular disease**

Cardiovascular disease is the first leading cause of death in the US, accounting for approximately 24% of all deaths in 2011 [36]. It is well recognized that oxidative stress is involved in cardiovascular disorders, which can cause congestive heart failure, atherosclerosis, myocardial ischemia, and chemical induced cardiac toxicity [37]. Therefore, agents with antioxidant property could be beneficial for preventing cardiovascular diseases. One recent study found that cardiomyocytes from neonatal rats treated with sulforaphane displayed lower production of reactive oxygen species (ROS) and higher viability [38]. Intake of broccoli sprouts, which is rich in sulforaphane, was also found to reduce oxidative stress in spontaneously hypertensive rats [39]. In addition, several studies demonstrated that dietary intake of broccoli sprouts can reduce inflammation of heart, kidney, artery, and central nervous system [40, 41]. Interestingly, maternal supplementation of sulforaphane to pregnant, spontaneously hypertensive rats lowered blood pressure and tissue inflammation of their adult offspring [41]. Taken together, these data suggest that sulforaphane may be a promising agent to prevent cardiovascular disease.

### **Sulforaphane and kidney diseases**

Clinical and epidemiological studies have shown that ROS plays a critical role in renal injury [42]. With its antioxidant function, sulforaphane is expected to protect kidney from these ROS-mediated deleterious effects. Indeed, HK2 renal tubular epithelial cells treated with sulforaphane significantly decreased phase 2 enzymes and protected cells from cytotoxicity [43]. Retinitis Pigmentosa is a hereditary disease that kills photoreceptor cells within the eyes and causes blindness to approximately 100,000 Americans [44]. Results from several studies suggest that impaired expressions of thioredoxin (Trx), thioredoxin reductase (TrxR), and NF-E2-related factor-2 (Nrf2) are involved in the pathogenesis of retinal degeneration in this disease. Administration of sulforaphane significantly up-regulated the retinal levels of Trx, TrxR, and Nrf2, and protected photoreceptor cells in homozygous tubby (tub/tub) mutant mouse model [45].

### **Sulforaphane and diabetes**

Studies testing whether sulforaphane has an effect on diabetes are scarce. A recent study demonstrated that intraperitoneal injection (ip) of 40 µg/kg sulforaphane prevented streptozotocin (STZ)-induced pancreatic islet apoptosis and diabetes [46]. Consistent with this *in vivo* finding, sulforaphane also protects against cytokine-induced clonal and islet β-cell apoptosis and dysfunction, which are mediated via activation of transcriptional factor Nrf2, leading to subsequent suppression of the transcriptional factor NF-κB-mediated pathway [46]. Another animal study demonstrated that sulforaphane can protect damage of the aorta in STZ-induced T1D mice. This study also showed that aortic Nrf2 expression and transcription are up-regulated by sulforaphane [47].

Oxidative stress and inflammation are the major causes of diabetic peripheral neuropathy [48]. Several studies demonstrated that sulforaphane may have protective action against diabetic complications. Past studies showed that sulforaphane provided neuroprotective effects by activating Nrf2 and inhibiting NF-κB. Nrf2 is regulated by redox involved in the antioxidant defense system regulation and NF-κB is a transcription factor, which plays a central role in regulating various pro-inflammatory cytokine and chemokine production. It was found that sulforaphane can reverse the endothelial cells dysfunction by activating Nrf2 and inhibiting the hyperglycemia-induced activation of hexosamine and protein kinase C in human microvascular endothelial cells cultured in either normoglycemic or hyperglycemic cultures [49].

### **Significance of this research**

The benefits of sulforaphane and the potential underlying mechanisms are summarized in Table 1.

Table 1. Health benefits of sulforaphane and underlying mechanisms

Disease	Mechanism of sulforaphane treatment
Cancer	Modulation of drug metabolizing enzymes [16, 50], induction of cell apoptosis [51-54], cell cycle arrest [51, 55-57]
Respiratory disorders, oxidant-induced respiratory diseases	Induce the mucosal phase 2 enzymes expression, up-regulation of phase 2 enzymes glutathione-S-transferases and quinone reductase in the airway epithelial cells [58, 59]

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Neurodegenerative diseases, Alzheimer's disease, cerebral ischemia	Inhibition of acetyl cholinesterase [60], up-regulation of antioxidant gene expression [61]
Kidney diseases, renal fibrosis and ROS-mediated kidney damage	Nrf-2 mediated induction of phase 2 enzymes [62]
Diabetes, Diabetic micro- and macro-vascular disorders	Reduction of oxidative stress and restoration of endothelial function [46, 63]
Ocular diseases Retinitis Pigmentosa, Age-related macular degeneration	Up-regulation of retinal Trx, TrxR, and Nrf-2 Up-regulation of retinal GSH and QR [64, 65]
Cardiovascular disorders, hypertension and atherosclerosis	Nrf-2 mediated reduction in the pro-inflammatory state [66, 67]
Gastrointestinal disorders	Nrf-2 dependent eradication of <i>H. pylori</i> [68, 69]

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GSH: glutathione.

Data from *in vitro* and *in vivo* studies as well as clinical trials consistently show that sulforaphane may have preventive or therapeutic potential to reduce the risk factors of numerous

diseases, including cancer, cardiovascular disease, kidney damage, and ocular disease. Epidemiological studies also found that sulforaphane-rich diet is associated with low rates of those diseases in humans. However, whether this compound has an anti-diabetic action is unknown. In the present study, I tested whether sulforaphane has an anti-diabetic effect in the high-fat diet-induced obese and diabetic mouse models.

## Research Design and Methods

### Materials

Sulforaphane ( $\geq 97\%$  in purity) was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Stock solution of sulforaphane at 40 mM dissolved in Dimethyl sulfoxide (DMSO) for *in vitro* studies was stored at  $-80\text{ }^{\circ}\text{C}$  before use. Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone Laboratories, Inc. (Logan, UT). Heat-inactivated fetal bovine serum (FBS) was also obtained from HyClone Laboratories, Inc. (Logan, UT). Penicillin Streptomycin was purchased from Gibco (Grand Island, NY). All other chemicals were from Sigma-Aldrich (St Louis, MO). Glucose was dissolved in sterile water and stored at  $-80\text{ }^{\circ}\text{C}$ .

### Animals

Eight-month old male C57BL/6 mice (National Cancer Institute, Frederick, MD) were individually housed in an animal room maintained on a 12-h light/dark cycle under constant temperature ( $22\text{--}25\text{ }^{\circ}\text{C}$ ) with *ad libitum* access to food and water. After 1 week of environment acclimation, the following two animal studies were performed. The protocols of these studies were reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Tech.

### Animal study one

Mice were divided into 3 groups ( $n=8$ ) and fed either a low fat diet (LF) with 10% of calories derived from fat, a high fat diet (HF; Research Diets Inc., New Brunswick, NJ) with 60% of calories from fat, or HF supplemented with sulforaphane ( $0.25\text{ g/kg}$  diet) (HF+SU), for 7 weeks. Body weight (BW) and food intake were recorded weekly throughout the study. The fasting blood glucose levels in tail vein blood sample were measured using a glucometer (Roche) at 7th week of dietary sulforaphane supplementation; body composition was evaluated using an LF-90 instrument (Bruker Optics, Inc., Billerica MA). The LF-90 body composition instrument is based

on Time Domain Nuclear Magnetic Resonance (TD-NMR) technology, which provides an *in vivo* measurement of lean tissue, body fat, and body fluid in live mice without anesthesia. After 7 weeks of dietary treatment, glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed. For GTT, mice were fasted 12 hours and injected intraperitoneally (ip) with a single bolus of glucose (1 g/kg BW). Glucose levels were measured at time points of 0, 15, 30, 60 and 120 min, and plasma insulin concentrations were measured at 0 and 30 min, after glucose administration. For ITT, mice were injected i.p. with insulin (0.75 units/kg BW), and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after insulin administration. Area under the curve (AUC) was calculated using the trapezoidal rule. At the end of the study, blood samples were collected from overnight-fasted mice; plasma insulin concentration was measured using an ultrasensitive mouse/rat insulin ELISA kit obtained from Mercodia (Winston-Salem, NC); fasting plasma total cholesterol and triglycerides were measured in triplicate by enzymatic methods using a Pointer 180 Analyzer (Pointe Scientific, Canton, MI).

### **Animal study two**

For second animal study, male C57BL/6 mice (NCI, NIH, 7 months old) were fed HF diet as stated above for 4 weeks when mice developed obese and insulin resistance [70, 71]. Obese mice were then divided into 2 groups with 15 mice each group, with identical body weight, body composition, and non-fasting and fasting blood glucose. Both groups were fed with HF diet. Mice were administered via oral gavage 40 mg/kg sulforaphane dissolved in 2% cellulose or cellulose alone for 30 days. Mice body weights were measured every 3 days and food consumptions were measured weekly. The fasting and non-fasting blood glucose levels were measured using a glucometer (The Kroger Co., Cincinnati, Ohio, USA) bi-weekly throughout the study. At the end of this experiment, mice were fasted overnight and euthanized for collecting blood samples. Total cholesterol and triglyceride in plasma were measured using liquid reagents

(Teco Diagnostics, CA). For GTT, mice were fasted 15 hours. Then animals were injected with a single dose of glucose (1 g/kg BW). Blood glucose levels were measured before injection and at 15, 30, 60, and 120 minutes following glucose administration. For ITT, mice were fasted 4 hours and then injected with a single dose of insulin (1 unit/kg BW), and blood glucose levels were measured before injection and at 15, 30, 60, and 120 minutes after insulin administration. Area under the curve (AUC) was calculated using the trapezoidal rule. Body compositions of the mice (8 mice per group) were analyzed using a LF-90 instrument (Bruker Optics, Inc., Billerica, MA) as described above. Body temperature was measured using a thermometer probe placed in the rectum at 2.5 cm in depth. Energy expenditure was evaluated using the indirect calorimetry system by assessing oxygen (O<sub>2</sub>) consumption and carbon dioxide (CO<sub>2</sub>) output. Regarding this, mice were individually placed in a TSE LabMaster Calorimetry System cage (Columbus Instruments, Columbus, OH). Following acclimation for 24 hours, mice were hooked up to the TSE LabMaster System, in a closed environment, which allows for metabolic sampling. Mice had free access to food and water for the duration of this study. O<sub>2</sub>, CO<sub>2</sub> and locomotor activity were recorded for 48 hours. All data were normalized using lean mass of body weight.

### ***In vitro* study**

#### **Pyruvate and fatty acid oxidation in primary human muscle cells**

Primary human muscle cells were cultured for measuring free fatty acid and pyruvate oxidation. Cultures of primary human muscle cells were performed [72] and were obtained from subjects who provided written informed consent under approved protocols by Virginia Polytechnic Institute and State University Institutional Review Boards. All experiments were performed on day 7 of differentiation. Pyruvate and fatty acid oxidation was assessed by measuring and summing <sup>14</sup>C<sub>2</sub>O<sub>2</sub> production and <sup>14</sup>C-labeled acid-soluble metabolites from the oxidation of [U-<sup>14</sup>C] glucose and [1-<sup>14</sup>C] free fatty acid (American Radiolabeled Chemicals, St. Louis, MO) as



described [73]. Briefly, transfer 1 ml of incubation medium to a glass scintillation vial containing H<sub>2</sub>SO<sub>4</sub> and a microcentrifuge tube, which contained benzenthonium hydroxide. Generated <sup>14</sup>CO<sub>2</sub> was trapped in small microcentrifuge tube with benzenthonium hydroxide and then the tube was placed in scintillation vial and counted.

### **Glucose Uptake Cell-Based Assays in C2C12 myotubes**

To test if sulforaphane has direct effect on glucose uptake, Glucose Uptake Cell-based Assay Kit was obtained from Cayman (Ann Arbor, MI). C2C12 mouse skeletal muscle cells were cultured to the confluence of 80-90% in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin streptomycin (P/S). Cells were then differentiated into myotubes in DMEM with 2% horse serum (HS) and 1% P/S. All experiments were conducted between days 5-7 of differentiation at which time formation of myotubes became maximal. Briefly, the night before experiment the medium was removed and starvation medium (DMEM/1000 mg glucose/4 mM glutamine/110 mg sodium pyruvate/1% P/S) was added. The next day, sulforaphane treatments and vehicle control were added as instructed. The concentrations of sulforaphane treatment were 1 nM, 10 nM, 50 nM, 100 nM, 1 μM, 5 μM, and 10 μM prepared using DMEM and Dimethyl sulfoxide (DMSO). Cells were incubated for 30 min/1 h at 37°C. After the incubation period, centrifuge the plate for five min at 400 x g at room temperature, then aspirate supernatant. Wash cells with Cell-based Assay Buffer once then the cells were ready for analysis using plate reader.

### **Data Analysis**

All data were analyzed by one-way ANOVA using JMP program and are expressed as mean±SE. Comparisons of group means were assessed using Tukey HSD method, with p-value<0.05 considered significant.

## Results

### **Sulforaphane had no effect on body weight change in obese mice**

There was no difference in food intake and body weight gain between HF and HF+SU group after 7 weeks of dietary supplementation (Fig. 1). In addition, dietary supplementation of sulforaphane had no effect on fat mass and lean mass (Fig. 2), as determined by body composition measurements.

### **Sulforaphane ameliorated hyperglycemia in obese mice**

After 7 weeks of sulforaphane treatment, sulforaphane group displayed lower fasting blood glucose levels as compared with the HF diet-fed mice ( $P < 0.05$ ) (Fig. 3A). GTT was performed to detect whether sulforaphane can ameliorate hyperglycemia in obese mice. HF+SU group also had significant lower blood glucose levels ( $178 \pm 10.40$  mg/dl vs.  $226 \pm 9.36$  mg/dl in HF group,  $P < 0.05$ ) at 2 hours post-glucose challenge, which were comparable to those of LF diet-fed mice ( $179 \pm 12.49$  mg/dl,  $P < 0.05$ ). The results also showed that HF+SU group had significantly lower AUC than that of HF group ( $P < 0.05$ ), suggesting that sulforaphane supplementation improved glucose tolerance in obese mice (Fig. 3B).

### **Sulforaphane improved insulin sensitivity in obese mice**

We examined whether sulforaphane modulates insulin sensitivity. In that regard, ITT was performed. HF+SU group had lower blood glucose levels after injection of insulin at 30 minutes ( $132 \pm 4.44$  mg/dl), 60 minutes ( $61 \pm 6.22$  mg/dl), and 120 minutes ( $83.83 \pm 7.00$  mg/dl) as compared to those in HF group ( $121 \pm 5.94$  mg/dl,  $95.35 \pm 5.90$  mg/dl, and  $140.06 \pm 10.44$  mg/dl,  $P < 0.05$ , respectively), which were almost restored to the levels of those of LF group (Fig. 3C). AUC of HF+SU group was also significantly lower than that of HF group ( $P < 0.05$ ). These

results indicate that sulforaphane significantly promoted peripheral insulin sensitivity in obese mice.

### **Dietary intake of sulforaphane reduced blood insulin concentrations in obese mice**

To test whether sulforaphane had the effect of reducing insulin resistance in obese mice, we measured the fasting serum insulin concentration. Results showed that sulforaphane treated mice had significant lower serum insulin levels ( $1.93 \pm 0.11$   $\mu\text{g}/\text{dl}$ ) than those in obese mice without treatment ( $3.09 \pm 0.27$   $\mu\text{g}/\text{dl}$ ,  $P < 0.05$ ) (Fig. 3D).

### **Sulforaphane treatment improved blood lipid profiles**

We investigated whether dietary intake of sulforaphane modulates plasma lipid profiles in obese mice. As shown in Fig. 4A, dietary provision of sulforaphane for 7 weeks reduced total circulating cholesterol levels ( $190.67 \pm 20.31$   $\text{mg}/\text{dl}$ ) as compared to HF group ( $256.00 \pm 6.52$   $\text{mg}/\text{dl}$ ,  $P < 0.05$ ). Interestingly, HDL cholesterol levels were also significantly lower in mice fed with sulforaphane ( $124.67 \pm 9.55$   $\text{mg}/\text{dl}$ ) as compared to those in HF group ( $156.4 \pm 6.24$   $\text{mg}/\text{dl}$ ,  $P < 0.05$ ) (Fig. 4B). Sulforaphane had no effect on blood triglyceride concentrations in obese mice (Fig. 4C).

### **Sulforaphane administration enhanced weight loss without affecting food intake in HF diet-induced obese mice**

We then conducted second animal study to determine whether sulforaphane has beneficial effects at the time when mice are already obese. Male C57BL/6 mice (7 months old) were fed a HF diet for 4 weeks to induce obesity and insulin resistance [70, 71] and then treated with sulforaphane (40  $\text{mg}/\text{kg}$  in 2% cellulose) or vehicle by gavage for 30 days. Obese mice treated with

sulforaphane or vehicle had similar weekly food intake ( $15.24 \pm 1.98$  g vs.  $15.72 \pm 1.48$  g) (Fig. 5 B). However, sulforaphane significantly enhanced body weight loss after 6 days of gavage (Fig. 5A). The net weights of inguinal fat, brown fat, and visceral fat were measured. Sulforaphane treated group had more inguinal fat ( $4.22 \pm 0.25$  g) as compared to control group ( $3.52 \pm 0.12$  g,  $P < 0.05$ ). There were no differences with their visceral fat and brown fat weights (Fig. 5C).

### **Sulforaphane did not alter energy expenditure in obese mice**

Energy expenditure was evaluated by indirect calorimetry at the end of gavage study. Mice treated with sulforaphane tended to have slightly higher energy expenditure, as assessed using data recorded over 48 h, but this difference is not statistically significant. In addition, sulforaphane treatment did not alter locomotive activity both during the day and night period (Fig. 5D) and both groups had the same respiratory exchange ratio (RER) (Fig. 5E).

### **Sulforaphane enhanced insulin sensitivity without affecting blood glucose homeostasis**

Sulforaphane treated group had significantly lower blood glucose levels at 30 minutes ( $116.56 \pm 8.19$  mg/dl), and 60 minutes ( $125.33 \pm 9.41$  mg/dl) following insulin injection as compared to control group ( $141.00 \pm 6.10$  mg/dl,  $163.43 \pm 11.05$  mg/dl, respectively,  $P < 0.05$ ) (Fig. 6A). However, there were no significant differences in fasting (FG) and non-fasting (NFG) blood glucose levels, between sulforaphane-treated mice and the control group (Fig. 6B). In addition, glucose tolerance was not altered by administration of sulforaphane (Fig. 6C). Area under the curve (AUC) was calculated using the ITT blood glucose data from all time points. Results showed that sulforaphane group had significantly lower AUC than that of control group ( $P < 0.05$ ).

### **Sulforaphane improved blood lipid profile and increased fecal lipid content**

Total plasma cholesterol levels in sulforaphane group ( $19.97 \pm 1.31$  mg/dl) were significantly lower as compared with mice in control group ( $24.29 \pm 1.03$  mg/dl,  $P < 0.05$ ). Mice treated with sulforaphane also had lower triglyceride concentrations ( $26.37 \pm 1.46$  mg/dl) than those in control group ( $32.47 \pm 2.14$  mg/dl,  $P < 0.05$ ). Total lipids were extracted from fecal samples using Folch method [74]. Total cholesterol levels ( $14.97 \pm 1.39$  mg/g dried fecal sample) in sulforaphane group were higher as compared with control group ( $11.73 \pm 1.50$  mg/g dried fecal sample) but this difference is not statistically significant. Similarly, mice treated with sulforaphane non-significantly increased fecal triglyceride concentrations ( $51.65 \pm 7.75$  mg/g dried fecal sample) as compared with those from control group ( $41.18 \pm 4.20$  mg/g dried fecal sample).

### **Sulforaphane increased pyruvate and fatty acid oxidation in primary human muscle cells**

Exposure of primary human muscle cells to sulforaphane dose-dependently increased the rates of pyruvate oxidation into  $\text{CO}_2$ , with 100 nM sulforaphane increasing  $\text{CO}_2$  production by 28.85% over the control ( $P < 0.05$ ) (Fig 7A). Sulforaphane at 1  $\mu\text{M}$  and 2  $\mu\text{M}$  increased the rates of fatty acid oxidation into acid soluble metabolites (ASM) by 26.22%, 24.87%, respectively ( $P < 0.05$ ). In addition, sulforaphane at 100 nM concentration significantly increased the efficiency of fatty acid oxidation by 2.2 folds (Fig 7B).

### **Sulforaphane improved glucose uptake in C2C12 myotubes**

To determine whether sulforaphane has effects on regulating glucose uptake in the C2C12 myotubes, the cells were cultured with multiple concentrations of sulforaphane for 30 minutes and 1 hour, respectively. As shown in Fig. 8 A&B, sulforaphane treatment increased glucose uptake in the C2C12 myotubes in a dose-dependent manner. Treating cells with 10  $\mu\text{M}$  of sulforaphane for 1 hour significantly increased glucose uptake by 27% as compared to control

group ( $P < 0.05$ ). Consistently, cells treated with 5  $\mu\text{M}$  and 10  $\mu\text{M}$  of sulforaphane for 30 minutes improved glucose uptake in C2C12 myotubes by 11% and 17%, respectively ( $P < 0.05$ ).

## Discussion

Sulforaphane is a natural compound derived from cruciferous vegetables exerting many promising benefits against numerous diseases [75, 76]. However, no research was done as to my knowledge to investigate the potential anti-obese and anti-diabetic properties of sulforaphane. In the present study, we demonstrated that this compound may have potential anti-obese and anti-diabetic effects by using HF diet-induced mouse models and *in vitro* study. Our data showed that sulforaphane can improve insulin sensitivity in middle-aged obese mice induced by HF diet feeding and ameliorate hyperglycemia in obese mouse model.

Obesity, a leading risk factor of T2D, is associated with elevated blood glucose, as well as insulin, and insulin resistance [77, 78]. Chronic metabolic syndromes, such as insulin resistance [79], are often caused by HF diet-induced obesity in mouse models [80, 81]. Insulin resistance is a hallmark of metabolic disorders that can lead to T2D [82]. In the present study, we used middle-aged mice, because T2D usually occurs at middle and older ages in humans. In addition, sulforaphane reduced body fat content and increased body lean mass, confirming that sulforaphane prevented obesity in HF diet feeding mice. We further demonstrated that provision of sulforaphane ameliorated glucose intolerance, improved insulin sensitivity, and decreased blood glucose concentration and lipid profile in HF diet feeding mice. Importantly, these beneficial metabolic effects elicited by dietary intake of sulforaphane are not associated with alteration in food intake. Given that obesity and insulin resistance are the risk factors of development to T2D, sulforaphane could be an effective dietary supplement that is capable of preventing the pathogenesis of T2D.

It is well established that increased triglyceride and free fatty acids, and decreased HDL-cholesterol with HDL dysfunction represent dyslipidemia that is typical of obesity [83]. Our data

showed that consumption of HF diet with 40 mg/kg sulforaphane had decreased plasma triglyceride levels and total cholesterol concentrations. Recent research on sulforaphane showed that sulforaphane may attenuate obesity by decreasing adipogenesis-related genes in adipose tissue and increasing adiponectin expression, as well as suppressing lipogenesis in adipocytes [84]. Based on the result that food intake was not affected by sulforaphane supplementation, the decrease in total plasma lipid and increase in fecal lipid content in sulforaphane treated mice in our study may be due to the regulation of adipogenesis-related genes and/or the reduced lipid absorption in the intestine. Further experiments are needed to determine how sulforaphane caused a reduction in circulating cholesterol concentrations in HF diet-fed mice.

It is well known that obesity results from energy imbalance. As sulforaphane supplementation either through diet or gavage did not change calorie intake, we then assessed energy expenditure to determine whether the reduced body weight in sulforaphane treated group is due to an effect on energy expenditure. It is worth to note that, in the present study, energy expenditure was normalized to lean mass instead of body weight, because fat tissue may contribute comparatively small to the total energy expenditure compared with lean mass due to its relatively low metabolic activity [85]. Interestingly, we did not observe any significant difference in energy expenditure between control and sulforaphane treated group, as assessed using data obtained over 48 h, but sulforaphane treatment slightly increased energy expenditure. However, this small difference in energy expenditure as shown within 48 h between the control and sulforaphane treated mice could lead to the large differences in the accumulation of fat mass over long-term of HF-diet feeding. Indeed, it was estimated that, given the identical calorie intake, a 5% difference in daily energy expenditure in HF-diet fed mice can result in about 10 grams difference in body weight over 4-6 months [85].



Insulin resistance and hyperglycemia are related with the dysfunctions of several metabolic pathways including the impaired glucose transport and oxidation, increased glucose output by the liver, and altered lipogenesis and lipolysis [86]. While data from present study show that sulforaphane supplementation improved insulin sensitivity and glucose homeostasis, these beneficial effects may be not the secondary action whereby intake of sulforaphane prevented the obesity in mice fed with HF diet, because sulforaphane had no effect on body weight gain in these mice. Moreover, sulforaphane treatment significantly increased glucose oxidation in myotubes as determined by pyruvate oxidation assays. These results suggest that dietary sulforaphane can potentially lower the risk of developing metabolic syndrome.

In the present study, we further assessed *ex vivo* pyruvate and fatty acid oxidation in primary human muscle cells. Compared to the control group, 10 nM, 1  $\mu$ M, and 2  $\mu$ M of sulforaphane treatment did not alter fatty acid oxidation, but 100 nM of sulforaphane displayed higher efficiency of fatty acid oxidation to CO<sub>2</sub> while accumulated less ASM in the primary muscle cells, as determined by directly measuring the rate of conversion of radiolabeled pyruvate into CO<sub>2</sub> or ASM, which provide the index of complete and incomplete oxidation of fatty acid, respectively. These results suggest that the ability of mitochondria to oxidize fatty acid may be enhanced by sulforaphane treatment.

One recent research showed that sulforaphane can suppress adipocytes differentiation via down-regulating peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) in 3T3-L1 preadipocytes [87]. Suppressed expression of PPAR $\gamma$  and C/EBP $\alpha$  in adipocytes can effectively prevent HF diet induced obesity [88-90], which indicate the critical roles of PPAR $\gamma$  and C/EBP $\alpha$  in adipogenesis and regulate fat accumulation [91]. AMP-activated protein kinase (AMPK) activity in adipocytes suppresses lipogenesis and promotes fatty acid

oxidation [92, 93]. In accordance with present study that sulforaphane treatment reduced body weight and fat mass, the anti-obese effect of sulforaphane may through the inhibition of PPAR $\gamma$  and C/EBP $\alpha$  and stimulation of AMPK activity, which is intriguing for further investigation.

In summary, we provided evidence that dietary intake of sulforaphane can prevent HF diet-induced obesity in middle-aged mice, which is associated with ameliorated hyperglycemia and increased insulin sensitivity, muscle cell fatty acid oxidation, fecal lipid content and decreased plasma lipids. These results indicate that sulforaphane can be used as a dietary supplementation to ameliorate obesity and related metabolic syndromes. Further studies are needed to elucidate the molecular mechanisms underlying the beneficial metabolic effects of sulforaphane.

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## Figure Legends

**FIG. 1.** Sulforaphane supplementation had no influence on body weight gain. Body weights were measured each week. Data are shown as mean±SE (n=8). LF: low fat chow diet; HF: high fat diet; HF+SU: HF diet supplemented with sulforaphane. ....30

**FIG. 2.** Dietary supplementation of sulforaphane had no effect on fat mass (A) and lean mass (B). Body composition was measured following 7 weeks of HF diet feeding. Data are shown as mean±SE (n=8) (\* P<0.05). LF: low fat chow diet; HF: high fat diet; HF+SU: HF diet supplemented with sulforaphane. ....31

**FIG. 3.** Sulforaphane supplementation ameliorated hyperglycemia and plasma insulin concentrations, and improved insulin sensitivity in HF-diet fed obese mice. (A) Fasting blood glucose levels measured after 7 weeks of dietary treatment. GTT (B) and ITT (C) were performed as described in the Research Design and Methods. The area under the curve (AUC) for GTT and ITT were calculated. (D) Plasma insulin levels were measured using an ELISA kit. Data are shown as mean±SE (n=8) (\* P<0.05 vs. LF; # P<0.05 vs. HF+SU). LF: low fat chow diet; HF: high fat diet; HF+SU: HF diet supplemented with sulforaphane. ....32

**FIG. 4.** Sulforaphane supplementation decreased circulating levels of total cholesterol and HDL-cholesterol, but had no effect on triglyceride concentration in HF diet fed mice. At the end of the experiment, fasting plasma total cholesterol (A), HDL-cholesterol (B), and triglyceride (C) concentrations were measured in duplicated samples by using mouse ELISA kits. Data are



shown as mean±SE (n=5) (\* P<0.05). LF: low fat chow diet; HF: high fat diet; HF+SU: HF diet supplemented with sulforaphane. ....34

**FIG. 5.** Sulforaphane administration enhanced body weight loss without changing food intake, but had no significant effect on the respiration rate, energy expenditure or cage activity. The net weight of inguinal fat, brown fat, and visceral fat were measured after isolation the tissues. Body weight change (**A**), weekly food intake (**B**), body fat deposit (**C**), cage activity (**D**), and respiration exchange ratio (RER) (**E**), were measured as described in Research Design and Methods following 30 days of sulforaphane administration via gavage. Data are shown as mean±SE (n=15) (\* P<0.05). ....36

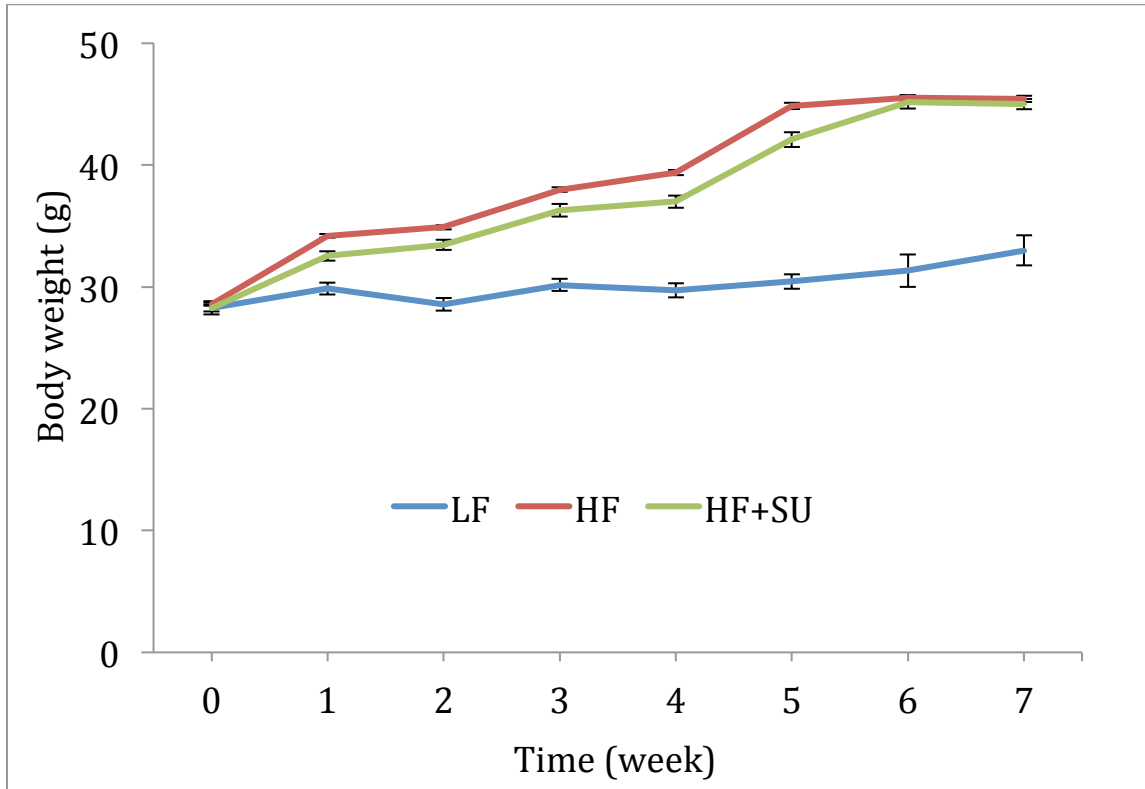
**FIG. 6.** Administration of sulforaphane via gavage enhanced insulin sensitivity in obese mice but did not affect glucose homeostasis. ITT (**A**) was performed as described in the Research Design and Methods. (**B**) Fasting (FG) and non-fasting (NFG) blood glucose levels were measured after 4 weeks of treatment. GTT (**C**) was performed as described in the Research Design and Methods. The area under the curve (AUC) for GTT and ITT were calculated. Data are shown as mean±SE (n=8) (\* P<0.05). ....39

**FIG. 7.** Sulforaphane (SU) improved pyruvate oxidation to CO<sub>2</sub> (**A**) and fatty acid oxidation efficiency (**B**) in primary human muscle cells. Data are shown as mean±SE (\* P<0.05). .....41

**FIG. 8.** Sulforaphane enhanced glucose uptake in C2C12 myotubes with 1 hour treatment (**A**) and with 30 minutes treatment (**B**). Data are shown as mean±SE (\* P<0.05). ....42

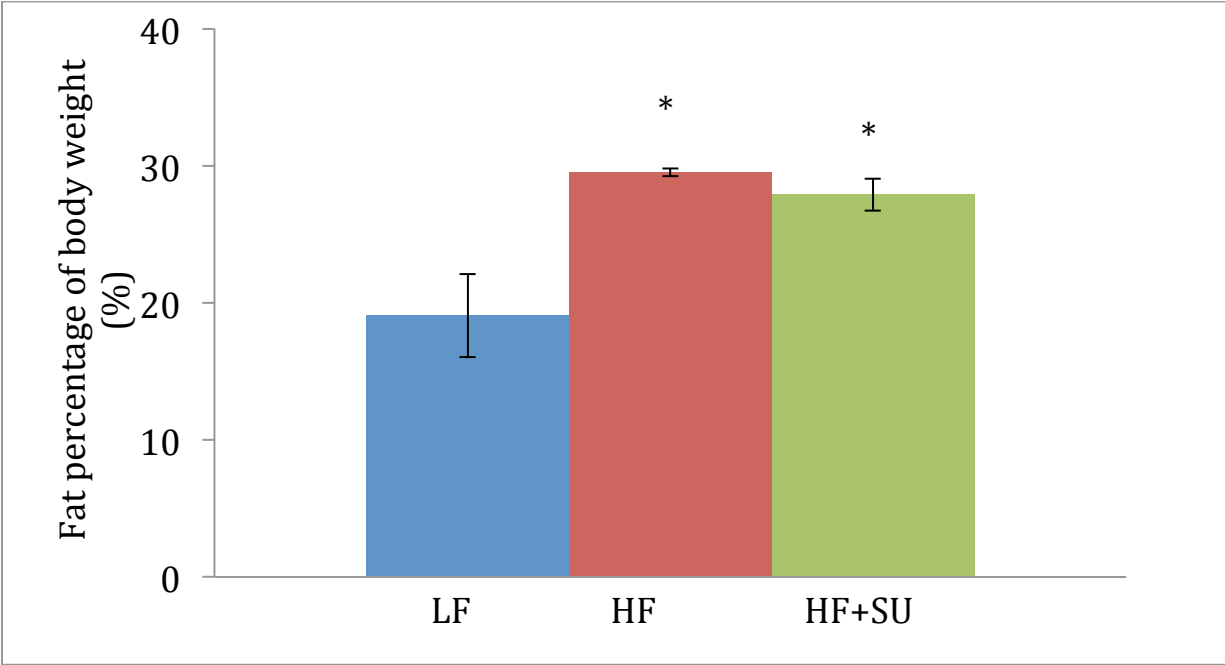
## Figures

**Figure 1. Sulforaphane supplementation had no influence on body weight gain.**

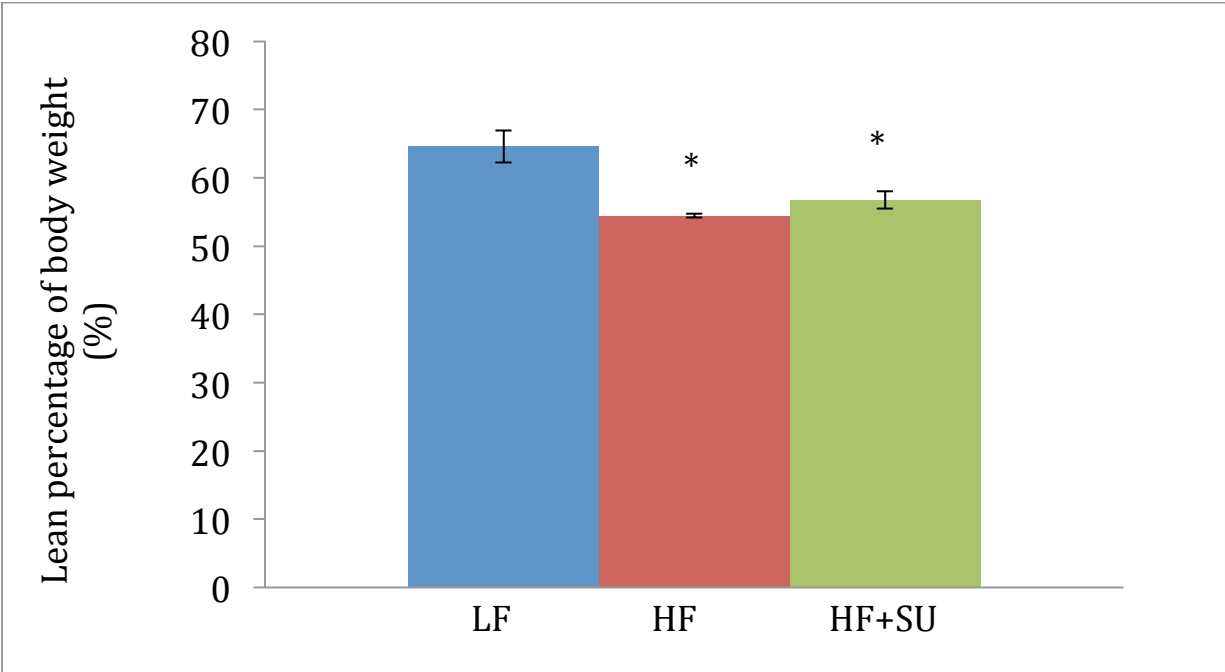


**Figure 2. Dietary supplementation of sulforaphane had no effect on fat mass (A) and lean mass (B).**

**A**

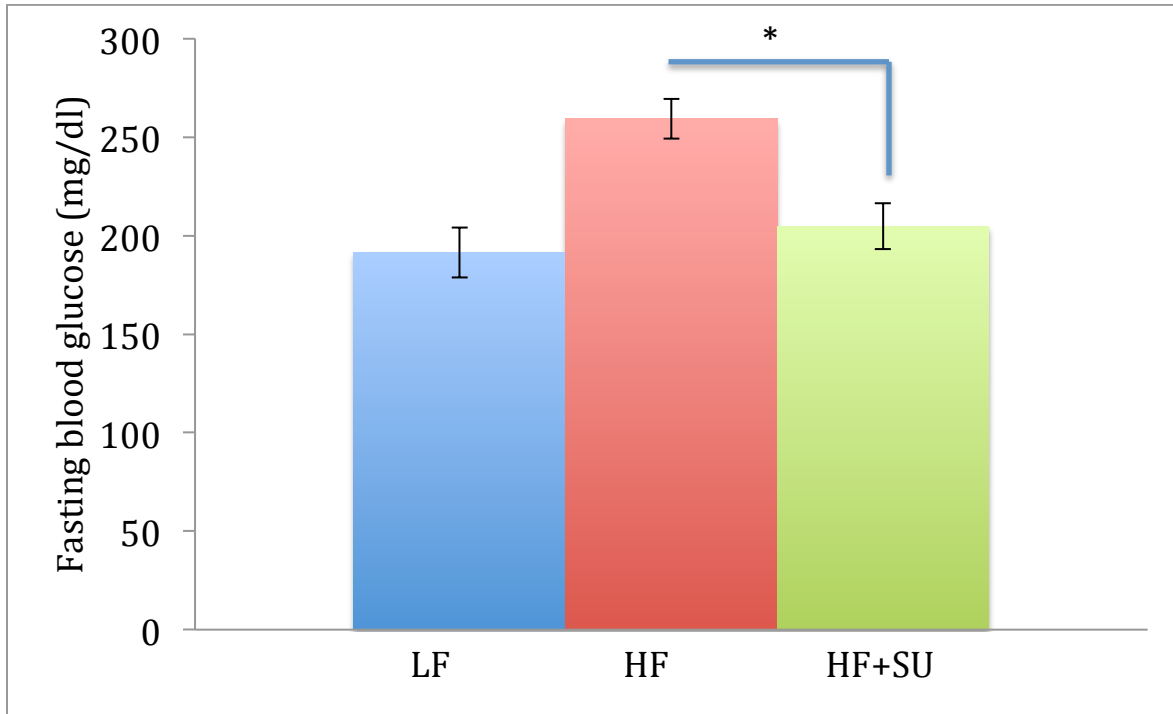


**B**

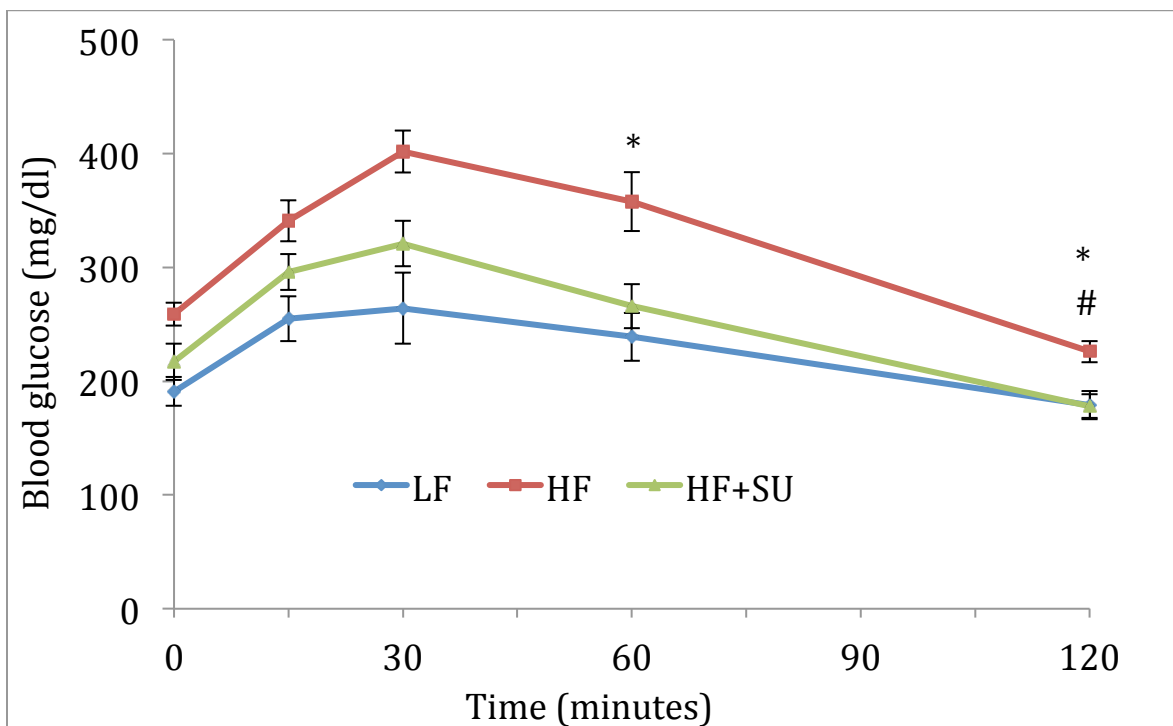


**Figure 3. Sulforaphane supplementation ameliorated hyperglycemia (A), improved insulin sensitivity (B, C), and ameliorated plasma insulin concentrations (D) in HF-diet fed obese mice.**

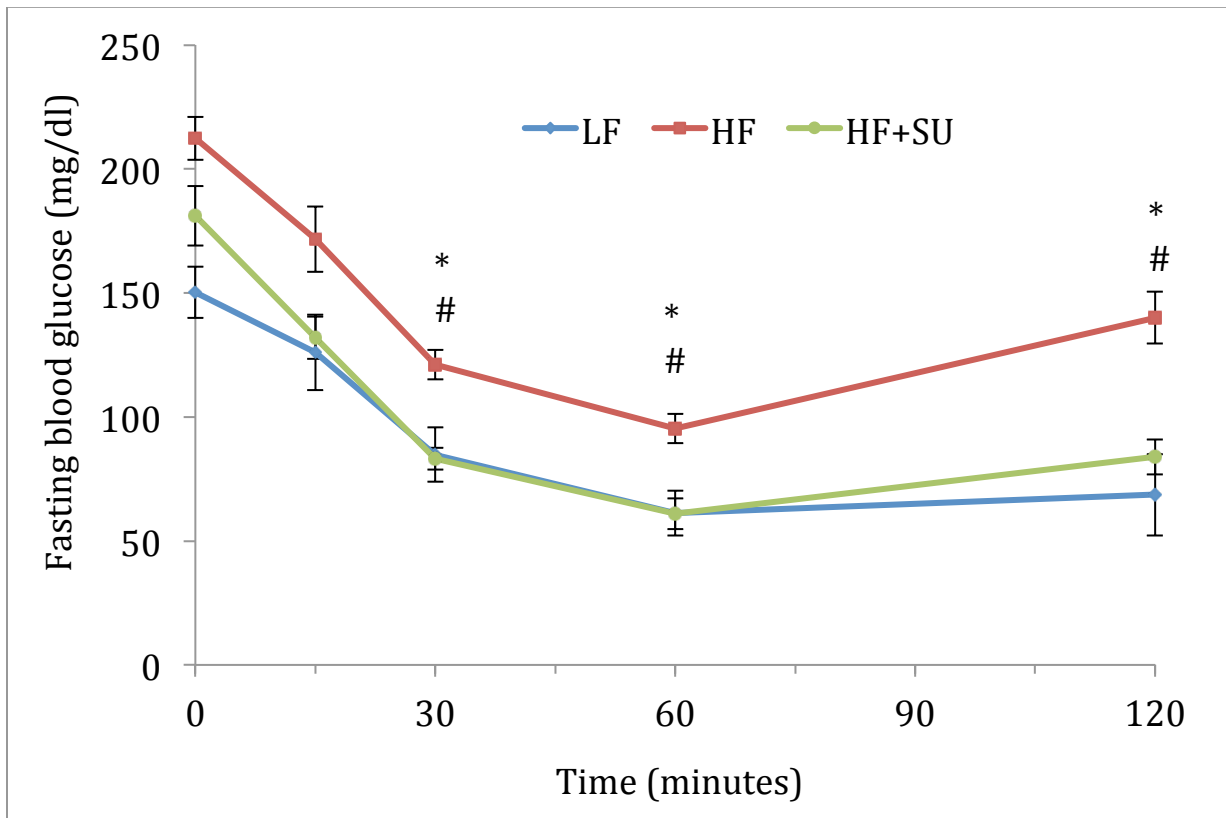
**A**



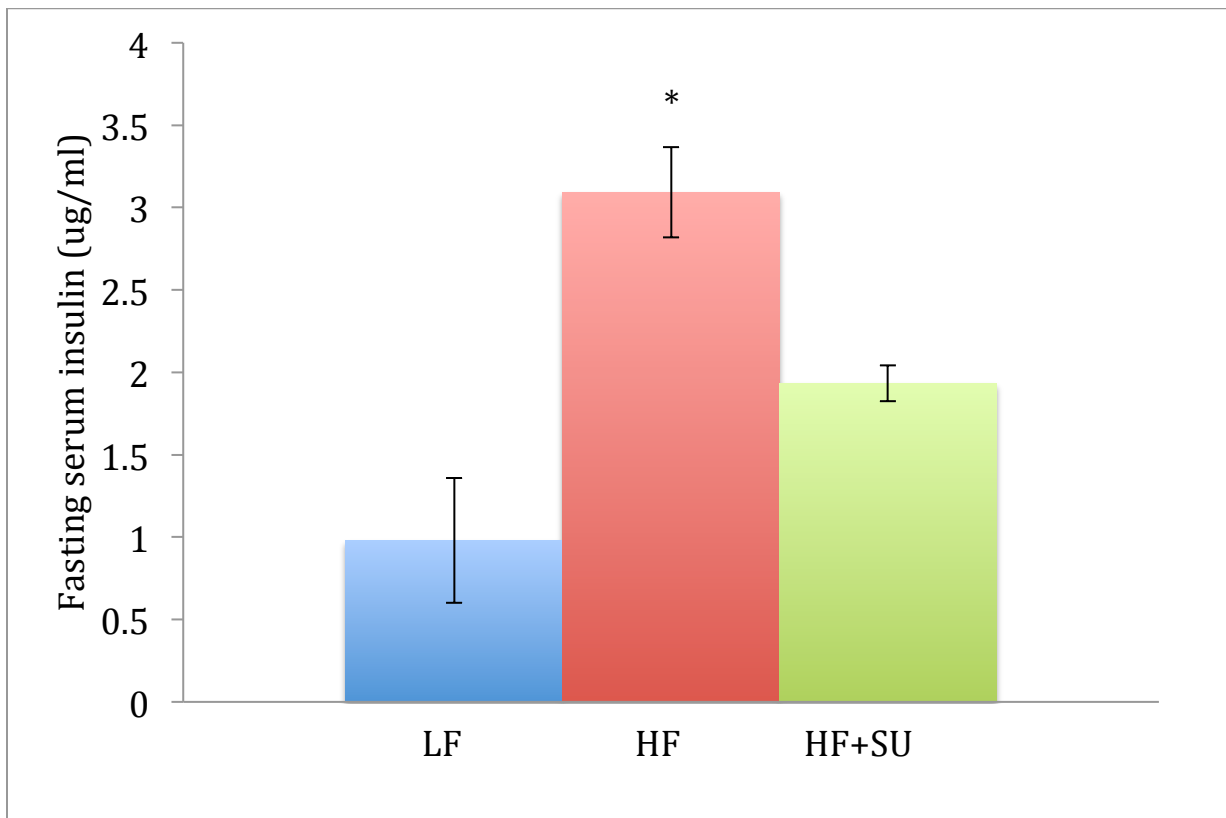
**B**



C

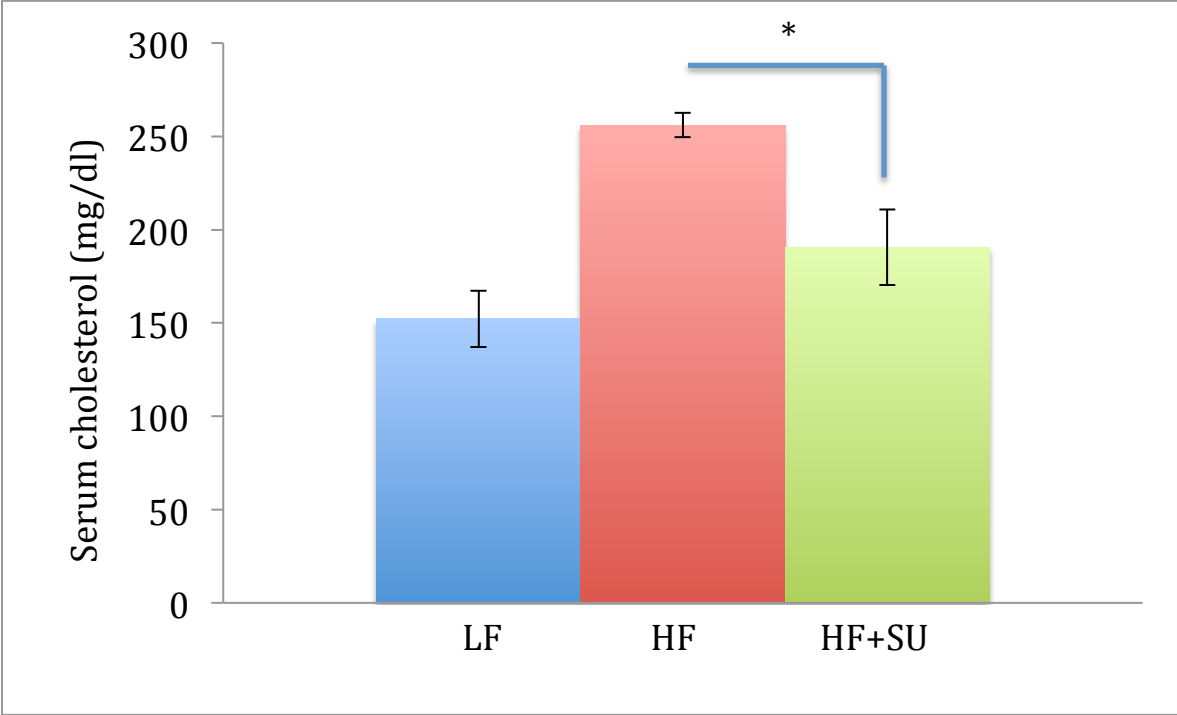


D

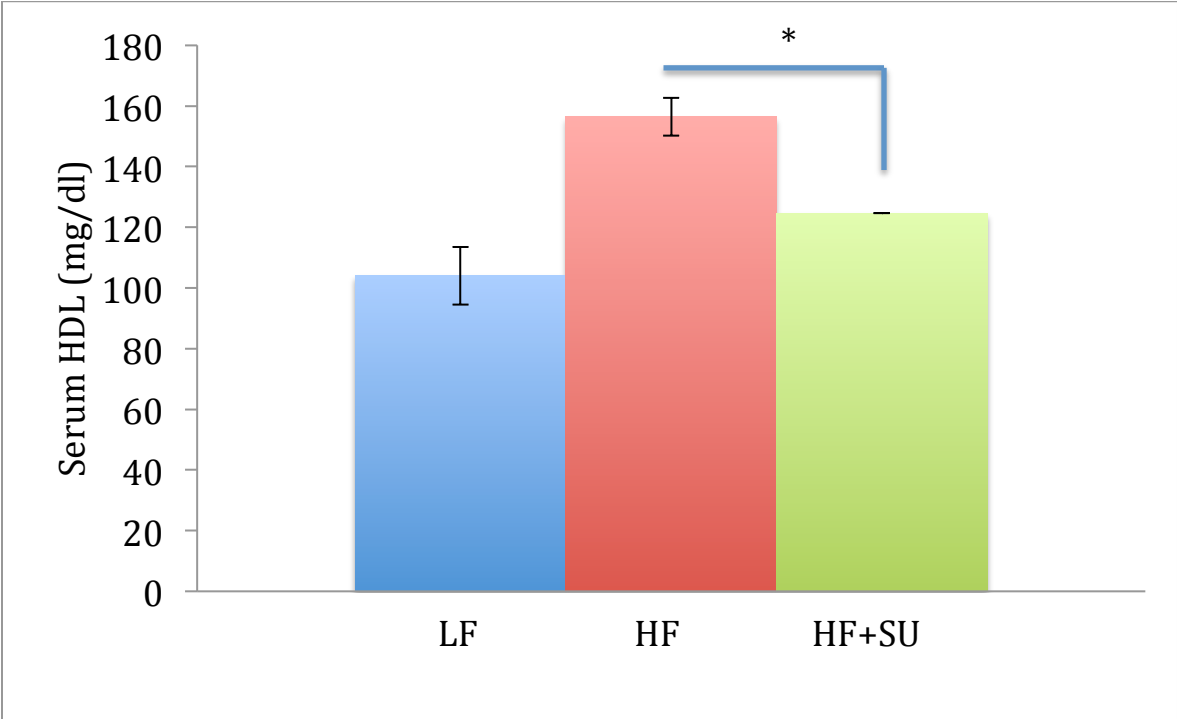


**Figure 4. Sulforaphane supplementation decreased circulating levels of total cholesterol (A) and HDL-cholesterol (B), but had no effect on triglyceride concentration (C) in HF-diet fed mice.**

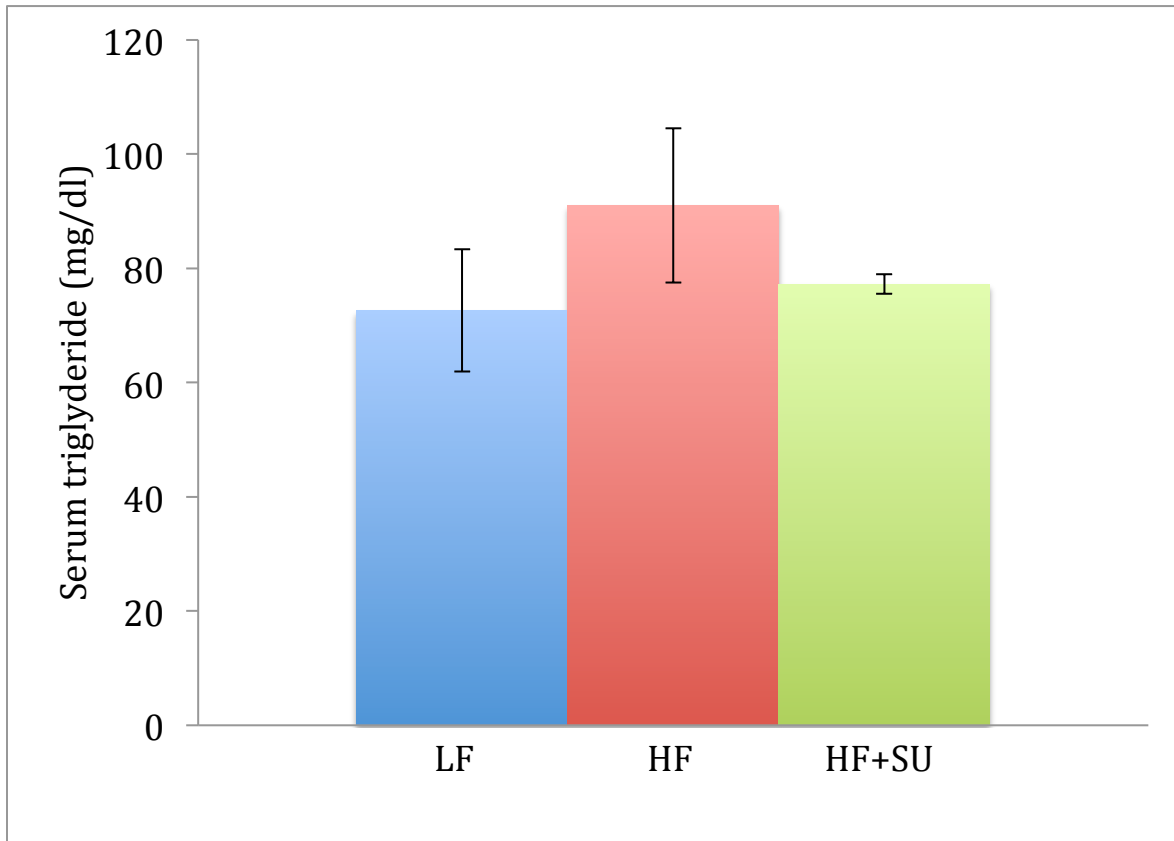
**A**



**B**

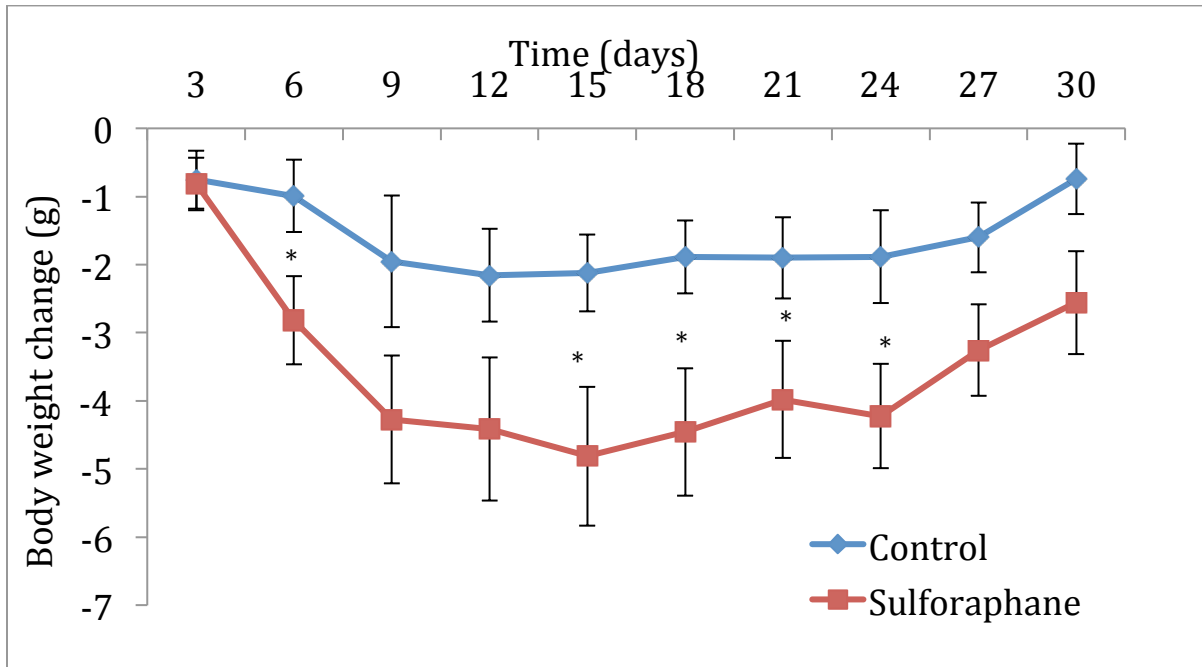


**c**

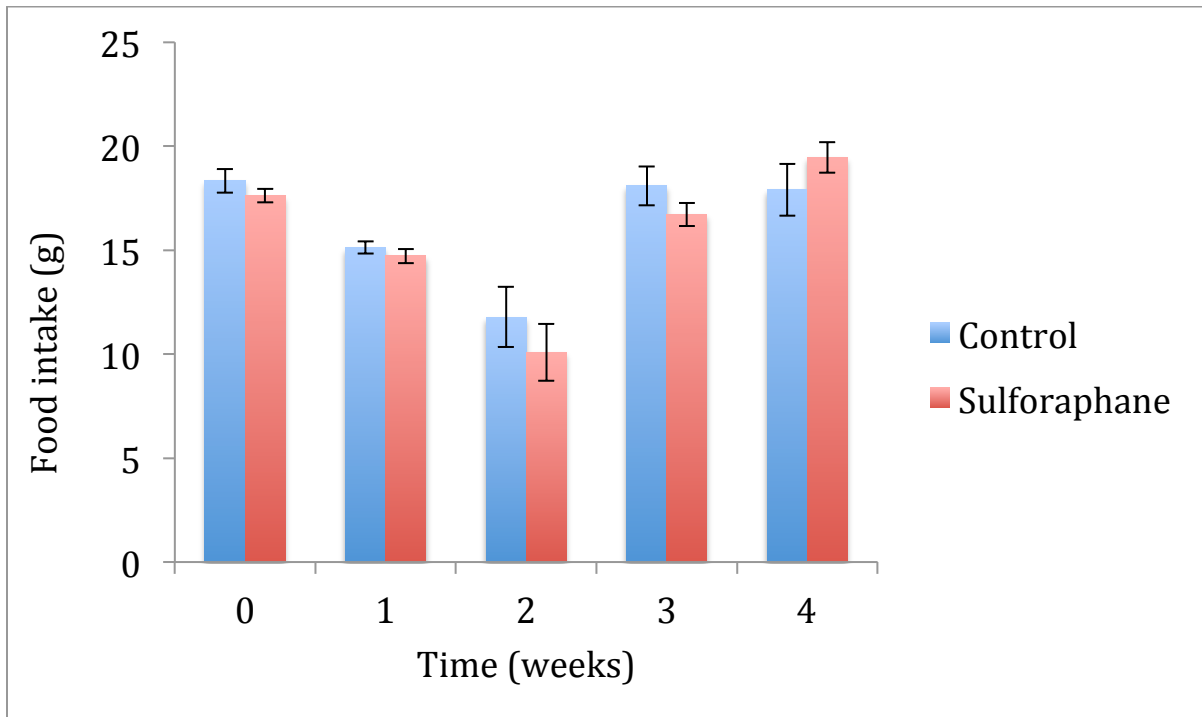


**Figure 5. Sulforaphane administration enhanced body weight loss (A) without changing food intake (B) and increased net weights of inguinal fat without changing neither visceral fat nor brown fat (C), but had no significant effects on neither cage activity (D) nor respiration rate (E).**

**A**

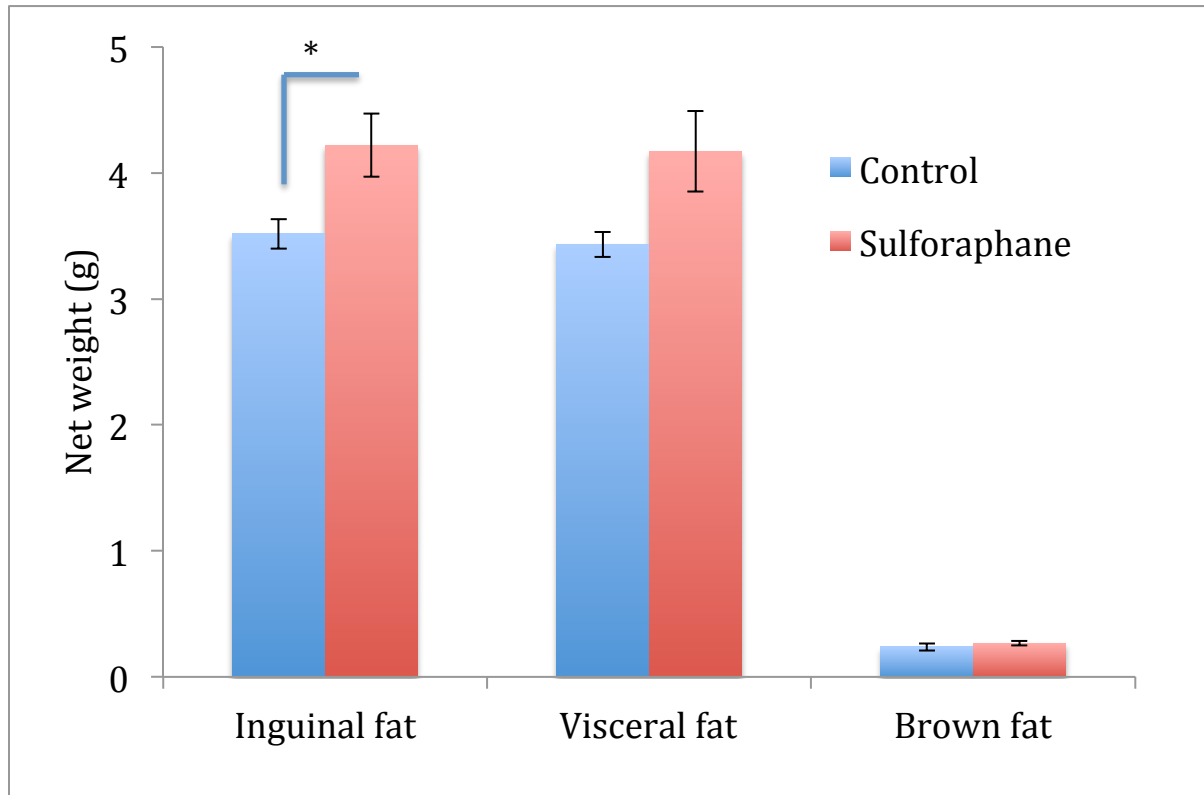


**B**

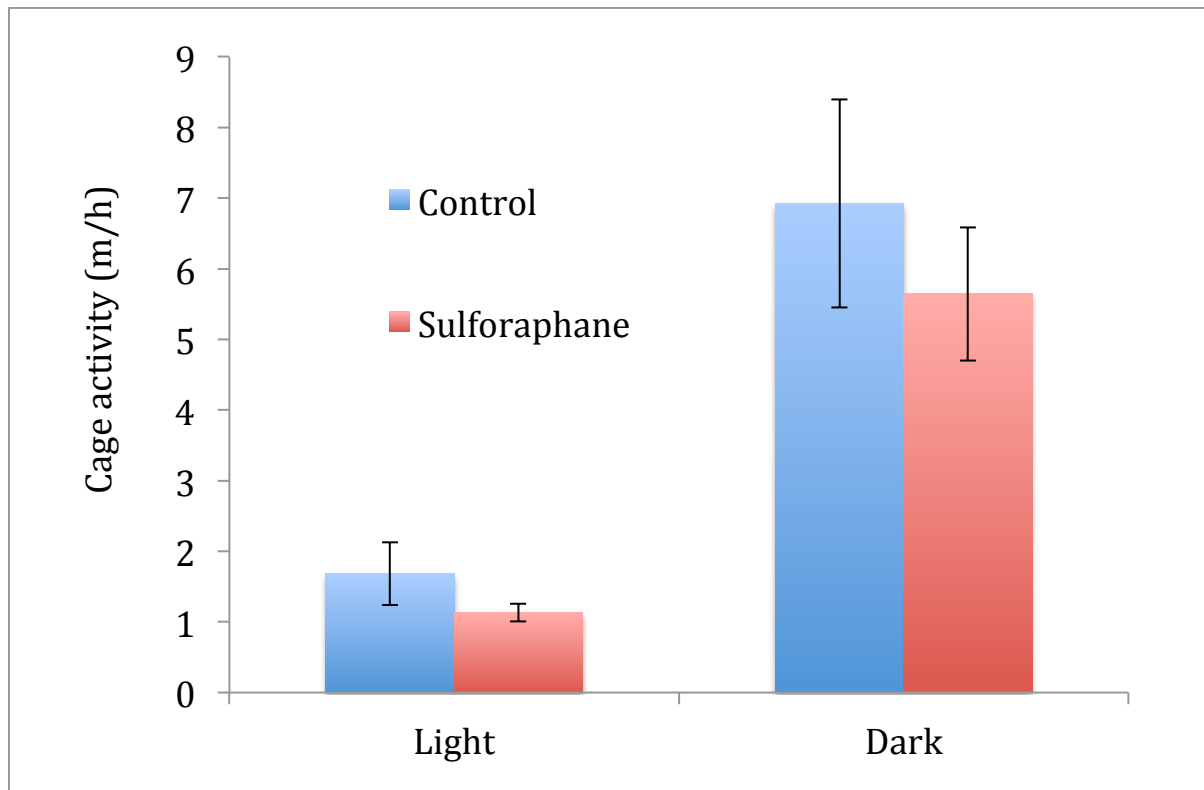




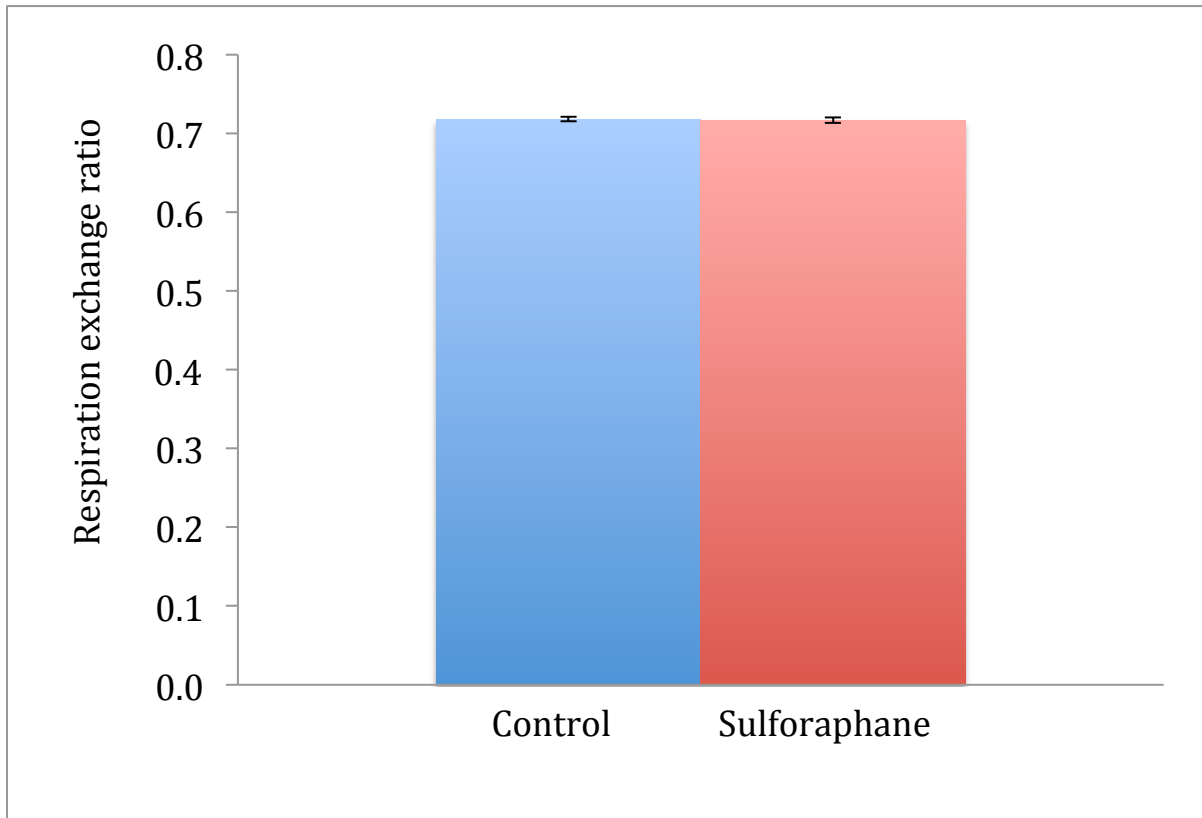
C



D

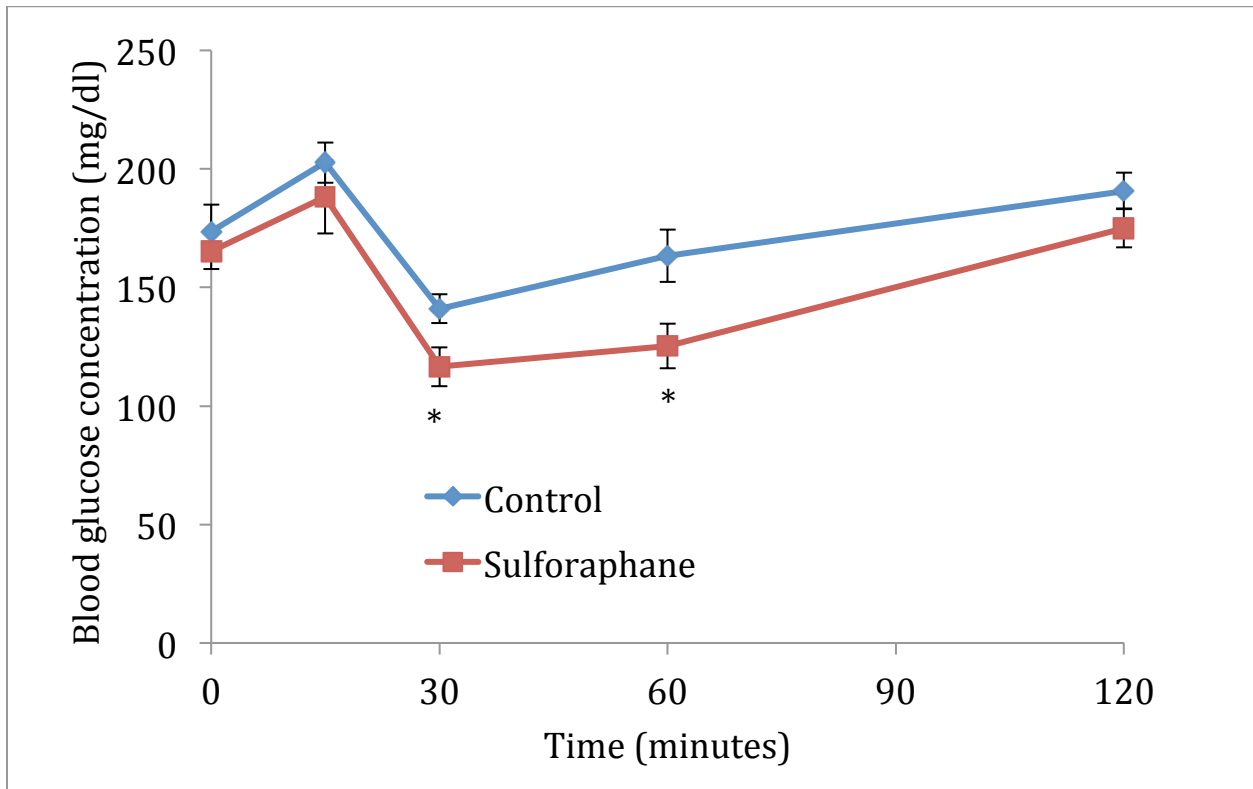


**E**

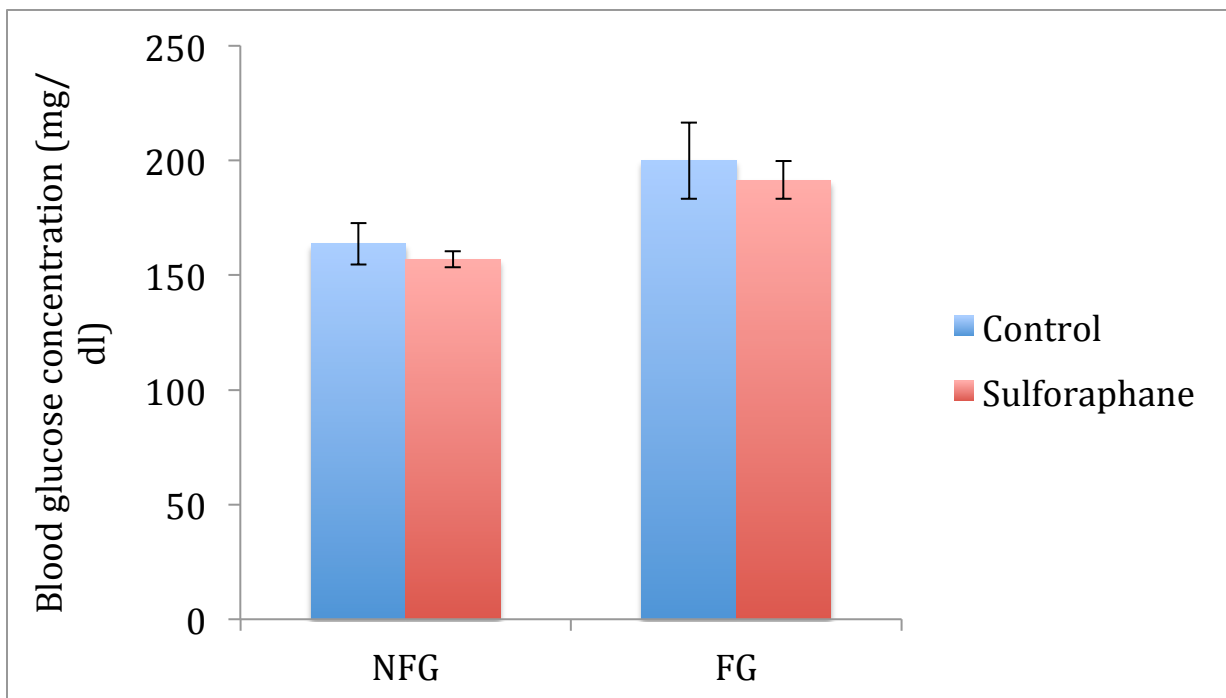


**Figure 6. Administration of sulforaphane via gavage enhanced insulin sensitivity in obese mice (A) but did not affect glucose homeostasis (B, C).**

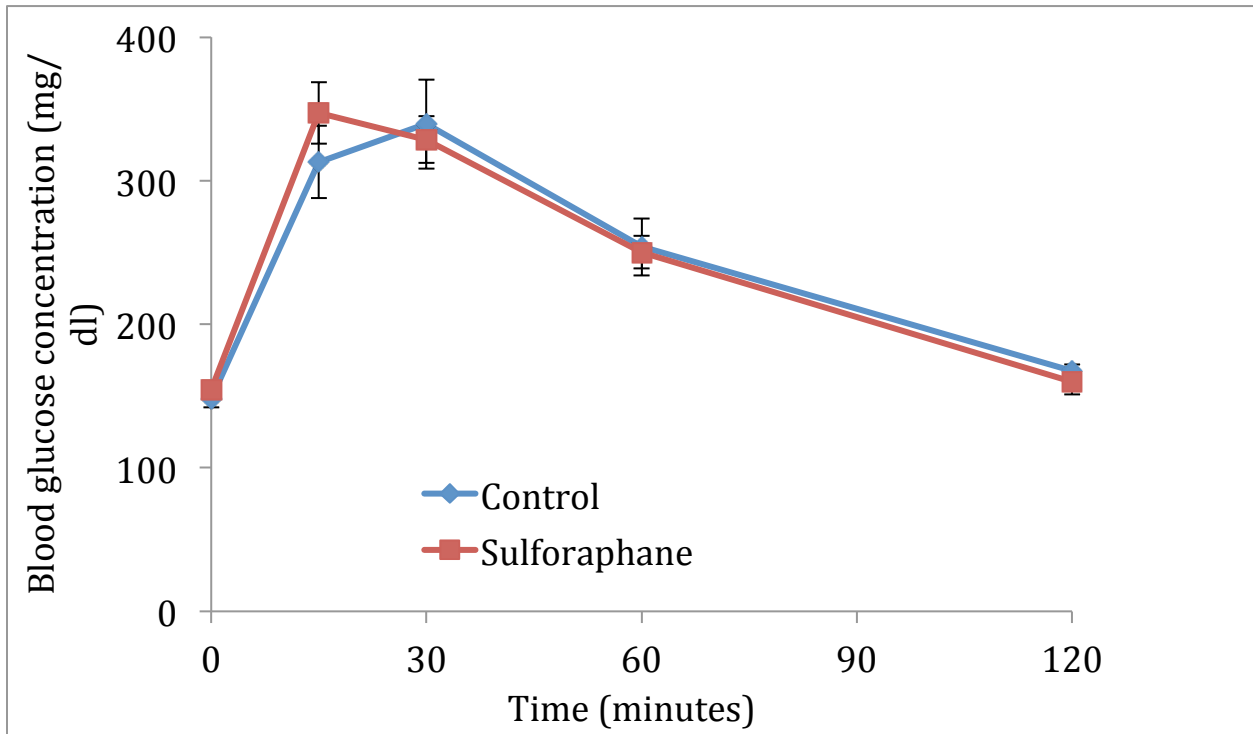
**A**



**B**

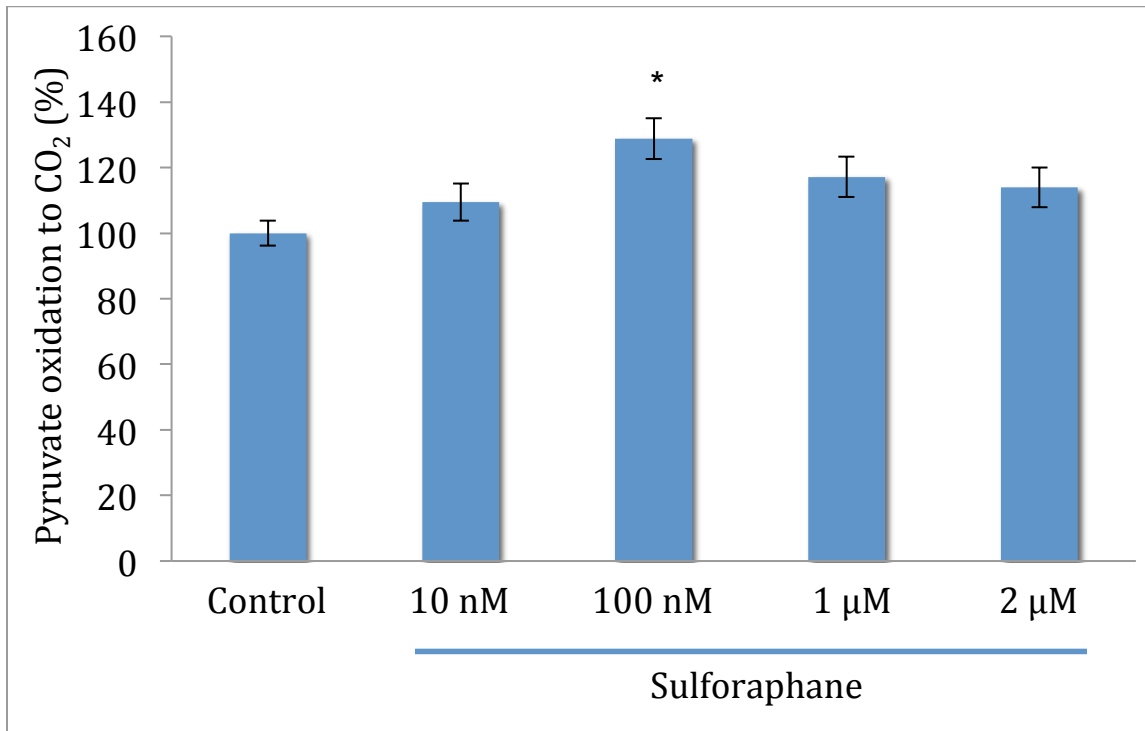


C

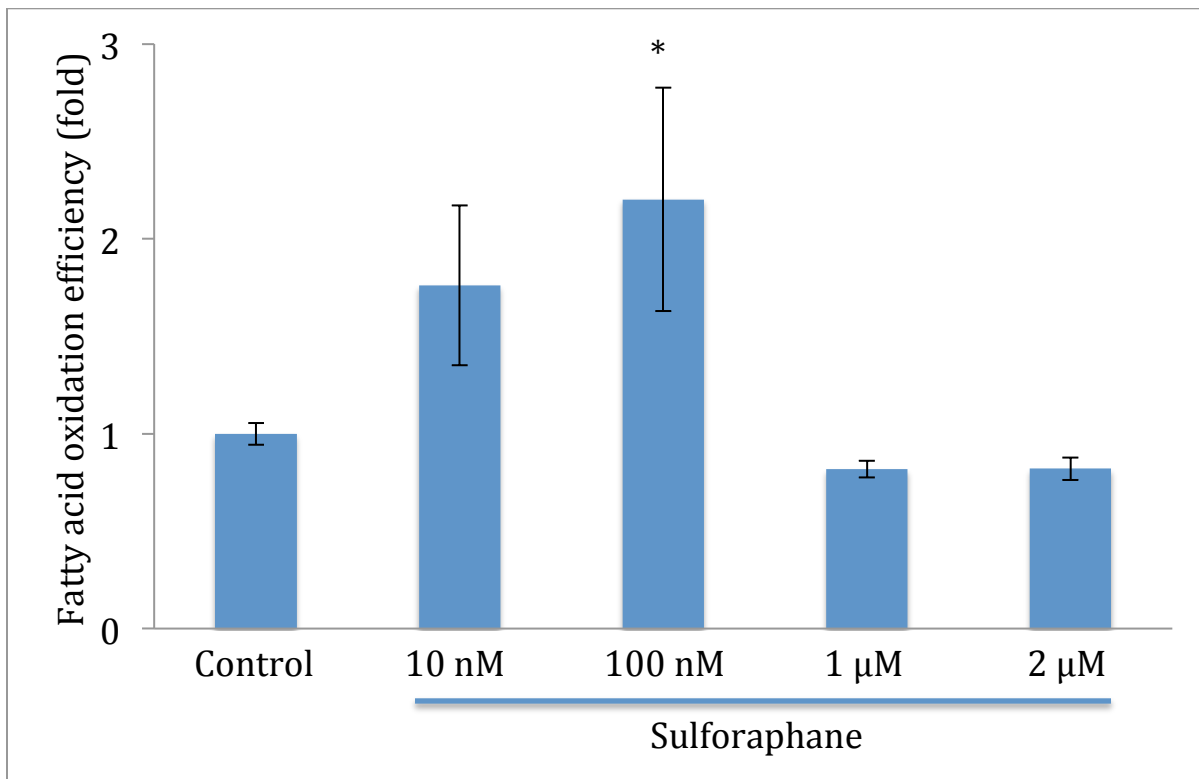


**Figure 7. Sulforaphane (SU) improved pyruvate oxidation to CO<sub>2</sub> (A) and fatty acid oxidation efficiency (B) in primary human muscle cells.**

**A**

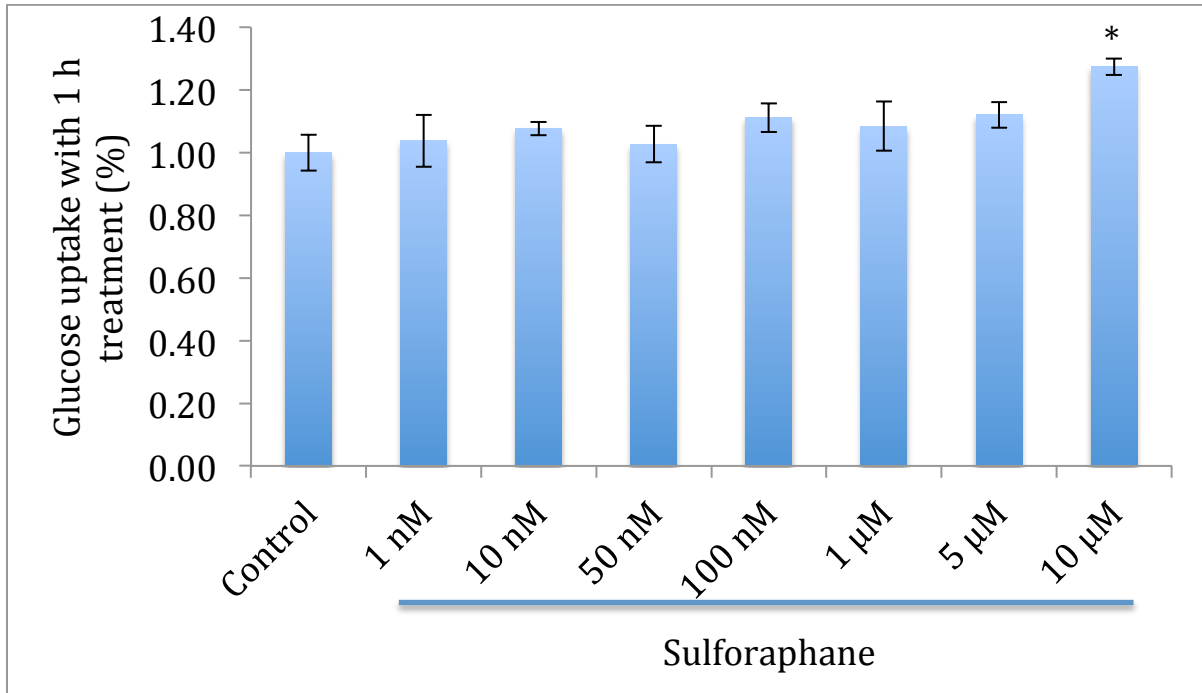


**B**



**Figure 8. Sulforaphane enhanced glucose uptake in C2C12 myotubes with 1 hour treatment (A) and with 30 minutes treatment (B).**

**A**



**B**

