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Original Article

N-Acetyl-L-Cysteine inhibits the development of glucose intolerance and hepatic steatosis in diabetes-prone mice

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Abstract: Oxidative stress is associated with different pathological conditions, including glucose intolerance and type 2 diabetes (T2D), however studies had failed to prove the benefits of antioxidants in T2D. Aim: On the assumption that the failure to demonstrate such anti-diabetic effects is a result of sub-optimal or excessive antioxidant dosage, we aimed to clarify the dose-response effect of the antioxidant N-Acetyl-L-Cysteine (NAC) on the progression of T2D in-vivo. Methods: Experiments were conducted on KK-Ay mice and HFD-fed mice given NAC at different concentrations (200-1800 and 60-600 mg/kg/day, respectively). Glucose and insulin tolerance tests were performed and plasma insulin and lipid peroxidation were measured. Insulin signaling pathway was followed in muscle and liver. Hepatic TG accumulation and mRNA expression of genes involved in glucose metabolism were measured. Results: While 600-1800 mg/kg/day NAC all improved glucose tolerance in KK-Ay mice, only the 1200 mg/kg/day treatment increased insulin sensitivity. Hepatic function was not affected, however; microsteatosis rather than macrosteatosis was observed in NAC-treated mice compared to control. Glucose tolerance was improved in NAC-treated HFD-fed mice as well; the best results obtained with a dose of 400 mg NAC/kg/day. This was followed by lower weight gain and hepatic TG. Plasma lipid peroxidation was not correlated with the glucose-lowering effects of NAC in either model. Conclusion: Identification of the optimal dose of NAC and the population that would benefit the most from such intervention is essential in order to apply preventive and/or therapeutic use of NAC and similar agents in the future.

Keywords: Antioxidant, oxidative stress, type 2 diabetes, N-Acetyl-L-Cysteine, steatosis

Introduction

Accumulation of reactive oxygen species (ROS) leads to oxidative stress, a common denominator in many diseases including type 2 diabetes (T2D) [1]. The metabolic overload, usually associates with T2D and manifested by elevated glucose and FFA, increases intracellular ROS accumulation in pancreatic β-cells and in insulin target tissues [2]. In addition, there is evidence indicating that hyperinsulinemia leads to the production of ROS [3]. Pancreatic β-cells are at relatively high risk for oxidative damage as a result of increased mitochondrial ROS production, elevated NADPH oxidase activity, and reduced antioxidant (AOX) defense mechanisms [2, 4, 5]. Oxidative stress leads to impaired β-cell function and reduced β-cell mass. Thus, there is a vicious cycle, in which hyperglycemia and increased FFA induce oxidative stress, which disturbs β-cell function, and accelerates the hyperglycemia. In addition, oxidative stress is suggested to be one of the major causes of aberrant insulin signaling in target tissues, by activating JNK and NFκB pathways as well as by other mechanisms [6-8]. Accordingly, increasing the AOX defense mechanisms either by inducing elevated levels of AOX enzymes [9, 10], or by administration of small molecules with anti-oxidative activity, such as N-acetyl cysteine (NAC), lipoic acid or vitamin E, should afford protection from ROS, thereby preserving the function and survival of β-cells [11, 12] and improving insulin sensitivity of muscle and adipose cells *in-vitro* and *in-vivo* [13-17]. NAC, a dietary supplement wide-

ly used as AOX in laboratory experiments, undergoes hydrolysis to cysteine, thus increasing intracellular GSH levels. Its anti-oxidative effects have been established [18] and several studies have shown a beneficial effect of chronic treatment with NAC on glucose tolerance in T2D mice [12, 19].

Unfortunately, major randomized clinical trials have yielded disappointing results. Meta-analysis of human intervention trials showing that supplementation with AOXs fail to reduce the prevalence of diabetes in a healthy population [6, 20-22]. Moreover, there is some evidence suggesting that effects may even be harmful, including increase in all-cause mortality with vitamin E, selenium, and other AOXs supplementations [23, 24].

We hypothesize that, as the optimal dose of AOX is difficult to determine, inappropriate concentrations of the AOX may lead to the failure of the intervention. It has become apparent that strictly regulated levels of ROS are not always harmful byproducts and are involved in several important physiological functions. ROS act as important molecules in insulin, MAPK, and JNK signaling pathways, and are involved in mediating gene expression, cell proliferation, differentiation and viability [7, 25]. Thus, tight regulation of the redox potential of cells is crucial for maintenance of normal cell function. In our previous study [26] we found that while low concentrations of H_2O_2 increased the viability and insulin secretion capability of pancreatic β-cells, NAC negatively affect these functions. These results suggest that although oxidative stress is involved in the pathophysiology of diabetes, complete neutralization of ROS may not necessarily be beneficial.

Based on the hypothesis that imbalance in redox state leads to disturbances both in the function of β-cells and in insulin sensitivity of target tissues [27], we propose that AOXs may improve glucose tolerance if consumed at an optimal dose, but may be ineffective at lower dose, or lead to undesired responses at higher doses. In this study we have attempted to clarify the dose-dependent effect of NAC supplementation on the onset of glucose intolerance and T2D in diet induced and genetic prone mice. This study highlights the efficiency of NAC in reducing the development of T2D and the importance of clarifying the optimal concentration of the antioxidant.

Materials and methods

Chemicals, kits and reagents

NAC was purchased from Calbiochem. Insulin, proteases and phosphatases inhibitors were purchased from Sigma. Anti-phospho PKB (Ser473), anti-PKB and anti phospho-GSK (ser9) were purchased from Cell-signaling Technology, anti-β-tubulin was purchased from Abcam, and secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (Peroxidase-AffiniPure Goat Anti-Mouse IgG antibody and Peroxidase-AffiniPure Goat Anti-Rabbit IgG antibody).

Methods

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Ariel (Permit Number: IL 25-07-09 and IL 44-11-12). Animals had been anesthetized by ketamine + xylazine as required, and all efforts were made to minimize suffering. Animal House operates in compliance with the rules and guidelines of the Israel Council for Research in Animals, based on the US NIH Guide for the Care and Use of Laboratory Animals.

KK-Ay mice were purchased from Jackson Laboratory (Bar Harbor, ME), and C57Bl/6J mice were purchased from Harlan Laboratories (Israel). The mice were housed in an animal laboratory with a controlled environment of 20-24°C, 45-65% humidity, and a 12 h light/dark cycle.

Study design

The study was performed on KK-Ay mice, a genetic model of T2D and on a model of diet-induced glucose intolerance, using high fat diet-fed C57bl/6 mice (HFD, 60% of total calories derived from fatty acids, 18.4% from proteins and 21.3% from carbohyrates. Harlan, Teklad TD.06414) [28, 29]. For these experiments, 6 weeks old male mice were separated into treatment groups, 8-10 mice each. KK-Ay mice were separated into 5 groups as follows: control-untreated mice and NAC-treated mice at the following doses: 200, 600, 1200 and 1800 mg/kg/day. The mice were fed with stan-

dard diet (STD, 18% of total calories derived from fat, 24% from proteins and 58% from carbohydrates. Harlan, Teklad TD.2018). C57bl/6 male mice were separated into 6 groups as follows: control mice fed with STD or HFD, and HFD-fed mice supplemented with 60, 200, 400 and 600 mg/kg/day NAC. The difference in NAC doses given to the two models was based on preliminary experiments (not shown). NAC was administrated daily in the drinking water starting at age of 6 weeks, before the onset of overt diabetes till age of 15 or 17 weeks in KK-Ay and C57BL/6 mice respectively.

GTT, ITT and PCT

Intraperitoneal glucose tolerance test (GTT) was performed at age of 12 weeks in the KK-Ay mice and 15 weeks in HFD-fed mice. Mice were injected with 1.5 mg glucose/g body weight after 6-h fast. Blood glucose was determined from tail blood using the ACCU-CHEK Go glucometer (Roche, Germany).

Insulin tolerance test (ITT) was performed at age of 13 weeks in KK-Ay mice and 16 weeks in HFD-fed mice following 6 h fast. Glucose was measured following intraperitoneal insulin injection (1 U/kg in KK-Ay mice and 0.5 U/kg in C57Bl/6J mice).

Pyruvate challenge test (PCT) was performed at age of 14 weeks in the KK-Ay mice following an overnight fast. 2 mg sodium pyruvate/g body weight was injected, and blood glucose was determined from tail blood.

At age of 15 or 17 weeks in the KK-Ay or C57Bl/6J, respectively, mice were anesthetized using ketamine + xylazine and euthanized by terminal bleeding followed by cervical dislocation. Blood was collected from the heart and serum was prepared. Insulin was measured by immunoassay, using commercial ELISA kit (Mercodia). Liver and soleus muscle were isolated. In order to follow insulin-induced PKB and GSK3β phosphorylation in liver and skeletal muscle, in some of the mice insulin was injected (1 mU/g body weight) 15 min before killing the animal. Liver and muscle were snapped freezed in liquid nitrogen, and preserved in -80°C for later protein and RNA extraction.

Western immunoblot analysis

Protein lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. The samples were homogenized and centrifuged at 14,000 rpm for 20 min. The supernatant was collected and protein concentration was measured using the Bradford method. 20 μg protein per lane was separated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked in 5% dry milk, incubated with the appropriate antibodies and proteins immunodetected using the enhanced chemiluminescence method.

Analysis of mRNA expression by PCR reaction

Total RNA was extracted from liver using TRI reagent according to manufacturers' instruction. 2.5 ng of total RNA were reverse transcribed by oligo-dT priming according to the manufacturers' instructions. Real-time PCR amplification reactions were performed using SYBRGreen Master mix (ROVALAB), by the Mx-Pro QPCR instrument (Stratagene). Primers for real time PCR reactions were synthesized (IDT, Israel).

Primer sequences were as follows: Phosphoenolpyruvate carboxykinase 1 (*Pepck,* Accession NM_011044): forward 5'-agcctttggtcaacaactgg-3', reverse 5'-gttatgcccaggatcagcat-3'. Glucose 6 phosphatase (*G6pase*, Accession NM_008061.3): forward 5'-gattccggtgttgaacgtc-3', reverse 5'-gtagaatccaagcgcgaaac-3'. Glucokinase (*Gck*, Accession NM_001287386): forward 5'-aaagatgttgcccacctacg-3', reverse 5'-accagcatcaccctgaagtt-3'. Hprt was used as housekeeping gene (Accession J00423.1): forward 5'-gttgttggatatgcccttg-3', reverse 5'-aaagcctaagatgagcgca-3'.

Lipid peroxidation analysis

Lipid peroxidation in serum was quantified using the thiobarbituric acid reactive substance (TBARS) assay as described before [26]. OD was measured at 532 nm by a Tecan Infinite F200 microplate reader (Tecan, Salzburg, Austria). Values were calculated as nmol MDA/mg protein according to a calibration curve of 1,1,3,3-tetraethoxypropane.

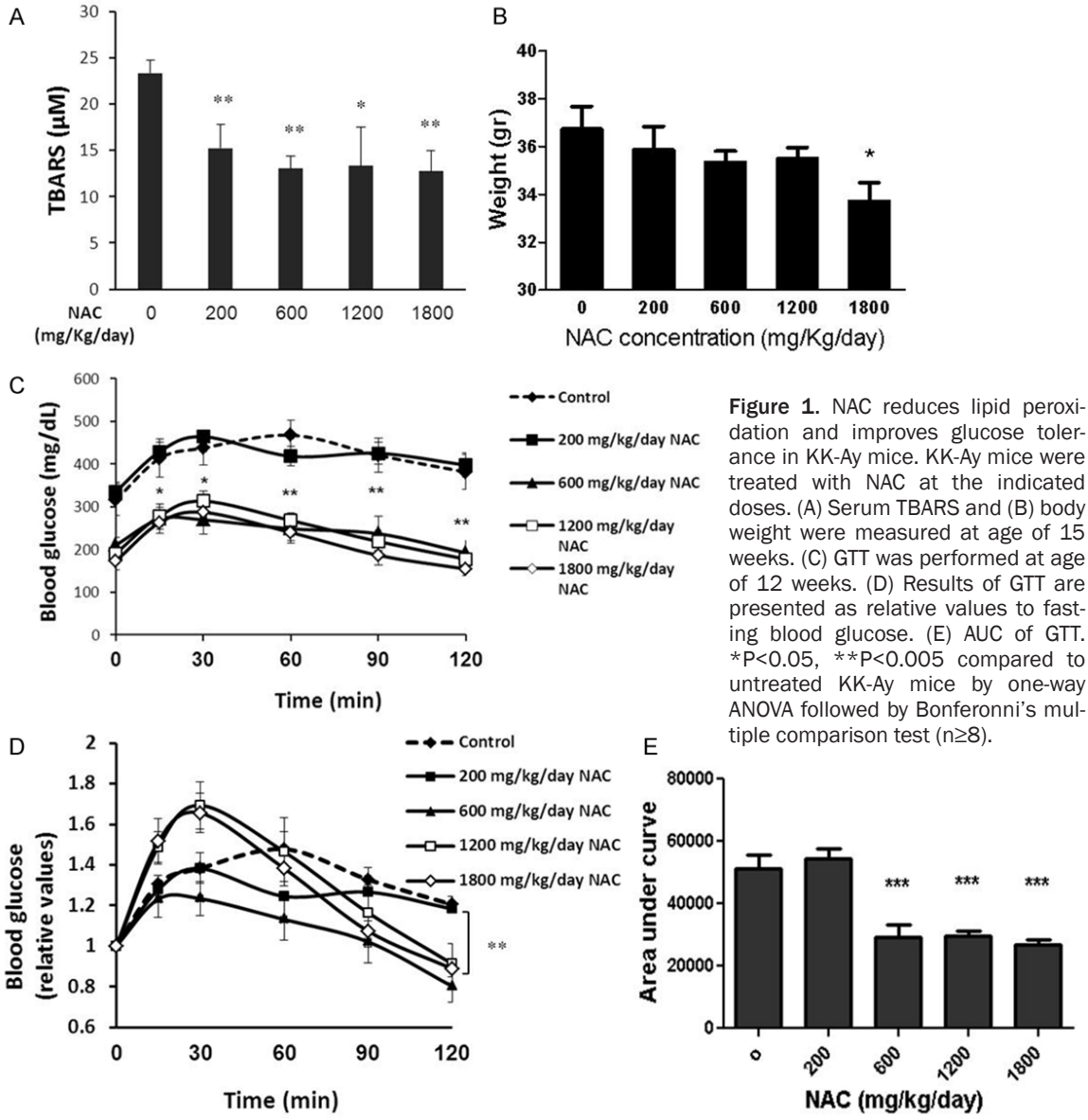

Figure 1. NAC reduces lipid peroxidation and improves glucose tolerance in KK-Ay mice. KK-Ay mice were treated with NAC at the indicated doses. (A) Serum TBARS and (B) body weight were measured at age of 15 weeks. (C) GTT was performed at age of 12 weeks. (D) Results of GTT are presented as relative values to fasting blood glucose. (E) AUC of GTT. *P<0.05, **P<0.005 compared to untreated KK-Ay mice by one-way ANOVA followed by Bonferonni's multiple comparison test (n≥8).

Hepatic triglyceride (TG) content

100 mg of liver was homogenized in 1 ml solution containing 5% NP-40 in water. The sample was twice heated to 80-100°C for 5 min and cooled to room temperature. The sample was centrifuged for 2 min and the supernatant was used for TG analysis using Triglyceride quantification kit (Abcam) according to manufacturer's instruction.

Histochemistry

Livers were isolated, fixed in 4% paraformaldehyde and embedded in paraffin. Consecutive 4 μm sections were cut and were stained with hematoxylin and eosin (H&E). Steatosis score was blinded evaluated by pathologist. Scoring of liver sections examined was done according to Modified Brunt criteria of staging and grading of non-alcoholic fatty liver disease (NAFLD) [30].

Data analysis

Values are presented as means ± SEM. Statistical differences were tested by one-way Anova followed by Bonferonni's multiple comparison test, or unpaired two-tailed Student's *t*-test as appropriate. Analysis was performed

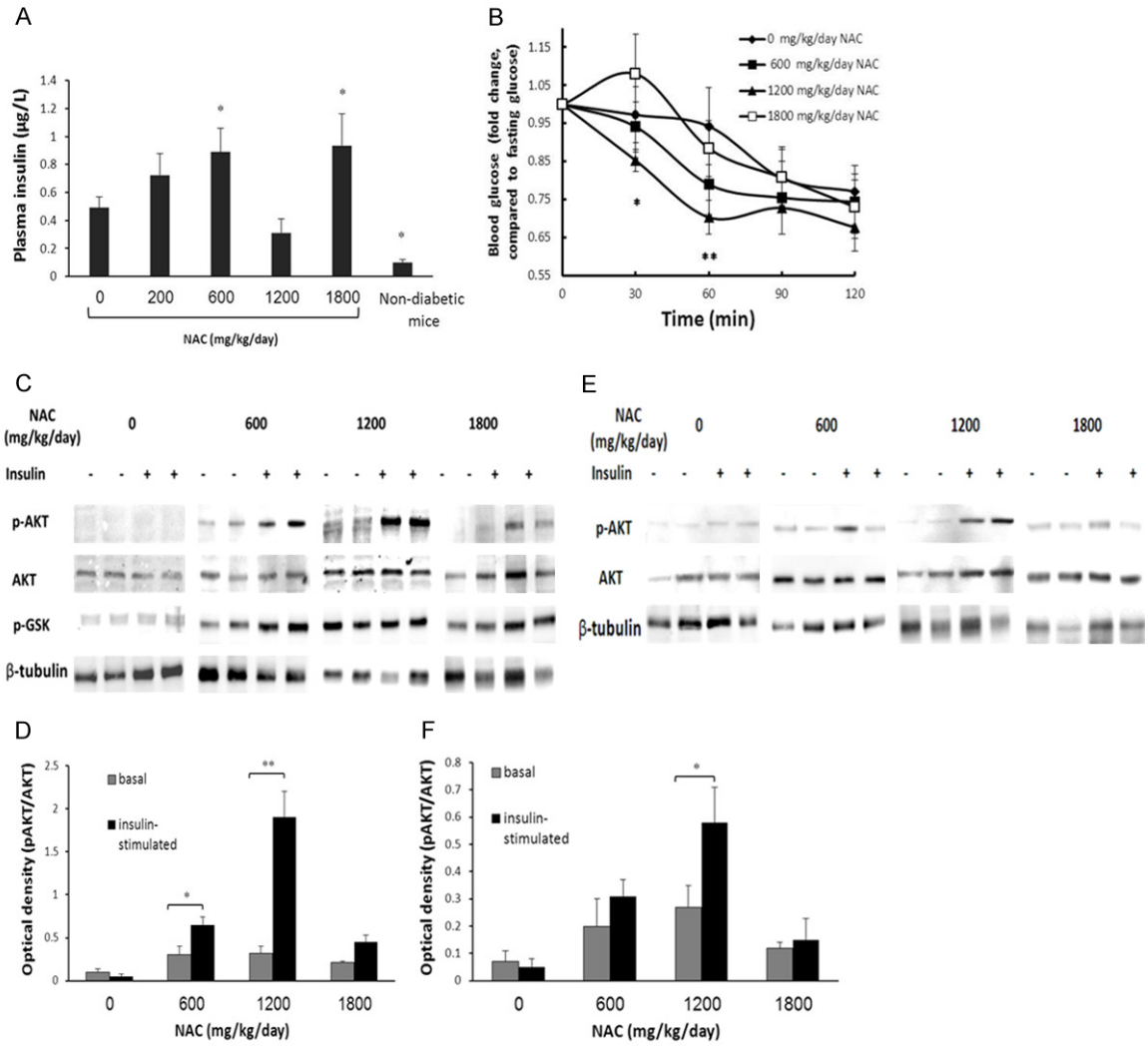

Figure 2. NAC increases insulin secretion and insulin sensitivity in KK-Ay mice at certain specific doses. Mice were treated with NAC at the indicated doses as described in *Methods*. (A) ITT. (B) Fasting serum insulin; results are presented as values relative to fasting blood glucose. *P<0.05, **P<0.01 compared to untreated KK-Ay mice by Student's *t*-test (n≥8). NAC increases PKB phosphorylation in skeletal muscle and liver in KK-Ay mice. Soleus muscles (C, D) and liver (E, F) were removed from mice with or without pre-treatment with insulin, protein extractions were prepared and Western-blot analysis performed. The bar graphs in (D and F) are results of optical density measurements of Western blots in (C and E), respectively.

using the GraphPad Prism 5.0 software. A difference of $P<0.05$ or less in the mean values was considered statistically significant.

Results

Effect of NAC on glucose tolerance in KK-Ay mice

NAC is suggested to support the AOX systems of the organism. In order to confirm the AOX capacity of NAC, serum levels of lipid peroxidation products were measured. All concentrations of NAC used in the study similarly reduced serum lipid peroxidation products (**Figure 1A**).

No signs of toxicity were observed in all treatment groups. Body weight was not affected by 200-1200 mg/kg/day NAC supplementation, while a significant reduction of 9% in body weight was found at dose of 1800 mg/kg/day (**Figure 1B**). Food consumption was not affected (data not shown). This significant, but minor, reduction in body weight indicates for the presence of physiological function of NAC as described before [31], rather than a toxic effect.

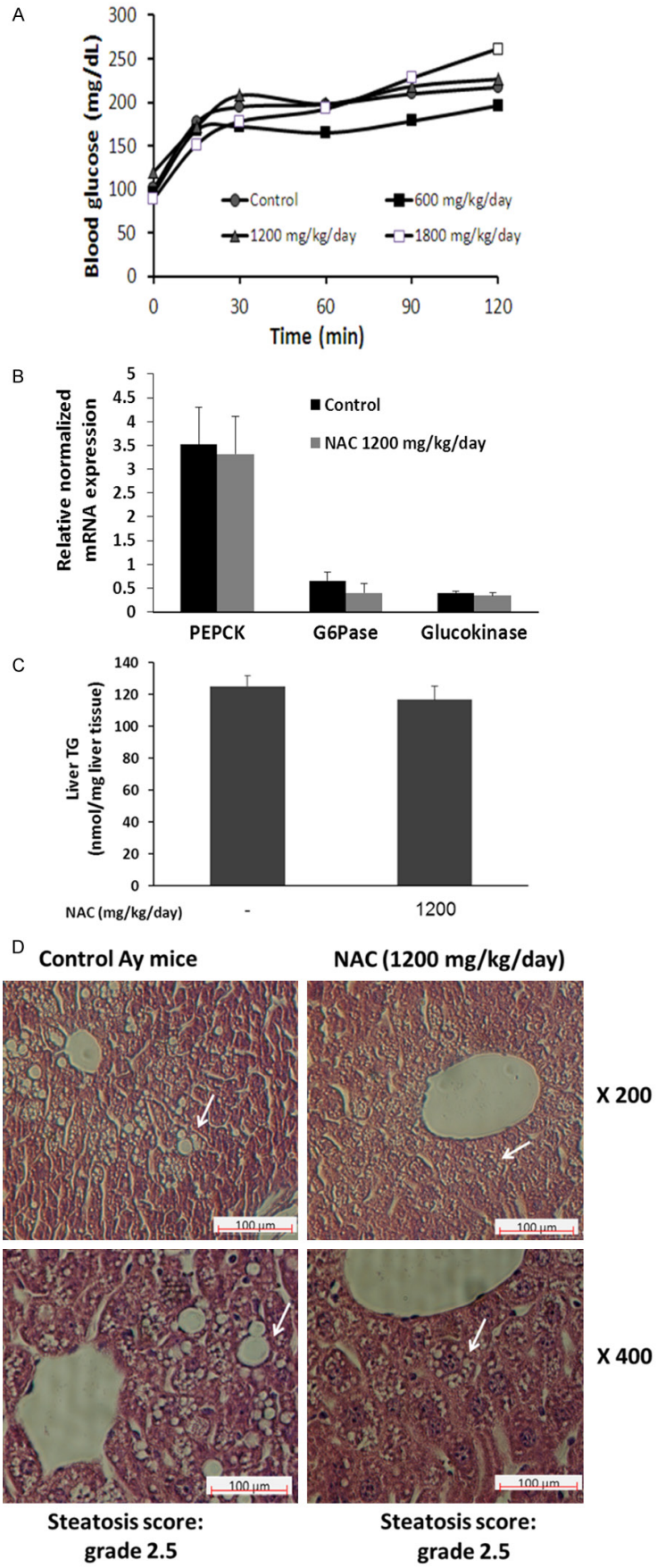

Figure 3. NAC does not affect hepatic function in KK-Ay mice. PCT (A) was performed at age of 15 weeks. Hepatic *G6pase*, *Pepck* and *Gck* mRNA (B) expressions were measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT (n≥6). (C) Hepatic TG was extracted and measured (n≥8). (D) Sections of liver were stained with H&E (n=3), and grade of steatosis was evaluated according to steatosis score; 0: None; 1: Minimal (<10%); 2: Mild (10-33%); 3: Moderate (33-66%); 4: Severe (>66%). Arrows point on representative macro-steatosis (control Ay) and micro-steatosis (NAC-treated).

Although all concentrations of NAC demonstrated elimination of oxidative stress, 600, 1200 and 1800 mg/kg/day-treated mice, except the 200 mg/kg/day treated group, displayed a marked reduction in fasting blood glucose as well as an improved glucose tolerance (**Figure 1C**). **Figure 1D** is a plot of the blood glucose levels relative to the fasting blood levels, and emphasizes that besides the prominent reduction in fasting glucose, glucose disposal rate was improved as well. The improvement in glucose tolerance is illustrated in **Figure 1E** showing the calculated area under curve (AUC). Despite the similar improvements in glucose tolerance found in mice supplemented by either 600, 1200 or 1800 mg/kg/day, insulin tolerance test demonstrated that insulin sensitivity was increased only in mice supplemented with 1200 mg/kg/day NAC (**Figure 2A**). In accord with this finding, fasting insulin was increased in the 600 and 1800 mg/kg/day NAC-supplemented groups, but not in the group of 1200 mg/kg/day NAC (**Figure 2B**). These results suggest that glucose lowering activity of NAC is mediated by improved β-cell

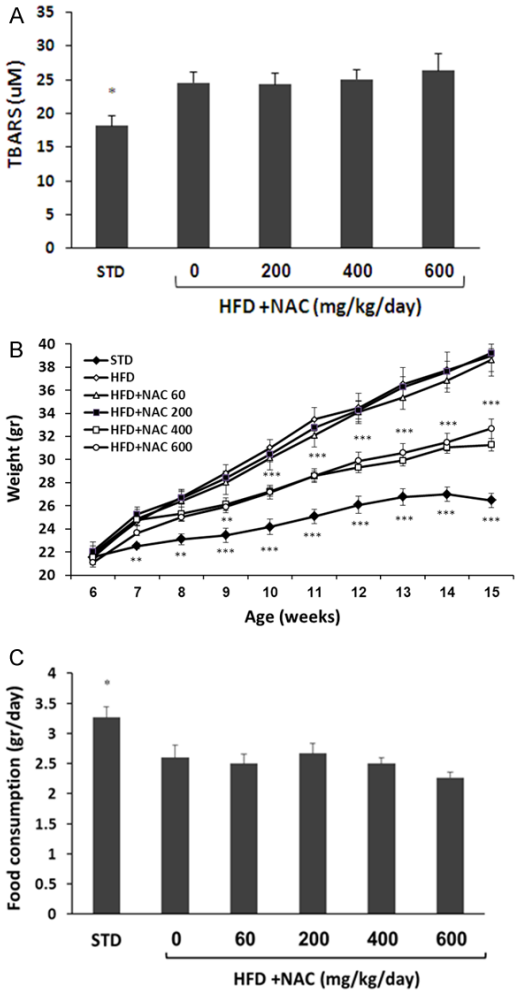

Figure 4. NAC does not affect oxidative stress but reduces body weight in HFD-fed mice. Mice were fed with STD or HFD ± NAC. (A) Serum TBARS were measured at age of 17 weeks. Body weight and average daily food consumption are shown in (B and C), respectively. *P<0.05, **P<0.01, ***P<0.0005 compared to HFD-fed mice by Student's *t*-test (n≥8).

function in relatively wide range of concentration, while the improvement in insulin sensitivity is presented in a narrow range of doses, as presented by the 1200 mg/kg/day dose.

PKB is a key enzyme regulating the transmission of the insulin signal widely used as a biomarker for the activation of insulin signaling. As expected, insulin-induced PKB phosphorylation is completely absent in skeletal muscle of untreated mice KK-Ay mice (**Figure 2C** and **2D**). All concentrations of NAC, found to improve glucose tolerance, increased this phosphorylation, NAC at a concentration of 1200 mg/kg/day being the most effective. Phosphorylation of GSK3β, a PKB target protein, was increased by insulin stimulation in all NAC-treated groups (600-1800 mg/kg/day). Interestingly, both basal and insulin induced GSK phosphorylation was higher in the 1200 mg/kg/day NAC treated mice.

The liver plays a central role in maintaining glucose homeostasis and is one of the major target organs of insulin. We followed PKB phosphorylation in livers of control and NAC treated mice (600-1800 mg/kg/day). Insulin-induced PKB phosphorylation was detected only in the 1200 mg/kg/day NAC treated mice (**Figure 2E** and **2F**); however, GSK3β was not phosphorylated in all groups (data not shown).

Elimination of hyperglycemia may indicate a positive effect of NAC on the regulation of hepatic glucose production. In order to investigate this possibility, PCT was performed. Glucose production was elevated in all groups, without any effect of NAC supplementation (**Figure 3A**). mRNA expression of key gluconeogenic enzymes *G6pase* and *Pepck* was measured and showed no difference in expression level of these genes among groups (**Figure 3B**), in accord with the results of PCT. mRNA expression of *Gck*, which phosphorylates glucose and enhances its uptake into hepatocytes, was not affected as well.

Hepatic steatosis is considered to be the hepatic manifestation of the metabolic syndrome. Despite the improvement in glucose tolerance and insulin sensitivity, NAC did not affect hepatic triglycerides content (**Figure 3C**). H&E staining of liver showed the presence of mild to moderate hepatic steatosis in both control and NAC-treated mice (**Figure 3D**). However histopathological evaluation revealed that although the steatosis score is similar among the groups, NAC-treated mice were characterized by the presence of micro-steatosis, while macro-steatosis was observed in untreated mice.

Effect of NAC on glucose tolerance in HFD-fed mice

In order to further validate the results obtained on KK-Ay mice which develop the disease on a genetic background, the dose-dependent effect of NAC supplementation was also inves-

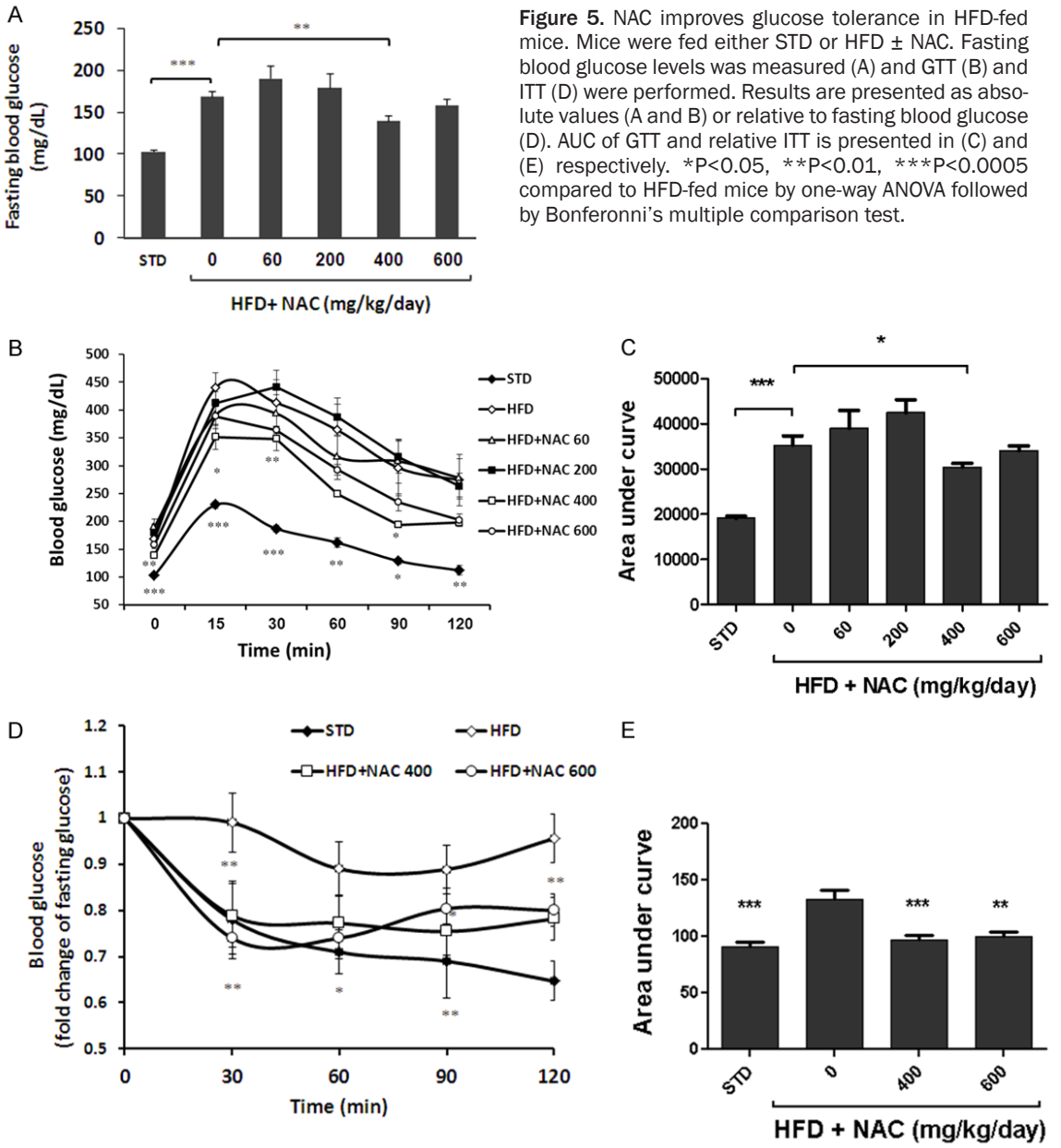

Figure 5. NAC improves glucose tolerance in HFD-fed mice. Mice were fed either STD or HFD ± NAC. Fasting blood glucose levels was measured (A) and GTT (B) and ITT (D) were performed. Results are presented as absolute values (A and B) or relative to fasting blood glucose (D). AUC of GTT and relative ITT is presented in (C) and (E) respectively. *P<0.05, **P<0.01, ***P<0.0005 compared to HFD-fed mice by one-way ANOVA followed by Bonferonni's multiple comparison test.

tigated on the HFD model of glucose intolerance.

Lipid peroxidation products were only moderately elevated in HFD-fed mice compared to STD-fed mice, without any effect of NAC supplementation at any dose (**Figure 4A**). Nonetheless, body weight-gain induced by HFD was significantly and similarly lower in HFD-fed mice supplemented with 400 and 600 mg/kg/day NAC (**Figure 4B**). This was not accompanied by lower food consumption compared to control HFD-fed mice (**Figure 4C**), suggesting that the reduction in body-weight gain was related to elevation in energy expenditure.

HFD-fed mice are glucose intolerant, presenting elevated fasting blood glucose and impaired glucose disposal following GTT. Mice given NAC (400 mg/kg/day) had significantly improved glucose tolerance, as shown by lower fasting glucose and glucose levels following GTT (**Figure 5A-C**). Lower or higher concentrations of NAC were ineffective. As expected, HFD-fed mice are insulin resistant, as shown by ITT (**Figure 5D** and **5E**). 60 and 200 mg/kg/day

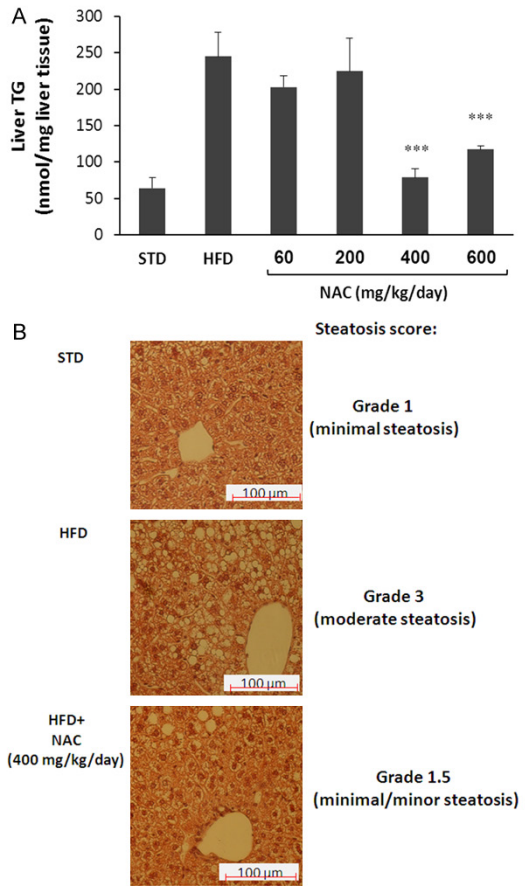

Figure 6. NAC reduces hepatic lipid accumulation in HFD-fed mice. Mice were fed STD or HFD ± NAC. Liver were removed, hepatic TG accumulation was measured (A) and sections were stained with H&E (B). **P<0.001, ***P<0.0005 compared to HFD-fed mice by one-way ANOVA followed by Bonferonni's multiple comparison test.

NAC supplementation did not affect insulin sensitivity (data not shown), while 400 and 600 mg/kg/day NAC significantly improved insulin sensitivity of HFD-fed mice.

Lower grade steatosis was demonstrated in HFD-fed mice supplemented with 400 and 600 mg/kg/day NAC, as indicated by TG measurement in liver extracts (**Figure 6A**). This was also validated by histopathological evaluation of liver of 400 mg/kg/day NAC-supplemented mice; using steatosis score (**Figure 6B**).

Discussion

This study highlights the efficiency of NAC in the management of blood glucose in two different models of glucose intolerance; genetic prone and HFD-induced mice, and the importance of clarifying the optimal concentration of the AOX. Although the antidiabetic properties of NAC were demonstrated before in several in-vivo models [12, 19], this study shows for the first time that the beneficial effects of NAC are highly dose-dependent, suggests an explanation for the failure of clinical trials to show benefits of NAC in balancing blood glucose and emphasizes the need to identify the optimal dose of NAC to be consumed by humans in order to earn the benefits of this agent.

The study shows that NAC significantly reduced the level of hepatic steatosis in the HFD model. These results support previous studies showing that NAC eliminates the severity of NAFLD and the progression of hepatic pathology into advanced stages of liver disease in rodents [32, 33]. Clinical data for the benefits of NAC to reduce hepatic steatosis in human also exist [34], although studies are very limited in number. Presence of microsteatosis rather than macrosteatosis was demonstrated in the genetic model following NAC supplementation. The metabolic consequences of the presence of macrosteatosis vs. microsteatosis had not yet been investigated. However, in relation to liver transplantation, macro-steatosis in the donor was found to be correlated with lower success, while micro-steatosis was comparable to non-steatotic liver with respect to graft survival rates [35], suggesting that the presence of microsteatosis in NAC-treated mice may be a marker of improved hepatic function.

In addition, a reduction in body weight was demonstrated in NAC-treated HFD-fed mice, and in more limited manner, also in NAC-treated KK-Ay mice. Food consumption was not altered, suggesting that the limited increase in body weight was related to elevation in energy expenditure, as was also reported before in sucrose-induced obesity in rats [31]. In KK-Ay mice, NAC dose affecting body weight was higher than the minimal dose found to improve glucose tolerance and hepatic steatosis, indicating that NAC regulates different pathways mediating these various effects.

Aside the potential therapeutic use of NAC, this study emphasizes the complexity of NAC action. The improvement in glucose tolerance observed in KK-Ay mice at different concentrations of NAC, seems to be the result of either enhanced insulin sensitivity or elevated insulin secretion.

While glucose tolerance was improved by 600-1800 mg/kg/day of NAC administration, insulin sensitivity was mostly enhanced by NAC given at concentration of 1200 mg/kg/day. Similarly, the efficacy of NAC supplementation in reducing blood glucose in the HFD-fed mice was found to be optimal at a specific dose, with reduced activity at both lower and higher concentration. It has been suggested previously, based on *in-vitro* experiments [25, 36], that insulin signaling is sensitive to redox balance. Our study reinforces this idea, showing the dose-dependent effect of NAC on insulin signaling *in-vivo* for the first time. In this study, we were able to show that while NAC enhanced insulin sensitivity at one dose, increasing NAC concentration further was less effective.

While insulin sensitivity was improved at very specific NAC concentrations, the wider dose ranges found to improve glucose tolerance in KK-Ay mice, together with elevated insulin secretion in 600 and 1800 mg/kg/day treated mice, suggest that NAC has a beneficial effect on β-cell function. This effect might be mediated via its activity to support the AOX defense system. Pancreatic β-cells have a low AOX capacity, leading to high vulnerability of these cells to develop oxidative stress and its associated damage [4]. We suggest that NAC supports the AOX system in β-cells, enabling neutralization of free radicals. By reducing oxidative stress, NAC might improve insulin secretion in order to accommodate to the peripheral insulin resistance.

In both models utilized in this study, lipid peroxidation level, although widely used as a marker of oxidative stress [37], did not correlate with the improvement in glucose tolerance. The failure of NAC given at low concentration to improve glucose tolerance in KK-Ay mice, despite reducing serum lipid peroxidation, emphasizes the complexity of NAC action. There are several suggested mechanisms for the AOX functions of NAC; in addition to being a precursor of glutathione production, there are evidence supporting the presence of a direct AOX activity of NAC, being more potent for neutralization of some radicals than others [38]. Most probably, NAC, a thiol-containing compound is able to directly reduce disulfides or to interfere in the formation of these bonds [38, 39], affecting protein structure, activity and ligand binding [40, 41]. Thus, in addition to being AOX, NAC might be involved in thiol post-translation modifications and can regulate protein function. The lack of correlation between the activity of NAC as an oxidative-stress neutralizing agent and its beneficial effects on glucose tolerance may indicate that at least part of NAC effects are exerted by redox regulation of signal transduction cascades. The effects of NAC on the activity and thiol modifications of key molecules involved in the transmission of insulin signaling are currently under investigation.

The difference in NAC doses improving glucose tolerance and insulin sensitivity between the two mice models supports the need to develop a strategy of "personalized supplementation", in which the dosage is matched to the patient needs. Accurate biomarkers should be identified in order to monitor the oxidative stress of the patient, the need for AOX supplementation, the dosage, and the efficacy of the pharmacological intervention.

NAC is used in the clinic mainly as a mucolytic agent [42] and for the replenishment of glutathione in paracetamol toxicity [38]. However clinical trials have shown that NAC is also effective for the treatment of several other conditions and to be a well-tolerated agent, with only minor side effects, detected mainly at doses >3 gr/day [43]. With regard to diabetes, clinical trials using NAC at a maximal dose of 1.2 g/day, demonstrate some efficacy in lowering diabetes-related complications such as high blood pressure and platelets-monocytes conjugation [44, 45]. Beneficial effects of NAC in blood glucose regulation have scarcely been reported, mainly in relation to PCOS, using NAC at dose of 1.8-3 gr/day [46]. In our *in-vivo* study, the minimal dose found to be effective was 400 mg/kg/day in the HFD-fed mice, and 600 mg/kg/day in KK-Ay mice. Using metabolic conversion factor, the dose for humans should be around 32-50 mg/kg/day, or 2.2-3.5 g/day for 70 kg person. We suggest that the limited evidence for the efficacy of NAC to improve glycemic control results from the use of NAC at suboptimal concentrations which are ineffective in regulation of blood glucose, despite reducing levels of oxidized molecules in circulation. On the other hand, the study suggests that NAC should not be consumed at higher doses than required, as it might be less effective in improving insulin sensitivity.

Conclusion

We suggest that the limited evidence for the efficacy of NAC to improve glycemic control results from the use of NAC at sub-optimal concentrations which are ineffective in regulation of blood glucose. As the benefits of NAC are not correlated with marker of oxidative stress, the mechanism of action of NAC should be investigated further. Hence, the lack of clear recommendations for NAC intake leads to inadequate consumption and failure of the intervention. The dose-effect relationship of other AOXs should be investigated in the future as well, in order to optimize the use of AOXs dietary supplements for the treatment of diabetes.

Disclosure of conflict of interest

None.

Abbreviations

AOX, Antioxidant; GTT, glucose tolerance test; HFD, high fat diet; ITT, insulin tolerance test; NAC, N-Acetyl-L-Cysteine; NAFLD, non-alcoholic fatty liver disease; PCT, pyruvate challenge; test; ROS, reactive oxygen species; STD, standard diet; T2D, type 2 diabetes.

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