

Reviewer's code: 03465463

SPECIFIC COMMENTS TO AUTHORS

The present study investigated the effects of allyl isothiocyanate (AITC) on lipid accumulation and inflammation during nonalcoholic fatty liver disease (NAFLD) development using the mice fed a high fat diet (HFD) and the AML-12 cells treated with palmitate acid (PA). I like to give the following comments. 1. In the introduction, previous report(s) mentioned the mediation of signals such as Sirt1 and AMPK in NAFLD would be more helpful. 2. Link of AITC with NAFLD seems not enough. Please add more in the introduction section. Additionally, chemical structure of AITC was not indicated. 3. Daily intake of AITC at 100 mg/kg needs the reference(s) to support. Additionally, purity of AITC is also required. 4. Hepatic and cellular TG contents were unclear, particularly for TG. 5. In Figure 1, one group shown normal control is required. Figure 1B means AUC of body weight or what? Additionally, total cholesterol in blood not modified by AITC but is it higher than normal control? Similar concerns are extended to another indicator including AST, ALT and uremic acid. 6. Pro-fibrotic signals were not investigated in mice. Why? How to know the presence of NAFLD? 7. In the conclusion, it seems better to show as: therapeutic agent for "the progress of" NAFLD in the last sentence. 8. Effective dose of AITC for clinical practice seems helpful even it was perspective. Additionally, limitation(s) of this report could be included.

Replies to Reviewer 03465463:

Comment 1: The present study investigated the effects of allyl isothiocyanate (AITC) on lipid accumulation and inflammation during nonalcoholic fatty liver disease (NAFLD) development using the mice fed a high fat diet (HFD) and the AML-12 cells treated with palmitate acid (PA). I like to give the following comments.

Reply: Thank you very much for your valuable comments and suggestions to improve our manuscript! The comments and suggestions are replied point-by-point below.

Comment 2: In the introduction, previous report(s) mentioned the mediation of signals such as Sirt1 and AMPK in NAFLD would be more helpful.

Reply: Thank you very much for your valuable comments!

We have added and made short introduction of relevant reports mentioned the mediation of signals such as Sirt1 and AMPK in NAFLD in the revised manuscript on Page 6.

Page 6, line 24-30: Previous studies revealed that Sirt1 is an important regulator of hepatic lipogenesis and fatty acid oxidation through multiple nutrient sensors, including sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor gamma coactivator1 α (PGC1 α) and peroxisome proliferator-activated receptor α (PPAR α)^[14-16]. Furthermore, Sirt1 was shown to regulate AMPK activation in NAFLD, resulting in enhanced lipolysis and β -oxidation as well as ameliorated hepatic steatosis^[17-19].

Comment 3: Link of AITC with NAFLD seems not enough. Please add more in the introduction section.

Reply: Thank you very much for this suggestion!

As reports about the link of AITC with NAFLD are limited, we mentioned some reports in the introduction section to show the relationship between AITC, NAFLD and lipid metabolism in the revised version on Page 7.

Page 7, line 37-41: Another study revealed that AITC can augment basal and epinephrine-induced lipolysis in adipocytes and intensify hydrolysis of TG in the blood serum of rats^[26]. Moreover, a previous study showed AITC effectively inhibits adipogenic

differentiation of 3T3-L1 preadipocytes and suppresses expression of genes up-regulated during adipogenesis^[27].

Comment 4: Additionally, chemical structure of AITC was not indicated.

Reply: We apologize for the confusion!

We have supplemented the essential information in results section of the revised version on Page 10.

Page 10, line 137: Fig. S1A shows chemical structure of AITC.

Comment 5: Daily intake of AITC at 100 mg/kg needs the reference(s) to support.

Reply: Thanks very much for the comments!

We did a pilot experiment before. We tested effects of 50 mg/kg, 100 mg/kg and 150 mg/kg AITC *in vivo* and found that daily intake of AITC at 150 mg/kg resulted in HFD-fed mice death seriously but 50 mg/kg and 100 mg/kg AITC had no effect on survival. What's more, compared to 50 mg/kg AITC, 100 mg/kg AITC could decrease the body weight, increase and reduce lipid accumulation of HFD-fed mice effectively. We didn't observe obvious toxic effects as the activity ability showed no difference between the control and 100 mg/kg AITC groups, and that 100 mg/kg AITC markedly decreased serum ALT and AST levels of the HFD-fed mice at the same time, so we chose 100 mg/kg as our final AITC concentration

Comment 6: Additionally, purity of AITC is also required.

Reply: We apologize for the confusion!

We have added information about the purity of AITC in the revised article on Page 7.

Page 7, line 58-59: AITC (Sigma-Aldrich, St. Louis, MO, USA), whose purity is 99.7%.

Comment 7: Hepatic and cellular TG contents were unclear, particularly for TG.

Reply: Thank you very much for this comments!

Results of hepatic and cellular TG contents were shown in Fig.1E and Fig.4C and indicated that 8-week HFD feeding increased TG level in the liver of mice, and AITC treatment significantly downregulated TG contents. We also verified that AITC could decrease TG level in PA-stimulated AML-12 cells in Fig.4C.

Fig.1E: The results of TG contents in standard chow diet (SCD)-fed control mice, HFD-fed control mice and HFD-fed mice with AITC treatment were presented.

Fig.4C: The results of TG contents in vehicle group, AITC group, PA group AND PA + AITC group were presented.

Comment 8: In Figure 1, one group shown normal control is required.

Reply: Thanks very much for the comments!

We have added related information about SCD-fed mice (normal control) into Fig.1.

Fig.1: The results of body weight change, liver weight, H&E, Oil Red O, liver photograph, TG contents, ALT, AST, cholesterol and uric acid levels of SCD-fed mice were presented.

Comment 9: Figure 1B means AUC of body weight or what?

Reply: We apologize for the confusion!

Fig.1B means the body weight of each group at week 8. We have supplemented this explanation in the labeling of the y-coordinate of Fig.1B and the corresponding figure legends.

Comment 10: Additionally, total cholesterol in blood not modified by AITC but is it higher than normal control? Similar concerns are extended to another indicator including AST, ALT and uremic acid.

Reply: Thanks very much for your comments!

We found that mice fed with HFD exhibited significantly higher ALT, AST and cholesterol levels in the serum compared to SCD-fed mice, and the level of uric acid in HFD-fed mice was slightly increased. We have added related information into Fig.1.

Fig.1F-I: The results of ALT, AST, cholesterol and uric acid levels of SCD-fed mice, HFD-fed mice and HFD-fed mice with AITC treatment were presented.

Comment 11: Pro-fibrotic signals were not investigated in mice. Why?

Reply: Thank you very much for the comments!

Herein, in this study, we mainly focused on lipid metabolism regulation and inflammation modulation role of AITC in NAFLD, and the influence of AITC on fibrosis were not involved. But this gives us insight to further explore the role of AITC in fibrosis and detect related pro-fibrotic signals in mice in the follow-up experiments.

Comment 12: How to know the presence of NAFLD?

Reply: Thanks very much for your comments!

Body weight and liver weight change, lipid accumulation in H&E and Oil Red O, hepatic TG levels as well as ALT, AST, cholesterol and uric acid variation could indicate the presence of NAFLD together, so we added this illustration in the revised manuscript on Page 10.

Page 10, line 139-142: HFD-fed mice exhibited higher body weight (Fig.1A-B) and liver weight (Fig.1D), more serious lipid accumulation (Fig.1C), much higher TG (Fig.1E),

ALT (Fig.1F), AST (Fig.1G), cholesteryl (Fig.1H) and uric acid (Fig.1I) levels compared to SCD-fed mice, showing characteristics of NAFLD *in vivo*.

Comment 13: In the conclusion, it seems better to show as: therapeutic agent for “the progress of” NAFLD in the last sentence.

Reply: Thanks very much for your comments!

We have added “the progress of “to the last sentence of the conclusion part on Page 15.

Page 15, line 306-307: This study indicates that AITC may be a potential therapeutic agent for the progress of NAFLD.

Comment 14: Effective dose of AITC for clinical practice seems helpful even it was perspective.

Reply: Thank you very much for the comments!

This study was a preliminary report about therapeutic value of AITC in NAFLD, sure, further experiments will extend to clinical circumstances, including therapeutic window exploration.

Comment 15: Additionally, limitation(s) of this report could be included.

Reply: Thanks very much for your comments!

We have supplemented some limitations of this report in the new version on Page 14 and 15.

Page 14, line 269-273: Furthermore, another study showed that sulforaphane-induced *Sirt1* activation inhibits endoplasmic reticulum (ER) stress and prevents cardiomyocytes from hypoxia/reoxygenation injury *in vitro*^[36]. As we have detected activation of *Sirt1* after AITC applyment, further studies should be carried out to explore mechanisms about ER stress in this model.

Page 15, line 290-303: Although several isothiocyanates have been proposed as chemopreventive agents for cancers, AITC has been reported to exhibit both carcinogenic and anticarcinogenic potential. A previous study demonstrated that AITC has abilities to cause Cu(II)-mediated DNA damage and induce 8-oxo-7,8-dihydro-29-deoxyguanosine (8-oxodG) formation, leading to carcinogenesis in human myelogenous leukemic cell lines^[40]. Moreover, impaired copper availability in obesity-related NAFLD was shown to predict early atherosclerosis as main cardiovascular risk^[41]. On the other hand, another study demonstrated that AITC could inhibit proliferation of human prostate cancer cells through inducing G2/M arrest and apoptosis^[22]. Our studies mentioned above have proposed many different mechanisms of AITC-induced amelioration of hepatic steatosis and inflammation in *in vivo* and *in vitro* model of NAFLD, none have explored whether AITC took part in Cu(II)-mediated DNA damage or the development of cancer. Further studies are then needed to clarify whether AITC induces oxidative DNA damage in our model and investigate the mechanism of AITC on cancer.

Reviewer's code: 03478516

SPECIFIC COMMENTS TO AUTHORS

Authors should comment on these specific points to give readers a more comprehensive view of the topic. Several isothiocyanates have been proposed as promising chemopreventive agents for human cancers. However, it has been reported that allyl isothiocyanate exhibit carcinogenic potential. Wauthors investigated whether these isothiocyanates could cause DNA damage, using (32)P-labeled DNA fragments obtained from the human p53 tumor suppressor gene and the c-Ha-ras-1 protooncogene. Allyl isothiocyanate caused Cu(II)-mediated DNA damage and formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) more strongly than benzyl and phenethyl isothiocyanates. Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited Cu(II)-mediated DNA damage by these isothiocyanates, suggesting involvement of H₂O₂ and Cu(I)...as evident in..Free Radic Biol Med. 2000 Mar 1;28(5):797-805. Mechanism of oxidative DNA damage induced by carcinogenic allyl isothiocyanate. This aspect is of main importance in the light of the impaired copper availability in obesity-related NAFLD and its comorbidity, i.e., atherosclerosis as presented in...Prediction of carotid intima-media thickness in obese patients with low prevalence of comorbidities by serum copper bioavailability. J Gastroenterol Hepatol. 2018 Aug;33(8):1511-1517. What about the process of activating SIRT1 and subsequently inhibiting ER stress, as studied in...Send to Acta Pharmacol Sin. 2016 Mar;37(3):344-53. Sulforaphane prevents rat cardiomyocytes from hypoxia/reoxygenation injury in vitro via activating SIRT1 and subsequently inhibiting ER stress. Authors should clearly state that this animal model of NAFLD does not completely mirror the human one, although quite similar.

Replies to Reviewer 03478516:

Comment 1: Authors should comment on these specific points to give readers a more comprehensive view of the topic.

Reply: Thank you very much for your valuable comments and suggestions to improve our manuscript! The comments and suggestions are replied point-by-point below.

Comment 2: Several isothiocyanates have been proposed as promising chemopreventive agents for human cancers. However, it has been reported that allyl isothiocyanate exhibit carcinogenic potential. Wauthors investigated whether these isothiocyanates could cause DNA damage, using (32)P-labeled DNA fragments obtained from the human p53 tumor suppressor gene and the c-Ha-ras-1 protooncogene. Allyl isothiocyanate caused Cu(II)-mediated DNA damage and formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) more strongly than benzyl and phenethyl isothiocyanates. Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited Cu(II)-mediated DNA damage by these isothiocyanates, suggesting involvement of H₂O₂ and Cu(I)...as evident in..Free Radic Biol Med. 2000 Mar 1;28(5):797-805. Mechanism of oxidative DNA damage induced by carcinogenic allyl isothiocyanate. This aspect is of main importance in the light of the impaired copper availability in obesity-related NAFLD and its comorbidity, i.e., atherosclerosis as presented in...Prediction of carotid intima-media thickness in obese patients with low prevalence of comorbidities by serum copper bioavailability. J Gastroenterol Hepatol. 2018 Aug;33(8):1511-1517.

Reply: Thanks very much for your comments!

As it has been reported that AITC could cause DNA damages, and oxidative DNA damages in obesity-related NAFLD was shown to predict early atherosclerosis as main cardiovascular risk, the mechanism of oxidative DNA damages is of main importance for our study. Meanwhile, some reports revealed that AITC reflects anti-inflammatory and anticancer activities. We discussed this issue and raised our limitations in the revised manuscript on Page 15.

Page 15, line 290-303: Although several isothiocyanates have been proposed as chemopreventive agents for cancers, AITC has been reported to exhibit both carcinogenic and anticarcinogenic potential. A previous study demonstrated that AITC has abilities to cause Cu(II)-mediated DNA damage and induce 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formation, leading to carcinogenesis in human myelogenous leukemic cell lines^[40]. Moreover, impaired copper availability in obesity-related NAFLD was shown to predict early atherosclerosis as main cardiovascular risk^[41]. On the other hand, another study demonstrated that AITC could inhibit proliferation of human prostate cancer cells through inducing G2/M arrest and apoptosis^[22]. Our studies mentioned above have proposed many different mechanisms of AITC-induced amelioration of hepatic steatosis and inflammation in *in vivo* and *in vitro* model of NAFLD, none have explored whether AITC took part in Cu(II)-mediated DNA damage or the development of cancer. Further studies are then needed to clarify whether AITC induces oxidative DNA damage in our model and investigate the mechanism of AITC on cancer.

Comment 3: What about the process of activating SIRT1 and subsequently inhibiting ER stress, as studied in...Send to Acta Pharmacol Sin. 2016 Mar;37(3):344-53. Sulforaphane prevents rat cardiomyocytes from hypoxia/reoxygenation injury in vitro via activating SIRT1 and subsequently inhibiting ER stress.

Reply: Thank you very much for the comments!

Actually we didn't detect ER stress in the study, and we have added this aspect as our limitations in the revised version on Page 14.

Page 14, line 269-273: Furthermore, another study showed that sulforaphane-induced *Sirt1* activation inhibits endoplasmic reticulum (ER) stress and prevents cardiomyocytes from hypoxia/reoxygenation injury *in vitro*^[36]. As we have detected activation of *Sirt1*

after AITC aplyment, further studies should be carried out to explore mechanisms about ER stress in this model.

Comment 4: Authors should clearly state that this animal model of NAFLD does not completely mirror the human one, although quite similar.

Reply: Thank you very much for the comments!

We added relative statement in the revised version on Page 10.

Page 10, line 137-139: To explore the effect of AITC on NAFLD, HFD model, which is quite similar to but does not completely mirror the human NAFLD was adopted.

We have also answered the other two reviewers' reports obtained from email.

Reviewer #1: Title: Allyl isothiocyanate ameliorates lipid accumulation and inflammation in nonalcoholic fatty liver disease via the Sirt1/ AMPK and NF-κB signaling pathways
Authors aimed to investigate the effect allyl isothiocyanate in a nonalcoholic fatty liver disease mouse model and in vitro. The concluded that allyl isothiocyanate decreased liver inflammation and steatosis by activating the Sirt1/AMPK and inhibiting the NF-κB pathways and propose allyl isothiocyanate as a potential therapeutic agent for NAFLD. This study is very interestingly with a degree of novelty and it seems that the results support their conclusions and that the methodology is robust. Unfortunately, they present only two groups in their results (diet and diet plus allyl isothiocyanate) and performed a student's two-tailed t-test. Authors need to show all the groups (including a vehicle, normal control and control of the allyl isothiocyanate) and then to perform an ANOVA with Turkey's test to see if the difference reach a significant value, otherwise their results lack relevance. I will be willing to re-review a new version.

Replies to Reviewer #1:

Comment 1: Authors aimed to investigate the effect allyl isothiocyanate in a nonalcoholic fatty liver disease mouse model and in vitro. The concluded that allyl isothiocyanate decreased liver inflammation and steatosis by activating the Sirt1/AMPK and inhibiting the NF- κ B pathways and propose allyl isothiocyanate as a potential therapeutic agent for NAFLD. This study is very interestingly with a degree of novelty and it seems that the results support their conclusions and that the methodology is robust.

Reply: Thank you very much for your valuable comments and suggestions to improve our manuscript! The comments and suggestions are replied point-by-point below.

Comment 2: Unfortunately, they present only two groups in their results (diet and diet plus allyl isothiocyanate) and performed a student's two-tailed t-test. Authors need to show all the groups (including a vehicle, normal control and control of the allyl isothiocyanate) and then to perform an ANOVA with Turkey's test to see if the difference reach a significant value, otherwise their results lack relevance. I will be willing to re-review a new version.

Reply: Thanks very much for your comments!

We added related data of standard chow diet (SCD) (normal control) group into our revised article and performed an ANOVA with Tukey's test to analyse the difference. What's more, we are willing to supply the AITC control group in our further study.

Page 10, line 137-142: To explore the effect of AITC on NAFLD, HFD model, which is quite similar to but does not completely mirror the human NAFLD was adopted. HFD-fed mice exhibited higher body weight (Fig.1A-B) and liver weight (Fig.1D), more serious lipid accumulation (Fig.1C), much higher TG (Fig.1E), ALT (Fig.1F), AST (Fig.1G), cholerestorol (Fig.1H) and uric acid (Fig.1I) levels compared to SCD-fed mice, showing characteristics of NAFLD in vivo.

Fig1: The results of body weight change, liver weight, H&E, Oil Red O, liver photograph, TG contents, ALT, AST, cholesterol and uric acid levels of SCD-fed mice were presented.

Reviewer #2: Dear Editor, I reviewed the manuscript titled “Allyl isothiocyanate ameliorates lipid accumulation and inflammation in nonalcoholic fatty liver disease via the Sirt1/AMPK and NF-κB signaling pathways”. This is very interesting manuscript. I think it can be accepted after these revisions. My comments are listed below: 1. How did The Authors choose the daily dosage of AITC (100 mg/kg)? 2. Some general informations about the methods and results of the manuscript should be moved from results to methods or discussion sections: such as: “Chronic inflammation characterized by increased proinflammatory cytokine levels and the activation of principal inflammatory pathways is closely associated with the development of NAFLD[28]. The IKK/NF-κB pathway can be activated by HFD challenge in vivo and by palmitate treatment in vitro, thus playing a crucial role in the development of metabolic disorders, including NAFLD[8, 9]. In response to numerous inflammatory stimuli, IKK complex activation induces IκB phosphorylation and subsequent degradation, which releases NF-κB and allows it to translocate into the nucleus[29].”

Replies to Reviewer #2:

Comment 1: This is very interesting manuscript. I think it can be accepted after these revisions. My comments are listed below.

Reply: Thank you very much for your valuable comments and suggestions to improve our manuscript! The comments and suggestions are replied point-by-point below.

Comment 2: How did The Authors choose the daily dosage of AITC (100 mg/kg)?

Reply: Thank you very much for the comments!

Actually, we did a pilot experiment before. We tested effects of 50 mg/kg, 100 mg/kg and 150 mg/kg AITC *in vivo* and found that daily intake of AITC at 150 mg/kg resulted

in HFD-fed mice death seriously but 50 mg/kg and 100 mg/kg AITC had no effect on survival. What's more, compared to 50 mg/kg AITC, 100 mg/kg AITC could decrease the body weight and reduce lipid accumulation of HFD-fed mice effectively. We didn't observe obvious toxic effects as the activity ability showed no difference between the control and 100 mg/kg AITC groups, and that 100 mg/kg AITC markedly decreased serum ALT and AST levels of the HFD-fed mice at the same time, so we chose 100 mg/kg as our final AITC concentration

Comment 3: Some general informations about the methods and results of the manuscript should be moved from results to methods or discussion sections: such as: "Chronic inflammation characterized by increased proinflammatory cytokine levels and the activation of principal inflammatory pathways is closely associated with the development of NAFLD[28]. The IKK/NF- κ B pathway can be activated by HFD challenge in vivo and by palmitate treatment in vitro, thus playing a crucial role in the development of metabolic disorders, including NAFLD[8, 9]. In response to numerous inflammatory stimuli, IKK complex activation induces I κ B phosphorylation and subsequent degradation, which releases NF- κ B and allows it to translocate into the nucleus[29]."

Reply: Thanks very much for your comments!

We have moved this part to the discussion section in the revised version on Page 14 and 15.

Page 14-15, line 274-281: Chronic inflammation is characterized by the abnormal production of proinflammatory cytokines, including TNF α , IL-1 β and IL-6, and the activation of inflammatory signaling pathways, such as the NF- κ B and JNK pathways;

moreover, it has been proposed to play a crucial role in the pathogenesis of NAFLD^[7, 37]. A number of recent studies have demonstrated a key role for the *IKK/NF-κB* signaling pathway in NAFLD development^[8, 9]. In response to numerous inflammatory stimuli, *IKK* complex activation induces *IκB* phosphorylation and subsequent degradation, which releases *NF-κB* and allows it to translocate into the nucleus^[38].

All of the revisions that I have made to the revised manuscript are cited below:

1. We added "Manuscript Number";
2. We added postcode and "Province";
3. We rewrote "Author contributions";
4. We rewrote the part of "Supported by";
5. We added email address to the corresponding author's information;
6. We changed some words in "Abstract" and "Core tip" parts;
7. We added and verified some content according to the reviewers' comments:

Page 6, line 24-30: "Previous studies revealed that *Sirt1* is an important regulator of hepatic lipogenesis and fatty acid oxidation through multiple nutrient sensors, including sterol regulatory element-binding protein 1 (*SREBP1*), peroxisome proliferator-activated receptor gamma coactivator1α (*PGC1α*) and peroxisome proliferator-activated receptor α (*PPARα*)^[14-16]. Furthermore, *Sirt1* was shown to regulate *AMPK* activation in NAFLD, resulting in enhanced lipolysis and β-oxidation as well as ameliorated hepatic steatosis^[17-19]."

Page 7, line 37-41: "Another study revealed that AITC can augment basal and epinephrine-induced lipolysis in adipocytes and intensify hydrolysis of TG in the blood serum of rats^[26]. Moreover, a previous study showed AITC effectively inhibits

adipogenic differentiation of 3T3-L1 preadipocytes and suppresses expression of genes up-regulated during adipogenesis^[27].”

Page 7, line 55-60: “In general, mice were given SCD or HFD feeding for total 8 weeks, and from the fifth week, SCD-fed mice began to receive corn oil (control) (n = 10), and HFD-fed mice were randomly divided into two groups to receive 100 mg/kg/d AITC (Sigma-Aldrich, St. Louis, MO, USA), whose purity is 99.7%, (n = 10) or corn oil (n = 9) daily by gavage for an additional 4 weeks while remaining on the SCD or HFD.”

Page 10, line 130-132: “Statistical analysis was carried out using Student’s two-tailed t-test and one-way ANOVA with Tukey’s post-test.”

Page 10, line 137-142: “Fig. S1A shows chemical structure of AITC. To explore the effect of AITC on NAFLD, HFD model, which is quite similar to but does not completely mirror the human NAFLD was adopted. HFD-fed mice exhibited higher body weight (Fig.1A-B) and liver weight (Fig.1D), more serious lipid accumulation (Fig.1C), much higher TG (Fig.1E), ALT (Fig.1F), AST (Fig.1G), cholestrol (Fig.1H) and uric acid (Fig.1I) levels compared to SCD-fed mice, showing characteristics of NAFLD *in vivo*.”

Page 14, line 269-273: “Furthermore, another study showed that sulforaphane-induced *Sirt1* activation inhibits endoplasmic reticulum (ER) stress and prevents cardiomyocytes from hypoxia/reoxygenation injury *in vitro*^[36]. As we have detected activation of *Sirt1* after AITC aplyment, further studies should be carried out to explore mechanisms about ER stress in this model.”

Page 15, line 279-281: “In response to numerous inflammatory stimuli, *IKK* complex activation induces *IκB* phosphorylation and subsequent degradation, which releases *NF-κB* and allows it to translocate into the nucleus^[38].”

Page 15, line 290-303: “Although several isothiocyanates have been proposed as chemopreventive agents for cancers, AITC has been reported to exhibit both carcinogenic and anticarcinogenic potential. A previous study demonstrated that AITC has abilities to cause Cu(II)-mediated DNA damage and induce 8-oxo-7,8-dihydro-29-deoxyguanosine (8-oxodG) formation, leading to carcinogenesis in human myelogenous leukemic cell lines^[40]. Moreover, impaired copper availability in obesity-related NAFLD was shown to predict early atherosclerosis as main cardiovascular risk^[41]. On the other hand, another study demonstrated that AITC could inhibit proliferation of human prostate cancer cells through inducing G2/M arrest and apoptosis^[22]. Our studies mentioned above have proposed many different mechanisms of AITC-induced amelioration of hepatic steatosis and inflammation in *in vivo* and *in vitro* model of NAFLD, none have explored whether AITC took part in Cu(II)-mediated DNA damage or the development of cancer. Further studies are then needed to clarify whether AITC induces oxidative DNA damage in our model and investigate the mechanism of AITC on cancer.”

Page 15, line 306-307: This study indicates that AITC may be a potential therapeutic agent for the progress of NAFLD.

8. We added content in “Article Highlights”:

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is an unmet medical need with no approved

therapies. Latest studies show that allyl isothiocyanate (AITC) has a potential protective effect on obesity and insulin resistance. The evaluation of the effect of AITC on NAFLD as well as the mechanism of action may provide a new therapeutic trend.

Research motivation

Emerging evidence suggests a beneficial role for AITC in inflammation, cancer, diet-induced obesity and insulin resistance. Enhanced lipolysis in adipocytes and intensified hydrolysis of triglyceride in the serum of rats treated with MS-275 was also reported. As little is known about its direct impact on liver or its underlying mechanism, it's imperative to characterize potential effect of AITC on NAFLD.

Research objectives

To validate the effect of AITC on NAFLD and clarify the possible mechanism of action.

Research methods

C57BL/6 mice were fed a high fat diet (HFD) for 8 weeks, and AML-12 cells were treated with 200 μ M palmitate acid (PA) for 24 h to establish *in vivo* and *in vitro* model of hepatic steatosis. Mice were administrated AITC (100 mg/kg/d) orally and AML-12 cells were treated with AITC (20 μ M) to detect the effect of AITC on NAFLD.

Research results

Our results show that AITC significantly ameliorates HFD-induced weight gain, hepatic lipid accumulation, inflammation, and PA-induced lipid accumulation as well as inflammation in AML-12 cells, accompanied by activated *Sirt1*/*AMPK* and inhibited *NF- κ B* signaling pathways. Curative effect of AITC on lipid accumulation is abolished by siRNA-mediated knockdown of either *Sirt1* or *AMPKa* in AML-12 cells.

Research conclusions



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AITC treatment protects against HFD and PA-induced lipid accumulation and inflammation *in vivo* and *in vitro*. These effects are associated with *Sirt1*/AMPK and *NF-κB* signaling pathways.

Research perspectives

Plant compounds, such as AITC, should be further explored for their potential effective activity in NAFLD.

9. We updated and added some references and also revised their form to meet our editor's request.
10. We rewrote all of the figure legends to meet our editor's request.
11. We added "47970 Supplementary material" containing Fig.S1 and its figure legends.