Peer Review File

Article information: http://dx.doi.org/10.21037/atm-19-4552

Comment of Reviewer A:

There already exists a lot of researches related to VitC in SAP. The novelty of this paper is to show us the deep mechanism of VitC in SAP.NRF-2/NQO1/HO-1 pathway is a new signaling pathway in SAP. However, the design of the paper cannot demonstrate this conclusion. Meanwhile, animal experiment is not a good tool to explore cell mechanism. More data in the cell level should be added.

Reply A: We have added some data in the cell level (see pages 10-11, lines 282-328)

Changes in the text:

3.6 Effect of different concentration of VC on AR42J cells

In order to choose the proper concentration of VC for the in vitro model, the viability of AR42J cells treated by different VC concentration (250, 500, 1000, 1500, 2000μM) was detected through CCK8 assay, and the results displayed that the activity and survival rate of AR42J cells did not decrease (Fig. 5a), suggesting that VC in this concentration range of 250-2000μM has no toxic effect on normal pancreatic acinar cells. Additionally, the effect of different VC concentration on sodium taurocholate-induced apoptosis of AR42J cells was further evaluated via TUNEL staining. As shown in Fig. 5b, a large number of apoptotic cells was observed in SAP group, however, concentration of 250, 500, 1000, 1500, 2000μM VC significantly reduced the proportion of apoptotic cells, suggesting VC alleviates sodium taurocholate-induced AR42J apoptosis. Meanwhile, there was no significant difference on reducing apoptotic cells between 1500μM and 2000μM VC, and then 1500μM VC was selected as the treatment concentration for the in vitro model.

3.7 Knockdown of NRF2 attenuated the protective effects of VC on sodium taurocholate-induced AR42J cell injury

To further explore the role of NRF2 in the protective effects of VC, small interfering RNA (siRNA)-mediated knockdown of NRF2 in AR42J cell was conducted. Our data demonstrated that the NRF2 siRNA sequence 1 (si-1) and 2 (si-2) significantly decreased NRF2 mRNA level compared with scramble group, but the NRF2 siRNA sequence 3 (si-3) had no obvious knockdown effect on NRF2 mRNA level (Fig. S1). Hence, si-2 with the highest knockdown efficiency was selected for subsequent experiments.

As shown in Fig. 6a, the increased expressions of Bax and cleaved caspase-3 induced by sodium taurocholate were significantly reduced after treatment with VC. Conversely, the expression of Bcl-2, Bcl-XL and MCL-1 was markedly decreased after sodium taurocholate stimulation, however, the alteration was reversed by VC treatment (Fig. 6a). These results suggested the VC reduced sodium taurocholate

induced apoptosis of AR42J cells, which similar results were also observed in TUNEL assay (Fig. 6b). However, these actions of VC on sodium taurocholate-injured AR42J were weaken by NRF2 knockdown (Fig. 6a and Fig. 6b), implying the involvement of NRF2 in the underlying mechanism of VC against sodium taurocholate induced AR42J cells apoptosis.

AR42J cells apoptosis linked to oxidative stress has been implicated in pancreatitis. Therefore, the effect of VC on oxidative stress in AR42J cells induced by sodium taurocholate was assessed. As displayed in Fig. 7a and Fig. 7b, the NRF2, HO-1, NQO1 were markedly decreased in SAP group compared with control group, but they were notably increased in SAP+VC group at both the gene and protein levels when compared with SAP group. Similar results were also confirmed by immunohistochemistry (Fig. 7c). Meanwhile, the levels of MDA, SOD, GPx, GSH/GSSG and T-AOC in cell culture supernatant were also detected, and the results showed the markedly decreased levels of SOD, GPx, GSH/GSSG and T-AOC and increased MDA level in SAP group when compared with control group (Fig. 7d). After treated with VC, these changes of MDA, SOD, GPx, GSH/GSSG and T-AOC in the SAP group were evidently reduced (Fig. 7d). However, these alterations in sodium taurocholate injured AR42J cells caused by VC treatment was attenuated by NRF2 knockdown (Fig. 7a, Fig. 7b, Fig. 7c and Fig. 7d).

Through ELISA, it found that sodium taurocholate significantly induced the release of amylase, lipase, IL-1β and IL-6 in AR42J cells, which was declined by VC treatment (Fig. 8a and Fig. 8b). Nevertheless, NRF2 knockdown undermined the effect of VC on the amylase, lipase, IL-1β and IL-6 in sodium taurocholate injured AR42J cells (Fig. 8a and Fig. 8b).

Comments of Reviewer B

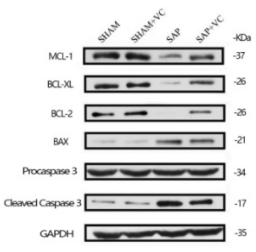
In this manuscript, the authors found that High-dose Vitamin C Alleviates Pancreatic Injury via the NRF2/NQO1/HO-1 pathway in a Rat Model of Severe Acute Pancreatitis.

Major critiques:

Comment 1： Please provide both procaspase 3 and active caspase 3 in one image.

Reply 1： Both procaspase 3 and active caspase 3 has been provided in one image (see Fig. 2b and Fig. 6a)

Changes in the text:



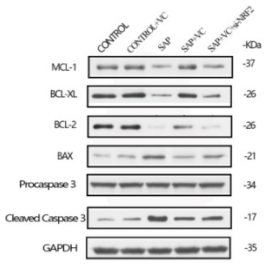


Fig. 2b Fig. 6a

Comment 2: The group of Vitamin C alone use should be supplemented in all figures.

Reply 2 : The group of Vitamin C alone use has been supplemented in all figures (see Fig. 2-Fig. 4, Fig. 6-Fig. 8

Changes in the text: There are 7 figures has been changed, please see the manuscript for details.

Comment 3: Does Vitamin C affect the expression of other mitochondrial proteins, for example: Bcl-XL, Bim, Bad, IAPs?

Reply 3: We add the data of Bcl-XL and MCL-1 protein expression in tissue of SAP rat and sodium taurocholate-injured AR42J cell (see page 8, lines 227-230; line 240; page 10, line 305-306). However, We did not add the data on the expression of Bim, Bad, IAPs. This is a limitation of our study, and we have addressed this in the Discussion section (see page 14, lines 405-406).

Changes in the text:

lines 227-230: the expression of apoptosis-related protein (Bcl-2, Bcl-XL, MCL-1, Bax, cleaved caspase-3 and procaspase 3) was detected, and the results showed that the increased expression of Bcl-2, Bcl-XL and MCL-1 and decreased expression of Bax and cleaved caspase-3 in pancreatic tissue from SAP group when compared with SHAM group (Fig.1d)

line 240: Moreover, the significantly increased expression of Bax and cleaved caspase-3 and TUNEL-positive cells and decreased expression of Bcl-2, Bcl-XL and MCL-1 in the pancreatic tissue of SAP rat were revealed when compared with those in the SHAM group

line 305-306: Conversely, the expression of Bcl-2, Bcl-XL and MCL-1 was markedly decreased after sodium taurocholate stimulation, however, the alteration was reversed by VC treatment (Fig. 6a)

lines 405-406: Third, the effect of VC on the expressions of other mitochondrial proteins, such as Bim, Bad, IAPs, has not been assess in this study.

Comment 4: Establishment of positive and negative control groups is needed. E.g. Octreotide.

Reply 4: In our study, the negative control groups were treated with the same dose of saline (in vivo) or PBS(in vitro) as VC. Additionally, vitamin C is not compared with other drugs for treatment in our study, only as a single treatment. Nevertheless, the lack of a positive control group is a limitation of our study. We have addressed it in our discussion (see page 14, lines 402).

Changes in the text:

see page 14, lines 402: First, this study lacks a positive control group is a limitation of our study.

Comments of Reviewer C

Vitamin C (VC) is one of the most important antioxidant. The effects of an intravenous administration of high-dose VC and the mechanisms by which it exerts its antioxidant function in an experimental model of SAP have not been determined. In this study, the authors aim to investigate the effects of intravenous administration of a high dose of VC and the potential mechanisms by which it exerts its antioxidant function. However, the partly symptoms such as the evidence of antioxidant activities need to modify, and the results were not enough to prove the conclusion. Therefore, I'd like to suggest the major comment of this manuscript in the current form.
Major comments on the manuscript and suggestions to authors:
Comment 1: In "Materials and methods" section, the author did not explain why the dose of Vc was chosen to be 500 mg/kg. The results show that 500 mg/kg exerted a better protective effect than 100 mg/kg (Fig.1a). However, there were only the results of SAP+LVC and SAP+HVC in Fig.1a. Does it mean that the effect of 1000 mg/kg will be better?

Reply 1: We investigated the effect of 1000mg/kg VC before this study. The results showed that the effect of 1000 mg/kg is not better than 500mg/kg. We have added these results in the revised manuscript (see page 8, lines 215-234). Some animal studies(Tyml K et al/ 2008; Tsai MS, et al. 2011;Zhao B, et al. 2014) that showed VC at a dose of 100–200 mg/kg is high. The maximum dose of VC used in vivo currently found is 200 mg / kg, and 500 mg / kg in our study is already the relative maximum dose. Additionally, we think that the dose of 1000 mg/kg is too large, which may cause side effects, such as kidney damage. Therefore, the effect of 1000 mg/kg VC has not been explored in detail.

Changes in the text: see page 8, lines 215-234 .

3.1 Effects of different dose of VC on pancreatic injury and apoptosis of pancreatic acinar cells in rats model of SAP

Pancreatic tissues from the SAP group showed mass edema and inflammation with necrosis when compared with the SHAM and SAP + VC groups (Fig.1a and 1b). When SAP rats were treated with 100, 500 and 1000 mg/kg of VC, it was found reduced edema, inflammatory cell infiltration and pancreatic necrosis in rat pancreatic tissue, which 500 mg/kg VC exerted the most obvious improvement on pancreatic injury as evidence of histological scores (Fig.1a and 1b). Then the effect of different dose of VC on apoptosis of pancreatic acinar cells in rats pancreatic tissue were further assessed. As shown in Fig .1c, the proportion of cells undergoing apoptosis in SAP group was higher than that in SHAM group, and 100, 500 and 1000 mg/kg of VC could reduce the proportion of apoptotic cells, which 500 mg/kg showed the

pronounced effect. Additionally, the expression of apoptosis-related protein (Bcl-2, Bcl-XL, MCL-1, Bax, cleaved caspase-3 and procaspase 3) was detected, and the results showed that the increased expression of Bcl-2, Bcl-XL and MCL-1 and decreased expression of Bax and cleaved caspase-3 in pancreatic tissue from SAP group when compared with SHAM group (Fig.1d). The alteration of apoptosis-related proteins in SAP rats were markedly weaken after receiving different dose of VC, especially 500 mg/kg of VC (Fig .1d). However, the expression of procaspase 3 showed no significant changes among these five groups (Fig .1d) . Based on the above results, 500 mg/kg VC was selected as the treatment concentration of in vivo model for further analysis.

Comment 2: The levels of antioxidants play an important role to evaluate the effect. The author measured the production of MDA, SOD, and GPx in this study. However, the experiments were not enough to prove the conclusion. The authors should provide more data such as GSH/GSSG and T-AOC as solid evidence to support the conclusion.

Reply 2: The production of GSH/GSSG and T-AOC has been added in our revised manuscript (see page 9, lines 246-249; page 11, line 317-322).

Changes in the text:

Lines 246-249 : MDA, SOD, GPx, GSH/GSSG and T-AOC, as indicators of lipid peroxidation and activity of antioxidant enzymes, were analyzed in pancreas tissue homogenates. As showed in Fig. 3a, induction of SAP was resulted in an elevation of MDA and decreasement of SOD, GPx, GSH/GSSG and T-AOC compared to the SHAM group.

Line 317-322: Meanwhile, the levels of MDA, SOD, GPx, GSH/GSSG and T-AOC in cell culture supernatant were also detected, and the results showed the markedly decreased levels of SOD, GPx, GSH/GSSG and T-AOC and increased MDA level in SAP group when compared with control group (Fig. 7d). After treated with VC, these changes of MDA, SOD, GPx, GSH/GSSG and T-AOC in the SAP group were evidently reduced

Comment 3: In this animal study, rats were randomly divided into three groups (n = 6). How can six animals in each group complete all the experiments in this study? The number of rats was not enough for all the animal experiment.

Reply 3: We feel sorry for our wrong statement. We have revised this mistake (see Page 5, line 134-137). Meanwhile, other wrong statement about the rats number has been revised (see Page 5, line147-148)

Changes in the text:

(n = 6 in each group)

line147-148: The following supplementary groups were also studied to assess mortality within 72h: SAP group (n=10) and SAP+VC group (n=10).

Comment 4: In this study, the author detected the protein expression of NRF2, HO-1, and NQO-1 using Western blot. Based on that, the author believed that high-dose VC alleviates pancreatic injury via the NRF2/NQO1/HO-1 pathway to inhibit oxidative stress. The conclusion cannot be deduced from these experimental results.

Reply 4: We have added some data in the cell level (see pages 10-11, lines 282-328)

Changes in the text: see pages 10-11, lines 282-328

3.6 Effect of different concentration of VC on AR42J cells

In order to choose the proper concentration of VC for the in vitro model, the viability of AR42J cells treated by different VC concentration (250, 500, 1000, 1500, 2000μM) was detected through CCK8 assay, and the results displayed that the activity and survival rate of AR42J cells did not decrease (Fig. 5a), suggesting that VC in this concentration range of 250-2000μM has no toxic effect on normal pancreatic acinar cells. Additionally, the effect of different VC concentration on sodium taurocholate-induced apoptosis of AR42J cells was further evaluated via TUNEL staining. As shown in Fig. 5b, a large number of apoptotic cells was observed in SAP group, however, concentration of 250, 500, 1000, 1500, 2000μM VC significantly reduced the proportion of apoptotic cells, suggesting VC alleviates sodium taurocholate-induced AR42J apoptosis. Meanwhile, there was no significant difference on reducing apoptotic cells between 1500μM and 2000μM VC, and then 1500μM VC was selected as the treatment concentration for the in vitro model.

3.7 Knockdown of NRF2 attenuated the protective effects of VC on sodium taurocholate-induced AR42J cell injury

To further explore the role of NRF2 in the protective effects of VC, small interfering RNA (siRNA)-mediated knockdown of NRF2 in AR42J cell was conducted. Our data demonstrated that the NRF2 siRNA sequence 1 (si-1) and 2 (si-2) significantly decreased NRF2 mRNA level compared with scramble group, but the NRF2 siRNA sequence 3 (si-3) had no obvious knockdown effect on NRF2 mRNA level (Fig. S1). Hence, si-2 with the highest knockdown efficiency was selected for subsequent experiments.

As shown in Fig. 6a, the increased expressions of Bax and cleaved caspase-3 induced by sodium taurocholate were significantly reduced after treatment with VC. Conversely, the expression of Bcl-2, Bcl-XL and MCL-1 was markedly decreased after sodium taurocholate stimulation, however, the alteration was reversed by VC treatment (Fig. 6a). These results suggested the VC reduced sodium taurocholate induced apoptosis of AR42J cells, which similar results were also observed in TUNEL assay (Fig. 6b). However, these actions of VC on sodium taurocholate-injured AR42J were weaken by NRF2 knockdown (Fig. 6a and Fig. 6b), implying the involvement of NRF2 in the underlying mechanism of VC against sodium taurocholate induced AR42J cells apoptosis.

AR42J cells apoptosis linked to oxidative stress has been implicated in

pancreatitis. Therefore, the effect of VC on oxidative stress in AR42J cells induced by sodium taurocholate was assessed. As displayed in Fig. 7a and Fig. 7b, the NRF2, HO-1, NQO1 were markedly decreased in SAP group compared with control group, but they were notably increased in SAP+VC group at both the gene and protein levels when compared with SAP group. Similar results were also confirmed by immunohistochemistry (Fig. 7c). Meanwhile, the levels of MDA, SOD, GPx, GSH/GSSG and T-AOC in cell culture supernatant were also detected, and the results showed the markedly decreased levels of SOD, GPx, GSH/GSSG and T-AOC and increased MDA level in SAP group when compared with control group (Fig. 7d). After treated with VC, these changes of MDA, SOD, GPx, GSH/GSSG and T-AOC in the SAP group were evidently reduced (Fig. 7d). However, these alterations in sodium taurocholate injured AR42J cells caused by VC treatment was attenuated by NRF2 knockdown (Fig. 7a, Fig. 7b, Fig. 7c and Fig. 7d).

Through ELISA, it found that sodium taurocholate significantly induced the release of amylase, lipase, IL-1β and IL-6 in AR42J cells, which was declined by VC treatment (Fig. 8a and Fig. 8b). Nevertheless, NRF2 knockdown undermined the effect of VC on the amylase, lipase, IL-1β and IL-6 in sodium taurocholate injured AR42J cells (Fig. 8a and Fig. 8b).

Comment 5: The English writing of this manuscript needs improvement. The author should have this manuscript reviewed by someone that is fluent in English.

Reply 5: The language of our revised manuscript has been polished and reviewed by someone that is fluent in English.

Changes in the text: please see the manuscript for details.

Minor comments on the manuscript and suggestions to authors:

Comment 1: The keywords did not represent the topic of this study.

Reply 1: The keywords has been revised (see page 2, line 36-37).

Changes in the text: Severe acute pancreatitis, pancreatic acinar cells injury, vitamin C, oxidative stress, NRF2/NQO1/HO-1 pathway, inflammation

Comment 2: In this manuscript, there were no spaces before brackets, such as 'within 72h were evaluated(n=10)', 'may reduce ascites in SAP rats(Fig.2a)', and so on.

Reply 2: Similar issues has been modified throughout this revised manuscript.

Changes in the text: please see the revised manuscript for details.