Dear Editors and Reviewers:

Thank you for your letter and for the reviewers comments concerning our manuscript entitled Paper Title **"Calycosin attenuates acute lung injury (ALI) in mice with severe acute pancreatitis (SAP) by curtailing High Mobility Group Box 1 (HMGB1)-induced inflammation" (NO.: 68241).**

Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in yellow in the paper. The main corrections in the paper and the responds to the reviewer's comments are as flowing:

Reviewer #1: Valeriu Marin Șurlin (No: 02534290):

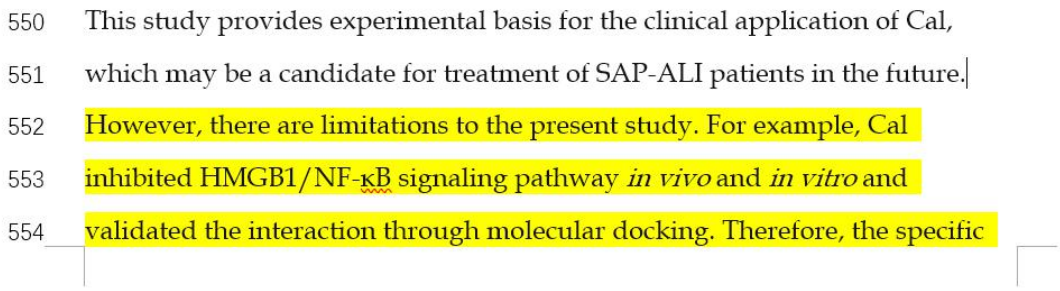
Responds to the reviewer's comments: However, there are limitations to the present study, the study shows that Calycosin inhibited HMGB1/NF-κB signaling pathway in vivo and in vitro, and validates the interaction by molecular docking. The future directions of this research topic should include a more thorough assessment of the specific interaction between Calycosin and HMGB1.

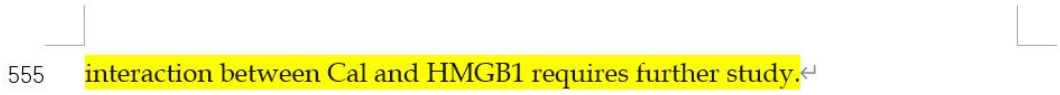
Response: Many thanks for your positive comments. It is my great honours receiving your recommendation. As Reviewer suggested that the specific interactions between Calycosin and HMGB1 are worth studying, that's what we need do next. Thanks again for your positive comments.

Reviewer #2 Saikat Samadder (No: 05915429):

1. Response to comment : ①The key problems or the limitation of this study is not revealed by the authors.

Response: We gratefully thanks for the precious time you spent making constructive remarks, we add the limitation of this study in the last paragraph and marked in yellow in line 552-555.


550 This study provides experimental basis for the clinical application of Cal,
551 which may be a candidate for treatment of SAP-ALI patients in the future.
552 However, there are limitations to the present study. For example, Cal
553 inhibited HMGB1/NF-κB signaling pathway *in vivo* and *in vitro* and
554 validated the interaction through molecular docking. Therefore, the specific


555 interaction between Cal and HMGB1 requires further study.

2. Response to comment : ②The minor limitation is One of the limitations of this research study is the pain caused by pancreatitis is not answered. Abdominal pain is reduced or not during pancreatitis is not understood from this study. In previous studies use of Cal reduced pain in animals is unknown or known can be mentioned in the discussion section. Markers from this existing study if have implications on pain can be discussed in just one sentence that pain might have been regulated in animals after Cal treatment. Pain is an important symptom of pancreatitis inflammation and pain goes hand-in-hand during pancreatitis. Hence, pain decreased or not in animals should also be discussed.

Response: It is really true as Reviewer suggested that 'Pain is an important symptom of pancreatitis inflammation'.

That's a new and good idea for our future study, Thanks to the reviewer for giving the interesting comments, we think the pain had been relieved after Cal treatment and added some sentences in line 545-548 in the paper.

544 dose-dependently inhibited the expression of HMGB1 and NF-κB signaling
545 activation both *in vivo* and *in vitro*. In addition, Studies have illustrated that
546 HMGB1 mediates pancreatic pain by targeting RAGE and CXCL12/CXCR4
547 signaling axis in mice with AP[40], therefore, pain may be relieved in animals
548 after Cal treatment.

3. Response to comment : ③Few minor changes needs to be made in the text are as follows; In figure 11 it is shown that "Serum IL-6, TNF-alpha, CXCL-1 and HMGB1 et al. increased" it should be etc not et al. Please change. In fig 3 Please align the results of ELISA in the sequence of mRNA levels (TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1). Place a bracket for p65 in Materials and methods last sentence.

Response: We gratefully appreciate for your carefully review and valuable comments, we had revised them in the paper.

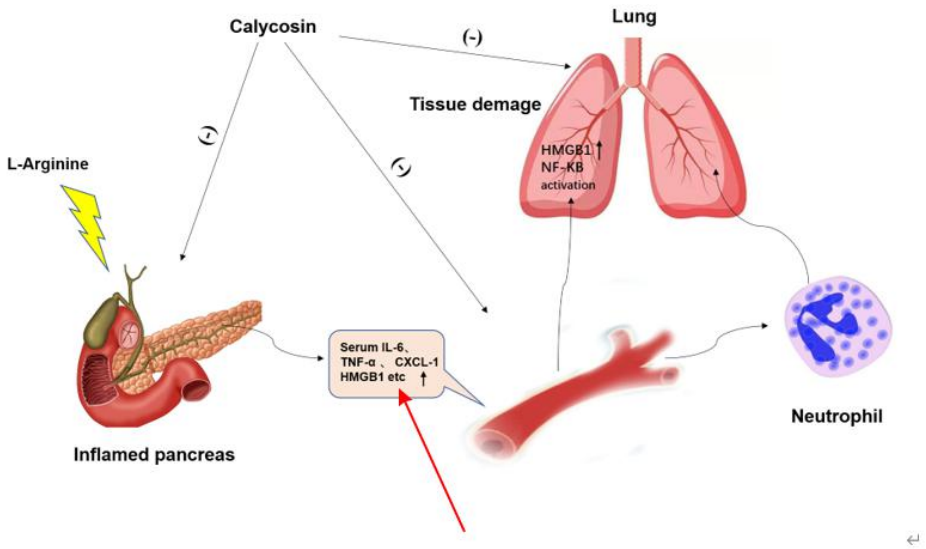


Fig. 11: Calycosin attenuates ALI in L-arginine induced SAP by curtailing High Mobility Group Box 1-Induced Inflammation

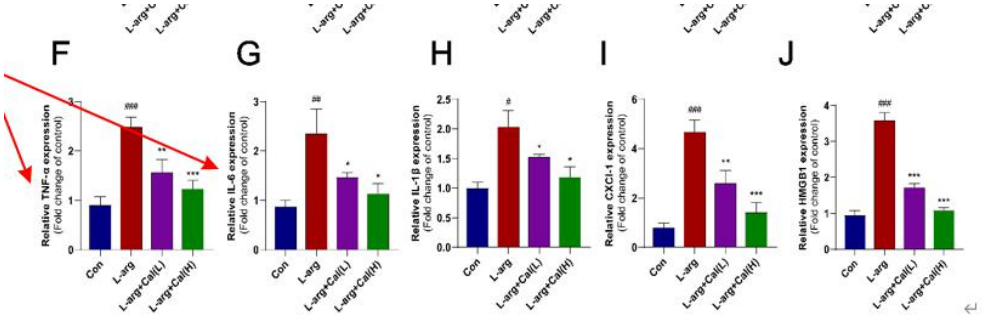


Fig. 3: Effect of calycosin on TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 in serum and mRNA levels. (A) TNF-α, (B)IL-6, (C)IL-1β, (D)CXCL-1, (E)HMGB1 in serum levels were determined by ELISA kits. (F) TNF-α, (G) IL-6, (H)IL-1β, (I)CXCL-1, (J)HMGB1 in mRNA levels. Data represent mean ± SD values. # p<0.05, ## p<0.01, ###P<0.001 vs. Con group, * p<0.05, ** p<0.01, *** p<0.001 vs. L-arg group.

MATERIALS AND METHODS

Chemicals and reagents

L-arginine (L-arg: purity > 98%, endotoxin-free), BCA Protein Assay Kit were purchased from Beijing Solarbio Science & Technology Co., Ltd.(Beijing, China). Calycosin (Cal: $C_{16}H_{12}O_5$, purity > 98%;) was from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, HMGB1, IL-1β and MPO were obtained from Wuhan Cloud-Clone Corp. (Wuhan, China). ELISA kits for TNF-α and CXCX-1 were procured from Proteintech Group (Rosemont, IL, USA). The amylase ELISA kit was bought from Shanghai BlueGene Biotech Co., Ltd. (Shanghai, China). Primary antibodies against NF-κB p65 (p65), phosphorylated NF-κB p65 (p-

4. Response to comment :

④Multiple bands in western blot results of P-P65 and p65 is being seen please explain the reason.

Response: Thank you so much for your careful check, Multiple bands in western blot results of P-P65 and p65 is being seen may due to the purity of the antibody, these bands are weakly visible and not in the correct position, we think they are meaningless bands.

5. Response to comment : ⑤What is the viability of pancreatic cell line in presence of Cal? It was not shown by this study if any other studies have showed can be explained in just one sentence.

Response: Thanks for your valuable suggestion, As shown in Figure 1A, Cal treatment significantly reduced the L-arg-induced necrosis in pancreatic acinar cells, The results sufficiently indicated that Cal has a significant protective effect on pancreatic acinar cells.

6. Response to comment : ⑥LPS induced inflammatory response on cell line is different from L-Arg induced inflammation or it is the same please explain in short in the introduction section.

Response: We gratefully appreciate for your valuable comment. There are three widely used pancreatitis models: cerulein, cerulein+LPS, and L-arg. Many studies have shown that they are differences AP models that determines the severity of AP[1-5]. Here, we cannot find an article that detailed describe whether the inflammation they induced is the same. LPS is a key component of the cell wall of gram-negative bacilli.

It is well known that LPS directly induces inflammation in various cell inflammatory model[7-9] and ALI model[10-12], Studies have shown that LPS has similar pathological characteristics to ALI and was the most suitable reagent for the preparation of ALI model[15]. Here we choose the LPS model.

Reference

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Reviewer #3 Anonymous (No: 03971255):

1. Response to comment : ④ Why were A549 cells chosen for the experiment?

①This article only detects A549 cells through CCK-8, and does not mention the changes in the cell activity of normal cells. The author is requested to make appropriate supplements.

Response: ④The pulmonary epithelium consists of two major cell types—alveolar type I (ATI) cells and alveolar type II (ATII) cells, also termed type I and type II pneumocytes. ATI together with ATII cells form a complete epithelial lining of the peripheral part of the lungs and play an important role in pulmonary homeostasis[1]. However, all these cells can be damaged during the course of acute lung injury. ATI cells are highly specialized for the key function of the lungs—the gas exchange between alveoli and capillary blood, which with very thin cytoplasm and a limited number of mitochondria cause extreme sensitivity of these cells to injury[2] and contribute to their vulnerability[2]. ATI cells were originally described as terminal differentiated cells without any ability to divide and change their phenotype[4]. While ATII cells are considered to be a multipotent cell with high plasticity and capability of self-regeneration and trans-differentiation into ATI cells. They are responsible for repairing the damaged tissue[5]. The main function of ATII cells is synthesis, secretion, and recyclation of the pulmonary surfactant, which is required for maintaining

sufficient respiratory surface area of the mammalian lungs at the end of expiration[6]. Potential mechanisms of ATII cell response to microbial infection have been studied in various models[7-9], the continuous cell lines such as human lung carcinoma A549 cells are extensively used as a model of ATII cells. So we chose the A549 cells for the experiment.

①Indeed, it will be more convincing if we get a comparative assessment on normal ATII cells. However, there are many difficulties with the isolation and maintenance of primary ATII cells in tissue cultures, associated with the loss of their morphological and biochemical characteristics[10, 11]. The reviewer's concern is of importance for us, and we will try to isolation and maintenance of primary ATII cells in our further study.

2. Response to comment: ② Why did the authors use only male mice?

Response: It is really true as Reviewer suggested that gender affects the susceptibility and course of diseases. In our study, We established the SAP model based on the studies of Kui[12] and Dawra[13]. In the study of Kui, they didn't detect any differences in AP severity of male and female mice. And according to Dawra's study who established the SAP model using male C57BL/6 mice, we successfully reproduce the

protocol.

RESEARCH ARTICLE

New Insights into the Methodology of L-Arginine-Induced Acute Pancreatitis

Balázs Kui[1‡], Zsolt Balla[1‡], Béla Vasas[2], Eszter T. Végh[1], Petra Pallagi[1] Eszter S. Kormányos[1], Viktória Venglovecz[3], Béla Iványi[2], Tamás Takács[1] Péter Hegyi[1,4], Zoltán Rakonczay Jr.[1]*

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Abstract

Animal models are ideal to study the pathomechanism and therapy of acute pancreatitis (AP). The use of L-arginine-induced AP model is nowadays becoming increasingly popular in mice. However, carefully looking through the literature, marked differences in disease severity could be observed. In fact, while setting up the L-arginine (2×4 g/kg i.p.)-induced AP model in BALB/c mice, we found a relatively low rate (around 15%) of pancreatic necrosis, whereas others have detected much higher rates (up to 55%). We suspected that this may be due to differences between mouse strains. We administered various concentrations (5–30%, pH = 7.4) and doses (2×4, 3×3, or 4×2.5 g/kg) of L-arginine-HCl in BALB/c, FVB/n and C57BL/6 mice. The potential gender-specific effect of L-arginine was investigated in C57BL/6 mice. The fate of mice in response to the i.p. injections of L arginine followed one of three courses. Some mice (1) developed severe AP or (2) remained AP-free by 72 h, whereas others (3) had to be euthanized (to avoid their death, which was caused by the high dose of L-arginine and not AP) within 12 h., In FVB/n and C57BL/6 mice, the pancreatic necrosis rate (about 50%) was significantly higher than that observed in BALB/c mice using 2×4 g/kg 10% L–arginine, but euthanasia was necessary in a large proportion of animals, The i.p. injection of lower L-arginine concentrations (e.g. 5–8%) in case of the 2×4 g/kg dose, or other L-arginine doses (3×3 or 4×2.5 g/kg, 10%) were better for inducing AP. We could not detect any significant differences between the AP severity of male and female mice. Taken together, when setting up the L-arginine-induced AP model, there are several important factors that are worth consideration such as the dose and concentration of the administered L arginine-HCl solution and also the strain of mice.

Am J Physiol Gastrointest Liver Physiol 292: G1009–G1018, 2007.
First published December 14, 2006; doi:10.1152/ajpgi.00167.2006.

Development of a new mouse model of acute pancreatitis induced by administration of L-arginine

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Department of Surgery, University of Minnesota, Minneapolis, Minnesota

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Dawra R, Sharif R, Phillips P, Dudeja V, Dhaulakhandi D, Saluja AK. Development of a new mouse model of acute pancreatitis induced by administration of L-arginine. *Am J Physiol Gastrointest Liver Physiol* 292: G1009–G1018, 2007. First published December 14, 2006; doi:10.1152/ajpgi.00167.2006.—The pathogenesis of acute pancreatitis is not fully understood. Experimental animal models that mimic human disease are essential to better understand the pathophysiology of the disease and to evaluate potential therapeutic agents. Given that the mouse genome is known completely and that a large number of strains with various genetic deletions are available, it is advantageous to have multiple reliable mouse models of acute pancreatitis. Presently, there is only one predominant model of acute pancreatitis in mice, in which hyperstimulatory doses of cholecystokinin or its analog caerulein are administered. Therefore, the aim of this study was to develop another mouse model of acute pancreatitis. In this study, C57BL/6 mice were injected intraperitoneally with L-arginine in two doses of 4 g/kg each, 1 h apart. Serum amylase, myeloperoxidase, and histopathology were examined at varying time points after injection to assess injury to the pancreas and lung. We found that injection of L-arginine was followed by significant increases in plasma amylase and pancreatic myeloperoxidase accompanied by marked histopathological changes. The injury to the pancreas was slow to develop and peaked at 72 h. Subsequent to peak injury, the damaged areas contained collagen fibers as assessed by increased Sirius red staining. In contrast, D-arginine or other amino acids did not cause injury to the pancreas. In addition, acute inflammation in the pancreas was associated with lung injury. Our results indicate that administration of L-arginine to mice results in severe acute pancreatitis. This model should help in elucidating the pathophysiology of pancreatitis.

pancreas; lung injury; fibrosis

ACUTE PANCREATITIS IS AN INFLAMMATORY disease of the pancreas resulting in significant morbidity and mortality (13). Various causes, including gallstones, alcohol, trauma, infections, and genetic alterations, have been implicated in the causation of this disease (1, 23). Although our understanding of the cell biology of the exocrine pancreas and epidemiology of pancreatitis has increased greatly in recent years, our knowledge of its pathophysiology and the ability to prevent or treat pancreatitis remain limited (23). This can partially be attributed to the paucity of clinical material from the early stages of the disease available for research. To overcome this and to study the effect of new therapeutic agents, different experimental animal models of pancreatitis have been developed. Our present limited understanding about the events associated with the development of the disease is based on the use of these experimental models (21). Among the animals used for developing experimental models, the mouse is ideal because of the availability of several genetic manipulations, accessibility of its complete genome, and ease of maintenance. It appears that the future of experimental pancreatitis is inextricably linked to the mouse. Administration of hyperstimulatory doses of cholecystokinin or its analog caerulein results in the development of pancreatitis in mice. This model has been extensively used for studying pancreatitis (7, 18). However, in this model, a relatively mild form of the disease develops, which resolves quickly and is not accompanied by any mortality. The other model that has been used is the choline-deficient, ethionine-supplemented (CDE) diet-induced mouse model, which results in hemorrhagic pancreatitis resembling the human disease; however, this model has several limitations (use of only young female mice, variable response, and high mortality) (19). In fact, this model has been used in relatively few studies in recent years. Clearly, therefore, the need exists to develop another mouse model of acute pancreatitis to supplement the information gained from the caerulein-induced model.

Previously Mizunuma et al. (20) established that intraperitoneal administration of a high dose of L-arginine to rats results in acute necrotizing pancreatitis. However, for reasons stated above, a mouse model of acute pancreatitis is far more advantageous. There are two previous reports in which L-arginine was used for induction of pancreatitis in mice (4, 31). In these studies, the dose and protocol followed were similar to those reported for use in rats. However, despite our repeated attempts to reproduce these protocols with the same dose of L-arginine, we were unable to induce pancreatitis in either Balb/c or C57Bl/6 mice. Other groups have also tried to induce pancreatitis in mice by administration of L-arginine using this protocol but did not succeed (personal communication). Therefore, the aim of this study was to develop a reproducible model of L-arginine-induced pancreatitis in mice. We have successfully developed a protocol for induction of severe acute pancreatitis in mice by intraperitoneal injections of L-arginine and report here the dose required, the method followed, and its effect on the different markers of pancreatic and associated lung injury.

MATERIALS AND METHODS

Male C57BL/6 (25–30 g) and Balb/c (18–22 g) mice were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 ± 2°C and 12:12-h light-dark cycle. Animals were fed standard laboratory chow, given

* R. Dawra and R. Sharif contributed equally to this work.

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3. Response to comment: ③Please note the basic format requirements of the manuscript, such as "°C" and "℃".

Response: We gratefully appreciate for your valuable comment, we had made corrections in line 191 and line 218 where marked in yellow in the paper.

187 ***Animals*** ↵

188 Twenty-four male C57BL/6N mice (weight: 18-22g, age: 8–10 weeks) were
189 purchased from Charles River Company (Beijing, China). Mice were housed
190 in specific pathogen-free facility with a dark/light cycle of 12 hours in ambient
191 temperature of 22±2℃ and humidity of 50±10%. Mice were fed standard
192 rodent chow and clean water *ad libitum*. All animal experiments were
193 conducted in accordance with relevant guidelines and regulations and
194 approved by Animal Ethics Committee of The National Drug Clinical Trial
195 Institution of The First Affiliated Hospital of Zhengzhou University (Ethic
196 Review Number: 2019-KY-140). All mice received humane care and the study
197 were conducted pursuant to the ARRIVE guidelines.↵

217 histopathological assessment and the other half snap-frozen in liquid nitrogen
218 and stored at -80℃ for biochemical analysis. ↵

4. Response to comment: ⑤Authors need to make sure that the manuscripts they upload are all in English, especially references.

Response: Thank you so much for your careful check, I am sure that all my references are in English, I had made a change in line 619.

619 **REFERENCES**↵

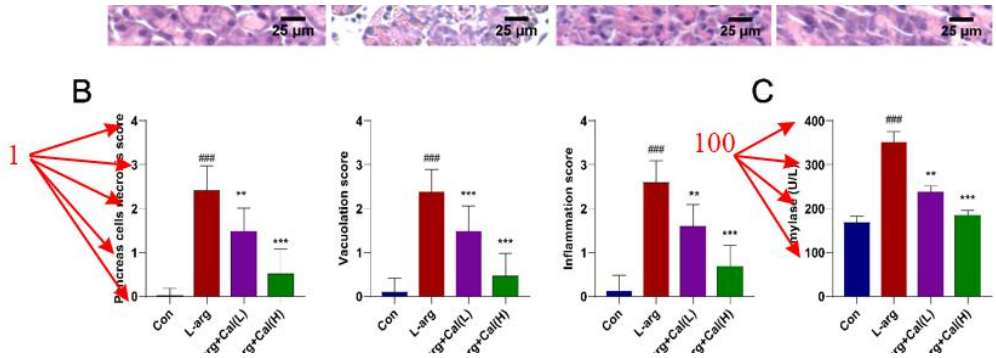
5. Response to comment: ⑥The scales of several fluorescent pictures are inconsistent. Is it necessary to modify them?

Response: Thank you for your consideration, the fluorescence pictures were taken from tissues and cells. In order to show the results more clearly, the fluorescence pictures of cells had a larger magnification than tissues, so the scales are not consistent. Maybe it is not necessary to modify them.

6. Response to comment: ⑦In figure 1B, figure 2B and figure 3F, some

standard deviations are too high, please adjust it.

Response: We gratefully thanks for the precious time the reviewer spent making constructive remarks, the pictures are about pathological scores, they were conducted according to the studies of Schmidt [14] and Vrolyk [15]. We can't revise it. The standard deviations of these pictures are high because the difference between the values on the ordinate axis is small, while others were 100 or more, so there will be a high standard deviation even if the difference of the date between one group is small.



7. Response to comment: ⑧Please unify the background of all Western Blot bands in the text.

Response: Thanks for your careful review, since the NF-κB signals were weak both in lung tissues and A549 cells, the contrast display must be enhanced to make it clear, so the background of Western Blot bands were not unified. If necessary, we can provide all the un-cropped western blot strips.

8. Response to comment: ⑨The quality of the strip in this paper is not

qualified; the author needs to modify it, for example: figure 5A GAPDH.

Response: Special thanks to you for your good comments. We had changed the picture to another high-quality strip in the three independent experiments.

9. Response to comment: ⑩The authors conclude that Cal protective and beneficial effects against ALI in SAP by averting local and systemic neutrophil infiltration and inflammatory response in part via suppression of HMGB1-NF-κB signaling activation. The authors should add NF-κB inhibitors for further testing.

Response: We derived much benefit from the reviewer's comment. While, first of all, Nuclear factor kB (NF-kB) is a ubiquitous inducible transcription factor responsible for mediating the expression of a large number of genes involved in inflammation, embryonic development, tissue injury, and repair[16]. If we add NF-κB inhibitors may affect other signals of the disease, the results are incredible. Second, Cal showed a protective and beneficial effects via suppression of HMGB1-NF-κB signaling activation, if we use a NF-κB inhibitors, will it be conflicted with the role of Cal? Last, the NF-κB signal were poorly expressed both in tissues and cells in our study, if we use a NF-κB inhibitors, it might be failure to get a result.

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Reviewer #4 Anonymous (No: 02445715):

1. Response to comment : ①There are so many typos in the main text.

Response: Thanks for your carefully reviewed. We carefully checked the article and made some changes, and we will pay more attention to the quality of the article in the future. Thank you very much.

2. Response to comment : ②The reviewer can not find the data of positive control drug such as montelukast or something like that.

Response: As Reviewer suggested that it's better to add a positive control drug, but to our knowledge, there is no clinically used drugs which could be used as positive control. We are looking forward to the clinical use of Cal.

3. Response to comment : ③Please show me the un-cropped WB data. How many WB did you perform?

Response: Three independent experiments were conducted in the study, we will provide all the un-cropped WB data in the supplementary materials.

4. Response to comment : ④There are so many figures. Please shorten the images/data by reducing or combining the current data.

Response: We gratefully appreciate for your valuable comment. The images/data are necessary in our study, we can't reduce them.

5. Response to comment : ⑤The corresponding author should use his/her email address as institutional one.

Response: It is really true as Reviewer suggested that we should use an institutional email to submitted the manuscript, we will use the public mailbox for the next submission.

6. Response to comment :⑥In Discussion section, the authors should describe more hyphothesis-based results and conclusion for the activity.

Response: Special thanks to you for your good comments. We have added a sentence in line 550-551.

550 This study provides experimental basis for the clinical application of Cal,
551 which may be a candidate for treatment of SAP-ALI patients in the future.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here the changes were marked in yellow in revised paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.