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Dear editor,

We would like to thank the editor and the reviewers for their conscientious reviews, and insightful comments and suggestions to improve the manuscript. In the response below, we have addressed all the concerns raised by the editor and the reviewers in the revised manuscript. We hope the editor and the reviewers will find that our revised manuscript has improved and is suitable for publication. All changes have been marked in blue.

I hope my paper could achieve the academic standards of your magazine and be published finally. Thank you very much.

Yours Sincerely,
Danan Wang and Changlong Lu

Response to Reviewers Comments

Manuscript Number: 30852-R1

Sodium selenite ameliorates the development of dextran sulfate sodium-induced chronic colitis in mice by decreasing Th1, Th17, and gamma delta T and increasing CD4(+)CD25(+)regulatory T-cell responses

Lixuan Sang; Bing Chang; Junfeng Zhu; Fangli Yang; Yan Li; Xuefeng Jiang;
Danan Wang; Changlong Lu; Xun Sun;

Reviewers' comments:

Reviewer #1(01434943)

This is an immunological study of selenite and its capacity to ameliorate DSS-colitis in mice. The statistical power (n=10 mice/group).

1. ABSTRACT: Aim: 'the protective effects' is a little presumptive. It implies that a protective effect was assumed before the start of the study. Methods: Very brief. How were the various end points (cytokines etc) 'measured'?

Response: Yes, it is very correct. The sentence has been modified to “This study assessed the effects of sodium selenite on the severity of DSS-induced colitis in C57BL/6 mice”. **METHODS:** Mice were randomly divided into four groups (n=10/group): normal group, Se group, chronic colitis group, and Se + chronic colitis group. The mice were sacrificed on day 26. Survival rates, clinical symptoms, colon lengths, and histological changes were determined. The percentage and absolute number of immune system cells in the lamina propria lymphocytes (LPL) of the colon, the expression of mRNA in colon tissue, and the concentrations of Th1, Th17, and Treg cytokines in LPL from the large intestine, were measured.

2. Results: Add p-values at least.

Response: Yes, it is very correct and p-values has been added in the results.

3. INTRODUCTION: An excellent summation of the field and where the selenite intervention fits in. Suggest deleting final sentence.

Response: Yes, it is very correct. The final sentence has been deleted.

4. METHODS: Include numbers of mice/group. Myeloperoxidase levels would be useful as a marker of neutrophil activation. Otherwise analyses are appropriate and well described.

Response: Yes, it is very correct. Numbers of mice/group were added (n=10). Oxidative stress is believed to play an important role in the pathogenesis of colitis-related intestinal tissue injury; Myeloperoxidase (MPO) is a marker of oxidative stress produced mainly by polymorphonuclear leucocytes and is associated with the severity of colitis. The assessment of MPO activity is well established for evaluating intestinal inflammation. We are adding animal experiments. Because the experiment has not been completed, we will report myeloperoxidase level in a future article. Thank you for your forgiveness.

5. RESULTS and tables/figures: Well described and presented. Figure 1C is unclear- suggest making larger or else, deleting it. DISCUSSION: A solid discussion of the work.

Response: Yes, it is very correct and Figure 1C making larger.

Reviewer #2(03254039):

The authors examined the effect of selenium in a murine DSS-induced colitis model. They showed that treatment with sodium selenite ameliorated the severity of colitis, with the concomitant suppression of various proinflammatory cytokine levels and increase in IL-10. They also demonstrated that Th1 and Th17 subsets were decreased and Treg cells were increased by sodium selenite treatment. Based on these results, the authors conclude that sodium selenite may be useful in IBD. Although various parameters are analyzed and the some results have interesting points, this study is phenomenological and no robust cause-to-effect relations are established. Thus, there are several points that should be addressed before publishing.

Response: Yes, this is a good idea.

Major comments

1. How do the authors detect the dosage of sodium selenite (2 ug/g body weight)? Please explain why this dosage selected in this study.

Response: Yes, this is a good question. Selenium content of the intestinal tissue and Serum selenium concentration have been tested and increase the literature. The study have confirmed:90 days of supplementation with 16 ppm(16 ppm*5ml=80µg sodium selenite/mouse/day) had no negative effect on growth and survival. We choose 2 µg/g (2µg/g*20g body weight = 40µg sodium selenite/mouse/ day) concentration is very safe for further research.

Table 3 Serum selenium concentration and content in colon tissue (n=10)

	Group			
	Control	Se	Chronic colitis	Se+Chronic colitis
Serum selenium concentration (µg/L)	368.8 ±15.1	416.6±13.2	311.5±12.1	341.6±11.3*
Selenium content in colon tissue (mg/kg)	2.29±0.21	3.41±0.18	1.71±0.12	2.10±0.14*

* $P < 0.05$, Se+Chronic colitis group versus Chronic colitis group;

Title: High selenium diet protects against TNBS-induced acute inflammation, mitochondrial dysfunction, and secondary necrosis in rat colon. Nutrition (Burbank, Los Angeles County, Calif.). 23(11-12):878-86, 2007 Nov-Dec. PMID:17936198

Paper content is as follows:

In vivo selenium supplementation

The rats were supplemented for 21 d with the following diets: a normal selenium diet (NSD) providing approximately 2 $\mu\text{g/g}$ of sodium selenite daily per animal, which is considered an acceptable excess supplementation level for this element [10,17].

The rationale for the use of a high selenium diet (HSD) in the form of 16 ppm of sodium selenite in the drinking water of rats was that such a diet should provide around 0.75 mg/kg of selenium per day (around 2 mg/kg of sodium selenite per day), according to a National Institutes of

Health toxicologic report (TOX-38, Toxicity Studies of Sodium Selenate and Sodium Selenite [CAS nos. 13410-01-0 and 10102-18-8] Administered in Drinking Water to F344/N Rats and B6C3F1 Mice). Thirteen weeks of supplementation with 16 ppm of sodium selenite in the drinking water had no negative effect on growth and survival.

Induction of colitis

Colitis was induced by administering 0.5 mL of 2,4,6-trinitrobenzene sulfonic acid (TNBS; 100 mg/mL dissolved in 50% ethanol) through the anal canal for a distance of 8 cm into the colon, just proximal to the splenic flexure. Animals were sacrificed 24 h after induction and the colon was removed [18]. This time frame was chosen because 72 h after induction already reflects a beginning of spontaneous remission in this model.

2. The authors indicate that sodium selenite alleviates the DSS-induced colitis due to the induction of Tregs. In figure 4 and 5, the number of CD4+CD25+ T cells and CD4+IL-10+ T cells in Se+Chronic DSS colitis group are higher than those in Chronic DSS colitis, however Treg number in Se group is similar to Control group. Why sodium selenite does not affect the population of Treg cells in the normal colonic lamina propria? Furthermore, there is no direct evidence that sodium selenite induces the Tregs. The authors should show how sodium selenite increases the population of Treg cells in the colonic mucosa. For example, the effect of sodium selenite on the differentiation of Treg cells in vitro experiment supports their conclusions.

Response: Yes, this is a good question, sodium selenite have little affect the population of Treg cells in normal colonic lamina propria(CD4⁺CD25⁺T cell: normal group *vs* Selenium group: 6.85±0.59% *vs* 7.05±1.13%; $P>0.05$).

This phenomenon may be due to the immune function of normal rats in state of relative balance, however, colitis of mice immune balance is broken. Sodium selenite is more likely to play a key role of the elevated Treg in colitis, but the exact mechanism is still not very clear. The effect of sodium selenite on the differentiation of Treg cells in vitro experiment are being added. Because of the experiment has not been completed, we will report the results in a future article. Thank you for your forgiveness.

3. In the manuscript, line 213, the mRNA expression of IL-6 is described, but figure 3 is missing IL-6 data. Furthermore, in line 216, the expression of IL-22 and IL-23 were not difference between Chronic DSS colitis and Se+Chronic DSS colitis group, however IL-23 mRNA expression is decreased in Se colitis group. Please describe correctly.

Response: Yes, IL-6 data has been added and IL-23 has been revised(Figure 3; Line201;Line203).

Minor comments 1. Line 236, the description of neutrophil and macrophages are same (CD11b+Gr1+F4/80-). Please correct.

Response: Yes, it has been revised in Line 548.

Flow cytometry of the populations of LPL in the colon in each group. (A)The frequency of neutrophil(CD11b+Gr1+F4/80-), macrophage (CD11b+Gr1+F4/80⁺), $\gamma\delta$ T cell ($\gamma\delta$ TCR), NK (NK1.1⁺), NKT(NK1.1+ $\alpha\beta$ TCR⁺), CD4⁺, CD4⁺CD44⁺ (Effective of T cell), CD4⁺CD25⁺(Regulatory T-cell), CD4⁺CD69⁺(Activation of T cell) T cell in

LPL of colon in each group. (B)The absolute cell number of all kinds of cells in each group. Data indicate mean \pm SD of six mice of obtained from a representative of three independent experiments (* P <0.05; ** P <0.01; *** P <0.001).