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**Cover Letter**

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Dear Editor:

Please find enclosed for your review an original medical hypothesis, "Lactulose: an indirect antioxidant ameliorating inflammatory bowel disease by increasing hydrogen production" by XiaoChen, QiaoZuo, et al. According to your " Instructions for Authors", we have included an original manuscript, an abstract. All authors have read this version of the article and approved the submission. Professor Xuejun Sun is the corresponding author, who can be reached at the address above.

Due care has been taken to ensure the integrity of the work. No part of this paper has been published or submitted elsewhere. No conflict of interest exists in the submission of this manuscript. We think that the most important point of this study is that we depicted a better picture for clinical IBD treatment with a promising indirect antioxidant, lactulose. We believe this paper may be of particular interest to the readers of your journal because our hypothesis not only provides a novel treatment for inflammatory bowel disease clinically, but also states the mechanisms of the therapeutic effects theoretically. It may answer in some aspect what exact roles the ROS play in the progression of IBD. Moreover, after this hypothesis is testified, this way of inducing endogenous antioxidant hydrogen could be widely employed and applied to many aspects in clinics. I believe if this hypothesis draws people's attention, researches related to it will be commenced soon.

We hope you will give favorable consideration to our manuscript, and we look forward to receiving comments from the reviewers. Please acknowledge receipt of this manuscript at your earliest convenience, and let us know if you need any further information.

Sincerely yours,
Xuejun Sun, M.D., PhD.

*Abstract

**Abstract:**

Lactulose, which cannot be digested and absorbed by body, is clinically widely used to treat constipation and hepatic encephalopathy. Fermented by gastrointestinal tract bacteria, lactulose can produce considerable amount of hydrogen, which is protective for DSS-induced colitis as a unique antioxidant. We propose that lactulose is an indirect antioxidant that mobilizes endogenous hydrogen production which in turn can reduce oxidative stress and ameliorate symptoms of inflammatory bowel disease in human beings.

***Revision Notes**

Dear editor,

Thank you for your thoughtfulness and carefulness. We have made some major changes according to your suggestions. The major changes are as follows.

1. I have changed the title from “Lactulose: an indirect antioxidant ameliorating DSS-induced colitis by increasing hydrogen production” to “Lactulose: an indirect antioxidant ameliorating inflammatory bowel disease by increasing hydrogen production”. I think this title matches better with the content.
2. I have changed the format of the references according to the sample.
3. I have corrected some scattered spelling and grammatical errors and some sentences with vague meanings.
4. I have reordered some sentences to make them more logical and reasonable.

Thank you so much again for your kind suggestions.

Sincerely yours

Xiao Chen

Title: Lactulose: an indirect antioxidant ameliorating inflammatory bowel disease by increasing hydrogen production

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**Key words:** lactulose; hydrogen; antioxidant; inflammatory bowel disease;

## 1. Introduction

Inflammatory bowel disease(IBD)-Crohn's disease and ulcerative colitis, whose causes are still not fully understood, exerts a substantially negative impact on the quality of life of affected individuals, among whom patients with chronic ulcerative colitis bear an 10% increased risk in developing colon carcinoma. Conventional medical treatments, inflammation inhibition, ie. corticosteroids, aminosalicylates, immunosuppression, ie. thiopurine, and alternative immunomodulatory medications, ie. methotrexate, TNF-α monoclonal antibodies, are introduced into IBD treatments from different aspects of IBD etiologic process[1]. Unfortunately, none of the drugs above is universally effective. Meanwhile, these medications could bring sever adverse effects.

A growing number of evidences support the notion that oxidant-mediated injury plays an important role in the pathogenesis of IBD[2]. Inflamed tissues generate hydroxyl radicals, the most cytotoxic reactive oxygen species(ROS), which up-regulate TNF-α expression through NF-κB signaling pathway[3] and activate NADPH-Oxygenase(NOX) expression, increasing ROS production[4] as a vicious circle.

Oxidation and inflammation are reciprocally related. In IBD, activated macrophages and neutrophils, which produce excessive ROS, aggregate in the inflaming gut. Subsequently, severe oxidative stress occurs[5]. When exceeding the antioxidative ability of the intestinal antioxidant defense system, ROS will cause substantial damages in protein, lipid and DNA, thus worsening the oxidative stress injuries in patients with ulcerative colitis[6]. Based on these findings, impaired antioxidant mechanism is implicated as one of the pathogenic causes of DSS-induced colitis[7]. Therefore, antioxidants are suggested as a novel therapy for IBD in recent years, which can significantly alleviate the symptoms, ie., body weight loss, visible fecal blood and diarrhea[8]. Thus, identifying new antioxidants in IBD treatment has recently attracted great attention worldwide.

## 2. Molecular hydrogen($H_2$) serves as a novel inflammation suppressor.

In recent years, experimental evidences have documented that without influencing other less potent ROS, important in intracellular signaling, molecular hydrogen($H_2$) possesses the ability to selectively neutralize $ONOO^-$ and ·OH, the most cytotoxic ROS, which can damage cellular macromolecules aggressively and indiscriminately. Thus, $H_2$ can protect cells from oxidative stress injuries[9]. Therapeutic effects of $H_2$ have been confirmed in the cell damage after stroke, ischemia-reperfusion injuries[10,11], transplantation injuries[12] and other injuries related to oxidative stress. In inflammation process, $H_2$ mediates suppression of pro-inflammatory cytokines, especially CCL2, IL-1β, TNF-α, IL-6 in inflammatory tissues. In experiments, hydrogen gas treatment has significant protective effects on schistosomiasis-associated chronic liver inflammation[13] and $H_2$-rich saline treatment significantly attenuates the severity of L-Arg-induced acute pancreatitis by ameliorating the increased serum amylase activity, inhibiting neutrophils infiltration and lipid oxidation[14].It was reported that $H_2$ mediated suppression of colon inflammation induced by dextran sodium sulfate(DSS) in 2009[15].

As a novel antioxidant, $H_2$ possesses a number of advantages. (1) Due to its high permeability, $H_2$ can easily penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus. (2)It is nontoxic to the organisms, which has been proven by

hyperbaric diving for decades. (3) Thanks to its selectivity as an antioxidant, it has less impact on other less active but very important ROS within the cells.

**3. Lactulose mediates $H_2$ production and ameliorates DSS-induced colitis in mice.**

Lactulose is a synthetic sugar used in the treatment of constipation[16] and hepatic encephalopathy. It is a disaccharide formed from one molecule each of the simple sugars fructose and galactose and cannot be absorbed by human bodies but can be digested by bacteria colonizing within the gastrointestinal tract, especially in the colon. One of the main byproducts is $H_2$. Oral administration of lactulose significantly increases $H_2$ production[17], which can be detected by $H_2$ breath test, introduced several decades ago as a diagnostic test for small bowel bacterial overgrowth[18].

In 2004, in an animal experiment, it was observed that lactulose had some protective effects on DSS-induced colitis. The authors attributed it possibly to alterations of colonic microflora[19], namely, increased number of beneficial microflora and decreased number of pathogenic microflora. According to the already known pathogenesis of IBD, host-microbiome interactions are quite complicated, which can be mutually beneficial or can be deleterious. Bacteria that can adhere to and invade the intestinal mucosa may be particularly important, as in the case of Escherichia Coli, one of the main bacteria colonizing in the colon[20]. Based on recent findings, we put forward some concerning questions. 1. New mechanisms of DSS-induced colitis were found by recent researches that the colon had an inner attached mucus layer devoid of bacteria[21]. DSS could cause alterations in the inner colon mucus layer and made it permeable to bacteria. Direct contact stimulates persistent immune response, causing inflammatory damage. Thus, the colitis occurred[22]. Under such circumstances, it is difficult to distinguish between beneficial and harmful microflora. This explained why most mouse models of colitis require intestinal bacteria for inflammation to occur[23]. Besides, antibiotics are effective in some patients with inflammatory bowel disease. Antibiotic therapy attenuates colitis in IL-10 gene-deficient mice[24] and long term treatment with nitroimidazoles or clofazimine are proved to be effective in patients with Crohn's disease[25]. 2. In the experiment, interestingly enough, the authors also observed that lactulose reduced the severity of colonic lesions induced by DSS treatment in a dose-dependent manner, the effect at 100 mg/kg being more potent than that of 5-ASA. Lactulose treatment also prevented the colon shortening and ameliorated the histological inflammation, together with significant attenuation of the increase in MPO activity as well as lipid peroxidation following DSS treatment. The lowered oxidative stress state could not be simply explained by alterations of colonic microflora.

Considering that gastrointestinal tract derived $H_2$, which is closely related to reduced cardiovascular events, could reduce general oxidative stress injuries[26], we assume that the induced $H_2$ by lactulose might be the key to the symptoms alleviation and lowered MPO level in DSS-induced colitis, which seems to be a more reasonable explanation.

According to the published data, patients with type 2 diabetes or impaired glucose tolerance were treated with 900 ml/day (300 ml three times a day) $H_2$-dissolved water. Drinking 300 ml of $H_2$-dissolved water lead to exhaled $H_2$ gas concentration reaching a maximum of 56 ± 27.8 ppm at 15 min, and returning to the baseline level at 150 min. This peak level of $H_2$ gas down-regulated oxidative stress biomarkers and improved glucose

metabolism in patients with type 2 diabetes or impaired glucose tolerance[27]. In previous study, it has been proven that 20g lactulose administration could increase the exhaled $H_2$ nearly to the same level of exhaled $H_2$ as compared to the consumption of 300ml $H_2$ dissolved water and had a longer maintenance time of $H_2$ concentration[16]. Thus, we consider that oral administration of lactulose may be better than drinking $H_2$-rich water in terms of maintenance of the appropriate $H_2$ gas levels in the body.

## 4. Hypothesis

Lactulose has been proved effective in DSS-induced mice colitis. Based on these observations and experiments, we hypothesize that lactulose may be a novel and promising therapeutic option for IBD as an indirect antioxidant. By increasing gastrointestinal tract derived $H_2$, it may significantly restrict inflammation and alleviate clinical IBD symptoms, improving the life quality of patients. What's more, it is noteworthy that lactulose probably has many other beneficial antioxidant effects on a wide range of aspects, such as cardiovascular diseases, cerebrovascular accidents, neurodegenerative diseases et al, which still needs further study.

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Conflicts of Interest Statement

None declared

***Reviewer Suggestions**

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