ANSWERING REVIEWERS
February 10, 2014
Dear Editor,

Please find enclosed the edited manuscript in Word format
File name: NADPH_oxidase_colon_inflammation_mice_8132_7178.docx

Title: Protective action of NADPH oxidase inhibitors and the role of NADPH oxidase in the pathogenesis of colon inflammation in mice

Authors: Rima Ramonaite, Jurgita Skieceviciene, Simonas Juzenas, Violeta Salteniene, Juozas Kupcinskas, Paulius Matusevicius , Vilmante Borutaite, Limas Kupcinskas

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The manuscript has been improved according to the suggestions of reviewers:

Format has been updated according to reviewers' remarks.

Revision has been made according to the suggestions of the reviewer:

Reviewer 1

Major comments:

1. **The Abstract should have more detailed explanations of the methods, such as describing "BALB/c mice were divided into three groups: 8 mice with acute DSS colitis *(3.5% DSS solution, 7 days)*, 8 mice with chronic DSS colitis *(four cycles totaling 44 days of 3.5% DSS solution, 5 days + water, 6 days)* and 12 mice *without DSS supplementation* as control group…" and stating that "…cells were cultivated in the presence of mediators *(20 ug/mL of LPS, 1 mM of apocynin, 20 ug/mL LPS + apocynin)*…"**

The Abstract section has been updated. We have introduced more detailed explanation of the methods in the abstract.

"**METHODS:** BALB/c mice were divided into three groups: 8 mice with acute DSS colitis (3.5% DSS solution; 7 days), 8 mice with chronic DSS colitis (3.5 % DSS solution for 5 days + water for 6 days; 4 cycles; total: 44 days) and 12 mice without DSS

supplementation as control group. The primary colonic epithelial cells were isolated using chelation method. The cells were cultivated in the presence of mediators (lipopolysaccharide (LPS), apocynin or diphenyleneiodonium). Viability of cells was assessed by fluorescent microscopy. Production of reactive oxygen species (ROS) by the cells was measured fluorimetrically using Amplex Red. Production of tumour necrosis factor- alpha (TNF- α) by the colonic epithelial cells was analysed by ELISA. *Nox1* gene expression was assessed by real-time (RT) PCR."

2. **Representative images of cell viability assays showing viable, apoptotic, and necrotic cells for all experimental groups should be provided.**

   We have introduced new representative images of cell viability, apoptosis and necrosis as proposed.

3. **For RT-PCR experiments, the data is presented as 2 x delta Ct which is 2 x (Ct of target - Ct of housekeeping Actb). Based on this calculation, Nox1 mRNA levels in control cells seems to be on par with Actb mRNA levels, even though Nox1 is supposed to be highly expressed in colon cells. What are the endogenous levels of Nox1 in colon cells compared to housekeeping genes?**

   Housekeeping gene Actb was chosen according to the experience of other studies *(Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004; 313(4):856-862).* In our study housekeeping gene Actb was expressed evenly in all experimental mice groups (Ct value average of control group was 19.11; acute DSS colitis group - 19.24; chronic DSS colitis group – 19.32); while Nox1 gene expression was not equivalent, i.e. average Ct value in control group was 26.42, acute DSS colitis group - 26.29, chronic DSS colitis group – 23.83.

4. **Nox1 produces superoxide, which the authors assert is rapidly converted to hydrogen peroxide. Amplex Red assay measures extracellular hydrogen peroxide release but complementary assays to confirm superoxide production (and therefore, more specifically Nox1 activity), such as ESR, and intracellular hydrogen peroxide assays, such as Amplite Green, would more convincingly show Nox1-mediated oxidant generation.**

   The literature data analysis revealed that Amplex Red assay is widely used to assess the Nox activity in cells (*Park HS, Lee SH, Park D, Lee JS, Ryu SH, Lee WJ, Rhee SG, Bae YS. Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H2O2. Mol Cell Biol. 2004 May; 24(10):4384-4394. Arnold RS, Shi J, Murad E, Whalen AM, Sun CQ, Polavarapu R, Parthasarathy S, Petros JA, Lambeth JD. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. Proc Natl Acad Sci USA. 2001;98(10):5550-5555. Ramonaite R, Skieceviciene J, Kiudelis G, Jonaitis L, Tamelis A, Cizas P, Borutaite V, Kupcinskas L. Influence of NADPH oxidase on*

inflammatory response in primary intestinal epithelial cells in patients with ulcerative colitis. BMC Gastroenterol 2013;13(1):159).

We agree that additional measurements to confirm superoxide or intracellular hydrogen peroxide production in colon epithelial cells would be valuable. Our study design did not include these experiments; however, existing data suggest that superoxide is rapidly converted to hydrogen peroxide and is less stable than hydrogen peroxide. Moreover, hydrogen peroxide can easily move through the cells membrane, therefore the exact evaluation of intracellular hydrogen peroxide might be inaccurate. This explanation has been added in the paper ("We believe that expression changes of other NADPH oxidase homologs and evaluation of superoxide in primary epithelial cells during colon inflammation should be identified in novel studies").

5. **Although Nox1 may be the highly expressed Nox protein in the colon, Nox4 expression and activity has been shown to be increased in the epithelial cells in colon cancer. Since Nox4 is primarily responsible for hydrogen peroxide production, Nox4 expression in both of these acute and chronic DSS colitis models should be evaluated.**

Numerous studies show that Nox1 is highly expressed in the colon, particularly in the colon epithelial cells (*Juhasz A, Ge Y, Markel S, Chiu A, Matsumoto L, van Balgooy J, Roy K, Doroshow JH. Expression of NADPH oxidase homologues and accessory genes in human cancer cell lines, tumours and adjacent normal tissues. Free Radic Res. 2009;43(6):523-532; Szanto I, Rubbia-Brandt L, Kiss P, Steger K, Banfi B, Kovari E, Herrmann F, Hadengue A, Krause KH. Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease.J Pathol 2005; 207(2):164-176). Valente AJ, Zhou Q, Lu Z, He W, Qiang M, Ma W, Li G, Wang L, Banfi B, Steger K, Krause KH, Clark RA, Li S. Regulation of NOX1 expression by GATA, HNF-1alpha, and Cdx transcription factors. Free Radic Biol Med 2008; 44(3):430-443).* We agree that measurement of other genes responsible for hydrogen peroxide production would be of interest within this setting. On the other hand, we didn't find information that Nox4 might be expressed in colon or primary colonic epithelial cells during inflammation. Accordingly, the design of our study did not include measurement of Nox4 in our mice model. Measurement of other Nox4 expression in acute and chronic DSS colitis models would be of interest in further studies. This explanation has been added in the paper ("The results of this study showed that epithelial NADPH oxidase is directly involved in chronic colon inflammation; however, stimulation by bacterial products is required for NADPH oxidase activation during acute colitis. In both cases, a molecular mechanism for activation of NADPH oxidase is similar. A signalling cascade for activation of NADPH oxidase in colonic epithelial cells might be associated with a toll-like receptor (TLR) pathway, where LPS strains potently stimulate ROS production by Nox1 through a TLR4 [9, 33-34]. Further studies should be designed to determine the role and exact mechanism of LPS/TLR4/TNF-α/NOX signalling in colon epithelium in acute and chronic colitis. We believe that expression changes of other Nox homologs and

evaluation of superoxide in primary epithelial cells during colon inflammation should be identified in novel studies").

6. **Neither apocynin nor DPI are specific inhibitors of Nox1. Studies show that apocynin has antioxidant properties and DPI is non-specific flavoenzyme inhibitor. Nox1-null BALB/c mice or Nox1 siRNA experiments would be more definitive in implicating Nox1 in DSS-mediated epithelial colon cell derangements.**

   We agree that apocynin and DPI are not specific inhibitors of Nox1 enzyme. These substances, however, are widely used as inhibitors of Nox in many experimental models involving phagocytic and nonphagocytic cells. Different studies show that antioxidant properties of apocynin and DPI can be associated with reduced level of ROS via Nox. *(Stefanska J, Pawliczak R. Apocynin: molecular aptitudes. Mediators Inflamm. 2008:106507; Zhang Y, Chan MMK, Andrews MC, Mori TA, Croft KD, McKenzie KU, Schyvens CG, Whitworth JA. Apocynin but not allopurinol prevents and reverses adrenocorticotropic hormone - induced hyper-tension in the rat. Am. J. Hypertens 2005; 18: 910–916; Barbieri SS, Cavalca V, Eligini S, Brambilla M, Caiani A, Tremoli E, Colli S. Apocynin prevents cyclooxygenase 2 expression in human monocytes through NADPH oxidase and glutathione redox- dependent mechanisms. Free Radic. Biol. Med 2004 37: 156 –165; Chen JR, Lazarenko OP, Shankar K, Blackburn ML, Lumpkin CK, Badger TM, Ronis MJ.Inhibition of NADPH oxidases prevents chronic ethanol-induced bone loss in female rats.J Pharmacol Exp Ther. 2011; 336(3):734-742; Kono H, Rusyn I, Uesugi T, Yamashina S, Connor HD, Dikalova A, Mason RP, Thurman RG. Diphenyleneiodonium sulfate, an NADPH oxidase inhibitor, prevents early alcohol-induced liver injury in the rat. Am J Physiol Gastrointest Liver Physiol. 2001;280(5):G1005-1012 Katsuyama M. NOX/NADPH oxidase, the superoxide-generating enzyme: its transcriptional regulation and physiological roles. J Pharmacol Sci 2010; 114(2):134-146).* Our results as well as other studies revealed that primary colonic epithelial cells have limited viability due to rapid necrosis and degeneration processes i.e. optimum cultivation period of primary cells was 24 hours with a maximum of 48 hours. Available data show that Nox1 siRNA experiments require considerably longer incubation than 24 or 48 hours (*Kaeffer B. Mammalian intestinal epithelial cells in primary culture: a mini-review. In Vitro Cell Dev Biol Anim. 2002;38(3):123-134. Pedersen G, Saermark T, Giese B, Hansen A, Drag B, Brynskov J. A simple method to establish short-term cultures of normal human colonic epithelial cells from endoscopic biopsy specimens. Comparison of isolation methods, assessment of viability and metabolic activity. Scand J Gastroenterol 2000;35(7):772-780. Seidelin JB, Horn T, Nielsen OH. Simple and efficient method for isolation and cultivation of endoscopically obtained human colonocytes. Am J Physiol Gastrointest Liver Physiol. 2003;285(6):G1122-1128).* Nevertheless, further more sophisticated studies including Nox1-null BALB/c mice and modified Nox1 siRNA experiments should be designed to elucidate the exact role of Nox1 in the pathogenesis of colitis.

7. **The authors use DPI in Amplex Red experiments because apocynin interferes with the assay, but does DPI have the same effects as apocynin on cell viability and TNF-alpha?**

Estimation of DPI effect on cell viability and TNF-alpha has not been performed, because DPI is less specific inhibitor of Nox than apocynin and can inhibit other oxidases e.g. mitochondrial complex I. Accordingly, we have not evaluated the effects of DPI on cell viability or TNF-alpha concentration. This explanation has been added in the paper ("Apocynin interferes with detection of ROS in assay systems selective for hydrogen peroxide or hydroxyl radicals. This inhibitor acts as a radical scavenger and inhibits Amplex Red oxidation. Thus ROS are not measured accurately and cannot reflect the effect of apocynin on the NADPH oxidase activity [16]. Therefore, for the assessment of NADPH oxidase activity we applied another large-spectrum inhibitor DPI. Estimation of DPI effect on cell viability and TNF-alpha has not been performed, because DPI is less specific inhibitor of Nox than apocynin and can inhibit other oxidases e.g. mitochondrial complex I [3]").

8. **The authors describe more severe clinical symptoms during acute DSS colitis compared to the chronic DSS colitis model, yet Nox1 expression was increased in the cells of the chronic DSS colitis model compared to the acute. Can the authors comment on the relative importance of Nox1 in the consequent clinical symptoms associated with colitis in these models? Are there other mediators of hydrogen peroxide generation that may be involved?**

Increased production of hydrogen peroxide in colon might be associated with mitochondrial electron-transport chain activity, lipoxygenase, cyclooxygenase and several other mediators, which generate ROS molecules as secondary products of biochemical reactions. (*Melstrom LG, Bentrem DJ, Salabat MR, Kennedy TJ, Ding XZ, Strouch M, Rao SM, Witt RC, Ternent CA, Talamonti MS, Bell RH, Adrian TA. Overexpression of 5-lipoxygenase in colon polyps and cancer and the effect of 5-LOX inhibitors in vitro and in a murine model. Clin Cancer Res. 2008;14(20):6525-6530; Alzoghaibi MA. Concepts of oxidative stress and antioxidant defense in Crohn's disease.World J Gastroenterol. 2013 Oct 21;19(39):6540-6547; Khansari N, Shakiba Y, Mahmoudi M. Chronic inflammation and oxidative stress as a major cause of agerelated diseases and cancer. Recent Pat Inflamm Allergy Drug Discov. 2009 Jan;3(1):73-80; Travica S, Pors K, Loadman PM, Shnyder SD, Johansson I, Alandas MN, Sheldrake HM, Mkrtchian S, Patterson LH, Ingelman-Sundberg M.Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins.Clin Cancer Res. 2013 Jun 1;19(11):2952-2961; Terada LS. Specificity in reactive oxidant signaling: think globally, act locally. J Cell Biol. 2006;174(5):615-623*). However, NADPH oxidase homologs generate a higher level of ROS in the colon during inflammation compared to other mediators. The literature data analysis showed that NADPH oxidase is involved in the molecular mechanism of colon inflammation. The results of this study showed that a molecular mechanism for activation of NADPH oxidase is similar in both acute and chronic colon inflammation. However, clinical symptoms of colitis in mice (body weight, diarrhoea, rectal bleeding) didn't correlate

with Nox1 expression. We hypothesize that increased level of ROS via Nox might be associated with the initiation and progression of chronic colon inflammation.

Minor comments:

1. **There is a typo error in the Discussion section (pg. 12) where "apocynin increased cell viability and *decreased* TNF-a…"**

   The note has been changed as proposed ("apocynin increased cell viability and decreased TNF-α" has been corrected to "apocynin increased cell viability and decreased TNF-α").

2. **The figure legends of Figs. 2 and 6 state that the mice are 6-8 weeks old, but they are actually older than that in the chronic DSS model. It is more appropriate to label these as "Figure 2. Expression of *Cd68*, *Cd3e*, *Ptprc*, and *Vill* in the colonic epithelial cells of male BALB/c mice." and "Figure 6. Expression of *Nox1* in the colonic epithelial cells of male BALB/c mice."**

   The legends of the figures have been changed as proposed:
   - "Figure 2. Expression of *Cd68*, *Cd3e*, *Ptprc*, and *Vill* in the colonic epithelial cells of 6-8 week-old male Balb/c mice" has been corrected to "Figure 2. Expression of *Cd68*, *Cd3e*, *Ptprc*, and *Vill* in the colonic epithelial cells of male Balb/c mice".
   - Figure 6. Expression of *Nox1* in the colonic epithelial cells of 6-8 week-old male Balb/c mice. has been corrected to "Figure 6. Expression of *Nox1* in the colonic epithelial cells of male Balb/c mice"

Reviewer 2

Major concerns:

1. **Why did the authors decide to use 12 animals for control whereas 8 animals in the treatment group? Also, how many animals per cage were housed?**

   The number of mice was chosen based on the design of other similar studies (*Axelsson LG, Landström E, Goldschmidt TJ, Grönberg A, Bylund-Fellenius AC.Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice: effects in CD4(+) -cell depleted, athymic and NK-cell depleted SCID mice.Inflamm Res. 1996; 45(4):181-191. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H.A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate.Cancer Sci. 2003; 94(11):965-973. Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N.Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis.J Clin Invest. 2008 Feb;118(2):560-570)* and animals ethics committee recommendations. We agree that higher number of mice within separate study groups

would add additional power to our study, however, statistical analysis of our data revealed significant differences between the groups.
The animals were kept in the vivarium of Lithuanian Veterinary Academy. Mice were housed in individual plastic cages (1 mouse per cage). This explanation has been added to the paper.

2. **Statistical analysis of histological analysis of colitis is required.**

Statistical analysis of histological examination of colitis has been performed.

"***Histological assessment of colon inflammation in mice.*** In mice with acute DSS colitis we observed major epithelium damage with loss of crypts in large areas (3.8±0.42, *P*=0.01) and inflammatory cell infiltration of *L. submucosa* (3.9±0.35, *P*=0.01) (Fig. 1B). In mice with chronic DSS, loss of goblet cells in large areas (2.9±0.19, *P*=0.02) and inflammatory cells infiltration of *L. muscularis mucosae* (2.2±0.30, *P*=0.02) was observed (Fig. 1C). Control mice had no histological alterations in the colon tissues (Fig. 1A)"

3. **The authors have mentioned the use of only one endogenous control gene for their RT-PCR experiment. Can the authors confirm if ACTB expression was constant in all samples?**

The same concern has been raised by Reviewer #1. Therefore, we refer to our response above (Reviewer #1; Major comments; no 3).

4. **The authors, in the introduction part, talk about the effect of pro-inflammatory cytokines on NADPH oxidase expression in intestinal epithelial cells. How does regulatory as well as anti-inflammatory cytokines affect NADPH oxidase?**

Several studies have shown that pro-inflammatory cytokines such as interleukin-18 (IL-18), interferon gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) can stimulate the NADPH oxidase expression and ROS production in the intestinal epithelial cell cultures *in vitro*. While, anti-inflammatory cytokines (interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β)) effectively block the stimulatory actions of pro-inflammatory cytokines and decreased NADPH oxidase activity colon cancer cell lines *(Kamizato M, Nishida K, Masuda K, Takeo K, Yamamoto Y, Kawai T, Teshima-Kondo S, Tanahashi T, Rokutan K.Interleukin 10 inhibits interferon gamma- and tumor necrosis factor alpha-stimulated activation of NADPH oxidase 1 in human colonic epithelial cells and the mouse colon. J Gastroenterol. 2009;44(12):1172-1184; Rokutan K, Kawahara T, Kuwano Y, Tominaga K, Nishida K, Teshima-Kondo S.Nox enzymes and oxidative stress in the immunopathology of the gastrointestinal tract. Semin Immunopathol. 2008; 30(3):315-327).* This explanation has been added to the paper ("Several studies have shown that bacterial products and pro-inflammatory cytokines such as interleukin-18 (IL-18),

interferon gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) can stimulate the NADPH oxidase expression and ROS production in the intestinal epithelial cell cultures *in vitro* [9, 10]. Anti-inflammatory cytokines (interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β)) effectively block the stimulatory actions of pro-inflammatory cytokines and decreased NADPH oxidase activity colon cancer cell lines [9, 10]").

5. **The authors do not specify the experimental unit in their statistical analysis section. This concern is also related to the point no. 2. If each cage was considered as an experimental unit then how many animals were housed per cage? Depending upon the experimental unit, the "n" used for the statistical analysis will change.**

   Experimental units have been included in the paper.

6. **In table 1, the authors state that the length of colon in control vs. chronic DSS colitis is significant. Please check. Also, it's the ratio of weight over length which has to be shown. A small description of Bristolscale in materials and methods or result section will be helpful for readers to understand the scale.**

   Table 1 has been updated according to the reviewer remarks. We have included the ratio "Weight of colon (mg)/ Length of colon (cm)" and short description of Bristol scale.

7. **In the section where the purity of isolated cells is assessed, the authors do not clearly mention details of the markers used. How is it possible to have Tcell marker and monocyte/macrophage marker expression levels same when compared between healthy controls vs. acute or chronic DSS colitis mice (Figure 2) since colitis is mediated by T cells? This is not explained by the authors anywhere in the text.**

   "Assessment of purity of isolated epithelial cell culture" section has been updated according to the reviewer remarks.

   "The purity of the primary colonic epithelial cell culture was assessed using the expression analysis of genes markers (*Ptprc* was hematopoietic cell marker; *Cd3e* - T cells marker; *Cd68* - monocytes/ macrophages marker; *Vill* - epithelial cell marker). These markers were expressed equally in cell cultures obtained from all three experimental groups. These findings confirmed the purity of the primary colonic epithelial cell culture *i.e.* inflammatory cells did not affect ROS and TNF- α production or Nox1 expression"

8. **It is difficult to keep isolated cells in good shape after 3 to 4 h culture. Experiments performed here are beyond this time lapse. The presentation of cell viability data (figure 3) is confusing. One easier way to present this data is setting unstimulated**

control as 100% and presenting other results relative to the unstimulated control results. The statistical significance presented in the figure is hard to follow.

Mice colonic epithelial cells were counted in at least 5 microscopic fields per well (three wells per treatment). Data are expressed as percentage of viable, necrotic or apoptotic cells of the total number of cells per field. We provided only percentage of viable cells in the figure 3. However, Figure 3 has been changed according to reviewer #1 remark (no. 2). We have introduced new representative images of cell viability (with the percentage of viable, necrotic and apoptotic cells) and explanations.

9. **The authors in their cell viability result section state that there is a significant increase in cell viability when LPS is compared to apoc+LPS. This function of Apoc is not discussed later. Does Apoc inhibit cell degeneration in chronic colitis? What mechanism could possibly be involved in this process? Also care should be taken in interpreting the increase in % of viable cells due to Apoc treatment. Unregulated increase in cell viability/ cell proliferation is unwanted as it may indicate cancer.**

We have included more detail interpretation of possible effects of apocynin on colon epithelium. We hypothesized that protective mechanism of apocynin might be associated not only with anti-inflammatory action of this inhibitor but also with the decreased ROS generation via NADPH oxidase and reduced oxidative stress in cells.

The literature data analysis shows that apocynin has very low toxicity *(Stefanska J, Pawliczak R. Apocynin: molecular aptitudes. Mediators Inflamm 2008; 2008:106507; Gambaro G, Ferraro PM, D'Addessi A. Ayurvedic medicine and NADPH oxidase: a possible approach to the prevention of ESRD in hyperoxaluria. Nephrol Dial Transplant 2011; 26(6):1759-1761).* In our study in chronic DSS colitis group, increased viability of apocynin-treated cells was related to decreased necrosis, *i.e.* apocynin increased the viability of cells (to 68%) and percentage of viable cells was similar to the control group (66%). Therefore, we believe that apocynin could not cause unregulated increase in cell viability.

10. **There is no analysis or discussion on possible regeneration of epithelium.**

Discussion on regeneration of epithelium has been included in the paper.

"Recently, a similar effect of apocynin was observed in the human primary colonic epithelial cells of patients with ulcerative colitis [31]. However, apocynin effect on colon epithelium regeneration must be investigated in the future."

11. **The presentation of data in figure 4 is very confusing as well as the statistical differences stated in the figure. It is not clear why the authors have evaluated the amount of hydrogen peroxide in cells and biopsies? Please explain.**

NADPH oxidase generates superoxide, which is rapidly converted to hydrogen peroxide; therefore our study design included evaluation of hydrogen peroxide production in order to assess NADPH oxidase activity in cells and biopsies. In this study, level of NADPH oxidase activity was not measured directly. However, we can assess the levels of NADPH oxidase activity indirectly *i.e.* measuring the production of hydrogen peroxide. It is known, that ROS generation activity correlates with NADPH oxidase activity *(Cariello M, et al.: Coagulation activation is associated with nicotinamide adenine dinucleotide phosphate oxidase-dependent reactive oxygen species generation in hemodialysis patients. Antioxid Redox Signal. 2012, 16(5):428-439)*; *Ramonaite R, Skieceviciene J, Kiudelis G, Jonaitis L, Tamelis A, Cizas P, Borutaite V, Kupcinskas L. Influence of NADPH oxidase on inflammatory response in primary intestinal epithelial cells in patients with ulcerative colitis. BMC Gastroenterol 2013;13(1):159; Park HS, Lee SH, Park D, Lee JS, Ryu SH, Lee WJ, Rhee SG, Bae YS. Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H2O2. Mol Cell Biol. 2004 May; 24(10):4384-4394. Arnold RS, Shi J, Murad E, Whalen AM, Sun CQ, Polavarapu R, Parthasarathy S, Petros JA, Lambeth JD. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. Proc Natl Acad Sci USA. 2001;98(10):5550-5555*).

12. **In figure 5, the data presented in each group is highly variable, as indicated by their error bars. Please explain this high variability within group. Unstimulated Chronic DSS colitis vs. LPS stimulated Chronic DSS colitis is significantly not different.**

Regardless of high variability, we identified a statistically significant difference between untreated and LPS treated cells in the chronic DSS colitis group. We believe that individual differences in sensitivity to LPS might be associated with high variability of our data within chronic DSS colitis group (The concentration average of TNF-α in untreated cells was 286.9 pg/ml/10^7 cells (Max=315.1 pg/ml/10^7cells; Min=241.8 pg/ml/10^7cells); while concentration average of TNF-α in LPS treated cells was 631.3 pg/ml/10^7 cells (Max= 897.7 pg/ml/10^7cells, Max=459 pg/ml/10^7cells)).

13. **The authors have seen no effect on TNF-a production by LPS treated control epithelial cells. Does this indicate that the cells were not stimulated by LPS? How do the authors explain this phenomenon, since LPS is a well-established agent to induce inflammation in…**

Intestinal barrier formed mostly by intestinal epithelial cells is a dynamic system, which comes into close contact with different commensal and pathogenic bacteria; therefore these epithelial cells evolutionary are adapted to interact with bacteria products and support the oral tolerance and intestinal homeostasis in the normal conditions. On the other hand, given the importance of the epithelium in intestinal immune regulation mechanisms, it is clear that loss of oral tolerance during inflammation contributes to maintenance of inflammatory response in gut and induced increased sensitivity to

bacterial products in intestinal epithelial cells *(Roda G, Sartini A, Zambon E, Calafiore A, Marocchi M, Caponi A, Belluzzi A, Roda E. Intestinal epithelial cells in inflammatory bowel diseases. World J Gastroenterol 2010; 16(34):4264-4271; Sharma R, Young C, Neu J. Molecular modulation of intestinal epithelial barrier: contribution of microbiota. J Biomed Biotechnol 2010; 2010:305879; Ismail AS, Hooper LV.Epithelial cells and their neighbors. IV. Bacterial contributions to intestinal epithelial barrier integrity. Am J Physiol Gastrointest Liver Physiol 2005; 289(5):G779-G784).*

Accordingly, colon epithelial cells were stimulated by LPS; however we did not determinate significantly changes of TNF-a production in the control group. We think that total concentrations of exogenous LPS in colon epithelial cells was too low.

Reviewer 3

1. **Abstract. This abstract comprehend 271 words. It is recommended that the authors mentioned in the abstract the name of the NADPH oxidase inhibitors that used in the experiment.**

   Abstract has been shortened as suggested (269 words).
   The names of NADPH oxidase inhibitors have been included in the abstract.

2. **Background:**
   In the title the authors present the role of NADPH oxidase in the pathogenesis of colon inflammation in mice. However, in the results, conclusions of the abstract, and also in the introduction they focus the attention in the treatment of NADPH oxidase inhibitors as a protective effect against pro-inflammatory action of lipopolysaccharides (LPS) in colonic epithelium cells of mice with DSS colitis. So it is recommended that the authors to consider a brief modification of the title of the manuscript. Because the title is not only focus is the pathogenesis of NADPH oxidase in the colon inflammation. The reasons are the following: 1) the problem statement (introduction last paragraph) the question of the molecular pathways that control the production of ROS through the products presented in NOX enzymes in the cells of the intestinal epithelium during acute and chronic inflammation.

   The notes have been changed as proposed ("The role of NADPH oxidase in the pathogenesis of colon inflammation in mice" has been corrected to "Protective action of NADPH oxidase inhibitors and the role of NADPH oxidase in the pathogenesis of colon inflammation in mice").

3. **Methods. It is advisable to more clearly describe the methodology definitions of acute and chronic inflammation for the times that were used in the experiment (Second paragraph of methodology, lines 5-7.) In the part of methodology, it is recommended that the authors describe, if the data followed normal distribution and the reasons why the results reported as standard errors and not confidence intervals or standard deviations.**

"Induction of DSS colitis" section has been updated according to the reviewer remarks. We have introduced more explanation on the induction of DSS colitis.

"***Induction of DSS colitis.*** Colon inflammation for BALB/c mice was induced by oral administration of 3.5 % DSS dissolved in the distilled drinking water and supplied *ad libitum* (molecular mass 40 kDa, TdB Consultancy, Uppsala, Sweden). We used a protocol that was established in Wirtz *et al.* study [11], which was slightly modified as follows: animals were divided into three study groups: 8 mice with acute DSS colitis (mice were given 3.5 % DSS in the drinking water over 7 days; 1 cycle; total number of days: 7), 8 mice with chronic DSS colitis (mice were given 3.5 % DSS in the drinking water over 5 days and water for 6 days; this cycle was repeated 4 times; total number of days: 44), and 12 mice as control group without DSS supplementation."

4. **Results. The title of Table 1, is very brief should be completed by noting more information. Writing in Table 1, if the data are presented as mean and SE of the mean. The results of assessment of faeces (points), rectal bleeding and mortality, describe the results in percentages in parentheses. Adequately indicate statistically significant differences with the values of "p". For example what is the value of "p" in the asterisk, and which is the # symbol? It is advisable to write at the bottom of Table 1, the initials that were used. For example, DSS.**
   Complete the subtitle of the second paragraph of results, noting that it is the inflammation of the intestinal epithelial cells.
   Explain in more detail how the statistical comparisons between groups of acute and chronic inflammation, and the control group were done. It is recommended to the authors write in all the figures, if the data are represented as mean values and standard errors.

   The notes have been changed as proposed:
   - Explanations of all figures and Table 1 have been updated according to reviewer remarks.
   - Subtitle of the second paragraph has been corrected ("Assessment of histological score in mice" to "Histological assessment of colon inflammation in mice").

5. **Discussion. The discussion is adequate**

6. **Conclusions. The findings would be largely appropriate if the title is amended as recommended above.**

   The title of paper has been changed as proposed (see above; Reviewer #3; no 2).

References and typesetting were corrected. We have been included new reference (Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. Scand J Gastroenterol 1997; 32(9):920-924 [PMID: 9299672])

We would like to thank reviewers for their constructive comments. We have revised the paper and we would like to resubmit the revised version for your consideration for publication in *World Journal of Gastroenterology*.

Sincerely yours,

Limas Kupcinskas, MD, Professor of Medicine
Institute for Digestive Research
Lithuanian University of Health Sciences
Mickeviciaus str. 9, LT-44307, Kaunas, Lithuania.
E-mail l.kupcinskas@gmail.com;