Response to Reviewers

We appreciate the comments and concerns of the reviewer.

MAJOR POINTS:

1. Author's main hypothesis is based on the idea that intracellular GSH levels in the intestinal mucosa are decreased after fasting and oral administration of GSH has been previously shown to restore its levels in deficient tissues. Conversely, they found that oral administration of two doses of GSH further decreases intracellular GSH levels in the jejunum, which correlates with a downregulation of Ggt1 (the enzyme that breaks down extracellular GSH to produce the cysteine used in the cells to resynthesize GSH). An action in extracellular ROS at the intestinal lumen has been proposed but there is not experiments in this study showing this fact, taken into account that every measurement has been performed in mucosal tissues.

(1) How can we explain that oxidative DNA damage (as indicative of intracellular ROS level) is reduced after GSH administration if intracellular GSH levels are lower than in control mice?

Response:

Fasting significantly decreased jejunal GSH concentrations and we found that they were further significantly decreased by oral GSH supplementation during fasting. The jejunal concentrations of the GSH oxidation product GSSG were not changed by any feeding regime. Furthermore, oral GSH treatment during fasting significantly decreased the fasting-induced ROS levels in the jejunum, but we do not consider that this depends on antioxidant effects given the depressed GSH concentration in the jejunum. Because oral GSH treatment during fasting significantly decreased both jejunal GSH concentration and *Ggt1* mRNA expression, the supplemented GSH in the intestinal lumen may not be involved in intracellular *de novo* GSH synthesis.

The ability of GSH to control extracellular redox provides another possible role for intestinal luminal GSH during fasting as the present study demonstrated that intracellular GSH of the jejunum mucosa was not induced by intestinal luminal GSH administration during fasting and that the GSH administration could attenuate the

fasting-induced ROS in the intestinal mucosa. We considered that intestinal lumen GSH might control the redox status.

We have revised the original manuscript as follows:

1) Original manuscript: DISCUSSION, P17, L483–484

……oxidation product GSSG was not changed by any feeding regime (Figure 10(B)). These results demonstrate that oral GSH administration……

Revised manuscript: DISCUSSION, P16, L480–483

…… oxidation product GSSG were not changed by any feeding regime (Figure 9B). Furthermore, oral GSH treatment during fasting significantly decreased the fasting-induced ROS levels in the jejunum (Figure 6), but we consider it unlikely that this effect depends on antioxidant activity given the depressed GSH concentration in the jejunum. These results demonstrate that oral GSH administration……

2) Original manuscript: DISCUSSION, P18, L500–505

……to such adverse conditions. Because ROS occurs in the jejunum during fasting, this may represent another factor underlying the up-regulation of *Ggt1* mRNA expression. However, considering that oral GSH treatment during fasting significantly decreased both jejunal GSH concentration and *Ggt1* mRNA expression, this suggests that the supplemented GSH in the intestinal lumen may not be involved in intracellular de novo GSH synthesis.

Revised manuscript: DISCUSSION, P17, L500–504

……to such adverse conditions. Similarly, we found that fasting increased jejunal ROS levels and up-regulated *Ggt1* mRNA expression in the jejunum in this study. However, considering that oral GSH treatment during fasting significantly decreased both jejunal GSH concentration and *Ggt1* mRNA expression, the supplemented GSH in the intestinal lumen may not be involved in intracellular *de novo* GSH synthesis.

3) Original manuscript: DISCUSSION, P18, L515

……thiol/disulfide pairs. In addition, the roles of……

Revised manuscript: DISCUSSION, P17, L514–520

…… thiol/disulfide pairs. Biliary GSH is an important intestinal luminal source of GSH and attenuates ROS of the intestinal mucosa by increasing the GSH levels of the intestinal mucosa. However, it has been suggested that biliary GSH controls the status of the intestinal lumen and contributes to protection of the intestinal mucosa[10]. The GSH of the intestinal lumen may act on the intestinal mucosa via both of these two pathways, and it is important to consider the control of the redox status of the intestinal lumen. In addition, the roles……

(2) What is the link between extracellular GSH actions and the expression of iNOS (and subsequent NO production)?

Response:

The luminal/extracellular redox environment is determined by the cysteine/cystine (Cys/CySS) redox pair with contributions from the GSH system, with the majority of Cys in the intestinal lumen originating from GGT enzymatic hydrolysis of GSH obtained from oral GSH administration and biliary supply. Intestinal cell proliferation and/or apoptosis is associated with quantitative changes in the redox potential (Eh) of the extracellular GSH/GSSG or Cys/CySS redox pair. iNOS expression and NO production are involved in the mechanism of FasL upregulation and Fas-mediated apoptosis. The iNOS–NO–ROS–FasL pathway represents a potential link between the apoptosis and intestinal atrophy observed during fasting. In addition, the change in the extracellular thiol/disulfide redox in response to peptide growth factors indicates an interaction of growth factor-activated pathways and thiol/disulfide metabolism during intestinal cell proliferation.

We have revised the original manuscript as follows:

1) Original manuscript: DISCUSSION, P19, L535–547

……apoptotic pathway including induced expression of FasL, Fas, and TNFR1.

In comparison, in the present study, fasting-induced intestinal mucosal atrophy resulting from increased apoptosis was caused by increased production of NO and ROS as apoptosis mediators following elevation of iNOS expression. Because ROS is induced by NO produced by iNOS and iNOS is induced by ROS, oral GSH

treatment during fasting might inhibit iNOS and NO generation following the decrease of ROS production via Fas and consequently leading to decreased apoptosis in the jejunum. Moreover Jonas et al.[53] showed that the extracellular thiol/disulfide redox state modulates cell proliferation rate and that this system interacts with growth factor signaling in a human colon carcinoma cell line. Thus, the change in extracellular thiol/disulfide redox in response to peptide growth factors indicated an interaction of growth factor-activated pathways and thiol/disulfide metabolism during intestinal cell proliferation.

Therefore, the oral GSH administration during fasting may regulate the redox state of the……

Revised manuscript: DISCUSSION, P18, L540–P19, L559

……FasL, Fas, and TNFR1. Selleri et al.[53] and Viard-Leveugle et al.[54] provided evidence that iNOS expression and NO production are involved in the mechanism of FasL upregulation and Fas-mediated apoptosis. The iNOS–NO–ROS–FasL pathway represents a potential link between the apoptosis and intestinal atrophy observed in the fasting.

In the present study, fasting-induced intestinal mucosal atrophy resulting from increased apoptosis was caused by increased production of NO and ROS as apoptosis mediators following elevation of iNOS expression. Because the iNOS–NO–ROS–FasL pathway is part of the apoptotic mechanism in the intestinal mucosa atrophy occurring after fasting[22,52-54], oral GSH treatment during fasting might inhibit Fas-mediated apoptosis following reduction of ROS levels in the jejunal mucosa resulting from Eh changes mediated by GSH and Cys in the intestinal lumen. Additionally, because iNOS protein expression was also induced by ROS produced from NO in fasting-induced intestinal mucosal atrophy in a previous study[20], the decrease in ROS levels in the intestinal mucosa reduces iNOS protein expression and NO production. Moreover, Jonas et al.[55] showed that the extracellular thiol/disulfide redox state modulates cell proliferation and that this system interacted with growth factor signaling in a human colon carcinoma cell line. Thus, the change in extracellular thiol/disulfide redox state in response to peptide growth factors indicated an interaction of growth factor-activated pathways and

thiol/disulfide metabolism during intestinal cell proliferation.

Therefore, oral GSH administration during fasting may regulate the……

53 Selleri C, et al. Induction of nitric oxide synthase is involved in the mechanism of Fas-mediated apoptosis in haemopoietic cells. Br J Haematol 1997; 99: 481-489

54 Viard-Leveugle I, et al. TNF-α and IFN-γ are potential inducers of Fas-mediated keratinocyte apoptosis through activation of inducible nitric oxide synthase in toxic epidermal necrolysis. J Invest Dermatol 2013; 133: 489-498

(3) Since there is not a measurement of luminal ROS and intracellular ROS and NO synthesis can only reflect the level of damage in the intestinal mucosa, we cannot assume a specific effect of oral GSH. In conclusion, more experiments (in the discussion authors proposed some cellular pathways that could be involved, such as Fas and growth factors signaling) would be necessary to understand the mechanism that leads oral GSH administration to ameliorate mucosal atrophy in the jejunum.

Response:

The GSH of the intestinal lumen, which considered to be biliary GSH, acts on the intestinal mucosa via two pathways, and it is important to consider the possibility that it controls the redox status of the intestinal lumen. We prepared a schematic diagram (added as Figure 11) to show the protective effects of GSH in the intestinal lumen against fasting-induced intestinal atrophy mediated through oxidative stress. The schematic summary diagram depicts a possible role of intestinal lumen redox status in the regulation of jejunal mucosa apoptosis and cell proliferation in fasting-induced intestinal atrophy, mediated through oxidative stress.

We have revised the original manuscript as follows:

1) Original manuscript: This figure was not present.

Revised manuscript: Figure 11

Figure 11. Schematic diagram of protective effects of GSH in the intestinal lumen against fasting-induced intestinal atrophy, mediated through oxidative stress.

The schematic diagram depicts a possible role of intestinal lumen redox status in the regulation of jejunal mucosa apoptosis and cell proliferation in fasting-induced intestinal atrophy, mediated through oxidative stress. Fasting causes increased

production of NO and ROS as apoptosis mediators following elevation of iNOS expression. The changes in apoptosis and cell proliferation in the intestinal mucosa resulting from oral GSH administration during fasting may derive from intracellular ROS removal by redox potential changes mediated by GSH and Cys (originating from enzymatic hydrolysis of GSH) in the intestinal lumen. Intracellular ROS removal is considered to inhibit Fas-mediated apoptosis and increase growth factor-mediated cell proliferation.

2) Original manuscript: DISCUSSION, P19, L548–L550

……intestinal lumen and consequently may both relieve apoptosis by Fas-mediated ROS removal and increase growth factor-mediated cell proliferation in jejunal epithelial cells. However, because our present study focused on intracellular GSH, further studies……

Revised manuscript: DISCUSSION, P19, L560– L562

……intestinal lumen and consequently may both relieve Fas-mediated apoptosis and increase growth factor-mediated cell proliferation by ROS removal in jejunal epithelial cells (Figure 11). However, because our present study focused on intracellular GSH, further studies……

2. For analysis of Ggt1 and Gapdh mRNAs a real-time RT-PCR must be performed. Amplification by conventional PCR does not assure to be in the linear phase of the reaction and then it is not accurate.

Response:

We understand that you recommend the use of quantitative RT-PCR because it provides results of higher quality. However, this study has objectively evaluated the expression of mRNA by using semi-quantitative RT-PCR. In this study, we considered the use of quantitative RT-PCR to provide additional data, but we were not able to because some jejunum samples were completely consumed in the original PCR and thus not available for further analysis. Therefore, quantitative RT-PCR is desirable, but we would like to use our semi-quantitative RT-PCR results for this

manuscript.

We have revised the original manuscript as follows:

Original manuscript: Abstract, P3, L54

……in the jejunum (by RT-PCR)

: MATERIALS AND METHODS, P11, L278

……by reverse transcription (RT)-PCR

: DISCUSSION, P18, L494

……rather than RT-PCR as used……

Revised manuscript: Abstract, P3, L54

……in the jejunum (by semi-quantitative RT-PCR)

: MATERIALS AND METHODS, P10, L277

……by semi-quantitative reverse transcription (RT)-PCR

: DISCUSSION, P17, L493

……rather than semi-quantitative RT-PCR as used……

MINOR POINTS

1. In the text, it should be avoided to refer treatments as "50 GSH" or "500 GSH". It would be better to write "50 mg/kg GSH" or "500 mg/kg GSH". This is particularly important in the abstract, where the groups are not defined (and named).

Response:

We inserted "mg/kg" in the necessary parts.

We have revised the original manuscript as follows:

Original manuscript: Abstract, P3, L58–69; Materials and methods, P8, L171–173; Results, P13, L346–347, P14, L358–359, L381; Results, P15, L392, 398

Revised manuscript: Abstract, P3, L58–71; Materials and methods, P7, L170–171; Results, P12, L345-346, P13, L361–362, L378–379; Results, P14, L389, 395

2. In the abstract, it is mentioned: "both GSH concentration and Ggt1 mRNA expression decreases in the jejunum were also attenuated in rats following oral administration of GSH during fasting as compared with fasting alone". This is not

correct. The decrease in GSH concentration it is not attenuated and a further decrease is reported. Ggt1 mRNA levels are increased (not decreased) after fasting, although attenuated with oral GSH. It should be changed.

Response:

We corrected this as you suggested.

We have revised the original manuscript as follows:

Original manuscript: Abstract, P3, L66–68

……Notably, both GSH concentration and Ggt1 mRNA expression decreases in the jejunum were also attenuated in rats following oral administration of GSH during fasting as compared with fasting alone……

Revised manuscript: Abstract, P3, L68–70

……Notably, both GSH concentration and Ggt1 mRNA expression in the jejunum were also attenuated in rats following oral administration of GSH during fasting as compared with fasting alone……

3. Values shown in the abstract must have mean ± SEM

Response:

We inserted "± SEM " in the necessary parts.

We have revised the original manuscript as follows:

Original manuscript: Abstract, P3, L58–69

……compared to SA-treated animals (527.2 for 50 GSH, 567.6 for 500 GSH *vs*. 483.1 (μm), $P < 0.01$ at 72 h). This effect was consistent with decreasing changes in……fasting alone (0.45 *vs.* 0.97 (nmol/mg tissue), $P < 0.01$; 1.01 *vs.* 2.79 (*Ggt1* mRNA/*Gapdh* mRNA), $P < 0.01$ for 500 GSH at 48 h, ……

Revised manuscript: Abstract, P3, L58–72

……compared to SA-treated animals (527.2 ± 6.9 for 50 mg/kg GSH, 567.6 ± 5.4 for 500 mg/kg GSH *vs*. 483.1 ± 4.9 (μm), $P < 0.01$ at 72 h). This effect was consistent with decreasing changes in GSH-treated animals compared to SA-treated animals for iNOS protein staining (0.337 ± 0.016 for 50 mg/kg GSH, 0.317 ± 0.017 for 500 mg/kg GSH *vs*. 0.430 ± 0.023 (area of staining part/area of tissue), $P < 0.01$ at 72 h) and NO (2.99 ± 0.29 for 50 mg/kg GSH, 2.88 ± 0.19 for 500 mg/kg GSH *vs*. 5.34 ± 0.35

(nmol/g tissue), $P < 0.01$ at 72 h) and ROS (3.92 ± 0.46 for 50 mg/kg GSH, 4.58 ± 0.29 for 500 mg/kg GSH *vs.* 6.42 ± 0.52 (8-OHdG pg/μg DNA), $P < 0.01$, $P < 0.05$ at 72 h, respectively) levels as apoptosis mediators in the jejunum. Furthermore, oral GSH administration attenuated cell proliferation decreases in the fasting jejunum (182.5 ± 1.9 for 500 mg/kg GSH *vs.* 155.8 ± 3.4 (5-BrdU positive cells/10 crypts), $P < 0.01$ at 72 h). Notably, both GSH concentration and *Ggt1* mRNA expression in the jejunum were also attenuated in rats following oral administration of GSH during fasting as compared with fasting alone (0.45 ± 0.12 *vs.* 0.97 ± 0.06 (nmol/mg tissue), $P < 0.01$; 1.01 ± 0.11 *vs.* 2.79 ± 0.39 (*Ggt1* mRNA/*Gapdh* mRNA), $P < 0.01$ for 500 mg/kg GSH at 48 h, ……

4. In the introduction, the sentences "In addition, these fasting states are also accompanied by a depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa" and "Intestinal mucosal antioxidants such as GSH in particular provide critical protection against oxidative tissue injury by ROS that are present in the intestinal mucosa" are repetitive.

Response:

We deleted the repeated sentence.

We have revised the original manuscript as follows:

Original manuscript: Introduction, P5, L99–104

……In addition, these fasting states are also accompanied by a depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa[5,6]. Intestinal mucosal antioxidants such as GSH in particular provide critical protection against oxidative tissue injury by ROS that are present in the intestinal mucosa[7]. Therefore, GSH constitutes……

Revised manuscript: Introduction, P5, L101–104

……In addition, these fasting states are also accompanied by depletion of the critical antioxidant glutathione (GSH), which functions to eliminate induced ROS in the intestinal mucosa[5,6,7]. Therefore, GSH is……

5. Some figures could be reassembled. Figures 7 and 8 and Table 1 are showing the same data.

Response:

We reassembled Figures 7 and 8 into one figure in reference to similar articles [1,2] and decided to just use Table 1. We corrected the text and the explanatory notes with the reconstruction of the figures. We also changed the other figure numbers accordingly.

1 Dahly EM et al. Alterations in enterocyte proliferation and apoptosis accompany TPN-induced mucosal hypoplasia and IGF-I-induced hyperplasia in rats. *J Nutr.* 2002; 132: 2010–4.

2 Ito J et al. Fasting-induced intestinal apoptosis is mediated by inducible nitric oxide synthase and interferon-γ in rat. *Am J Physiol Gastrointest Liver Physiol*. 2010; 298: G916–26.

We have revised the original manuscript as follows:

1) Original manuscript: Figures 7 and 8, P27, L766–782

Figure 7 Representative apoptotic changes by conventional light microscopy of hematoxylin and eosin (HE)-stained sections of jejunal mucosa.

A: A jejunal villus from a 72-h fasted ……and condensed chromatin (d). Bar = 20 μm.

Figure 8 Effects of fasting and GSH treatment on apoptotic index (AI) in the jejunal villus.

AI distribution curves are shown generated …… respectively. A total of 7–8 rats were tested in each group.

Revised manuscript: Figure 7, P26, L783–796

Figure 7. Effects of fasting and GSH treatment on apoptotic index (AI) in the jejunal villus and crypt.

Representative apoptotic changes determined by conventional light microscopy of hematoxylin and eosin (HE)-stained sections of the jejunal mucosa are shown in A. Left side: A jejunal villus from a 72-h fasted rat with 500 mg/kg GSH treatment. Apoptotic cells in the villus are indicated by an arrow showing an apoptotic corpuscle (a) and condensed chromatin (b). Bar = 100 μm (low magnification). Bar =

20 μm (high magnification). Right side: Jejunal crypts from 48- and 72-h fasted rats. Apoptotic cells in the crypt are indicated by an arrow showing an intensely eosinophilic cytoplasm and nuclear fragmentation (c) and condensed chromatin (d). Bar = 20 μm. AI distribution curves in the villus and the crypt are shown in B. AI is defined as the total number of apoptotic cells at each cell position and is expressed as a percentage of the total number of cells counted at that cell position. Cell position 1 is defined as the cell at the crypt-villus junction and the cell at the base of the crypt column for the villus and crypt data, respectively. 7–8 rats were tested in each group.

2) **Original manuscript:** Results, P13, L349–P14, L371

Evaluation of enterocyte apoptosis

The representative apoptosis changes as visualized by conventional light microscopy. ……was evident at 48- and 72-h fasting periods in SA-treated groups (Figure 8B), which was diminished by GSH treatment.

Revised manuscript: Introduction, P12, L348–P13, L369

Evaluation of enterocyte apoptosis

Apoptosis was determined by histomorphometry, which is preferable to terminal deoxynucleotidyl transferase dUTP nick-end labeling for quantitative assessment. Using histomorphometric assessment of jejunal cells, we evaluated the contribution of reduced apoptosis to the recovery from fasting-induced mucosal atrophy mediated by oral GSH treatment. The representative apoptosis changes as visualized by conventional light microscopy of H&E-stained specimens are shown in Figure 7A. AI distribution profiles in the villi and the crypt are also shown in Figure 7B. From the AI distribution profiles, increased apoptosis in the lower half of the villus (cell positions 1 to 40) was evident with 48 and 72 h of fasting in the SA-treated groups, and this increase was diminished by GSH treatment. In the SA-treated groups, fasting significantly increased jejunal villus AI with 48 and 72 h of fasting compared with that in the normally fed controls ($P < 0.01$, Table 1). GSH treatment significantly decreased the fasting-induced enhancement of villus AI compared with that in the respective SA-treated group for each fasting period ($P < 0.05$ for 50 mg/kg GSH vs. SA with 48 or 72 h of fasting, $P < 0.01$ for 500 mg/kg GSH vs. SA with 48 or 72 h of

fasting). From the AI distribution profiles, increased apoptosis in the lower two-thirds of crypts (cell positions 1 to 20) was evident with 48 and 72 h of fasting in the SA-treated groups (Figure 7B), and this effect was diminished by GSH treatment. In the SA-treated groups, fasting significantly increased jejunal crypt AIs with 48 and 72 h of fasting compared with the normally fed controls ($P < 0.01$, Table 1). GSH treatments significantly ameliorated the fasting-induced increase in crypt AI compared with the respective SA-treated group for each fasting period ($P < 0.01$).