RSC Advances

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Hydrogen peroxide-induced oxidative stress activates NF-κB and Nrf2/keap1 signals and triggers autophagy in piglets

Jie Yin[1,2#], Jielin Duan[1,2#], Zhijie Cui[1,2], Wenkai Ren[1], Tiejun Li[1*], Yulong Yin[1*]

[1] Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Hunan Provincial Engineering Research Center of Healthy Livestock, Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, Hunan 410125, China; E-Mails: yinjie2014@126.com (J.Y.); 791292383@qq.com (J.D.); xiaocui.huoying@qq.com (Z.C.); renwenkai19@126.com (W.R.); tjli@isa.ac.cn (T.L.); yinyulong@isa.ac.cn (Y.Y.)

[2] University of Chinese Academy of Sciences, Beijing 100039, China;

[#] These authors contributed equally to this work.

[*] Author to whom correspondence should be addressed; E-Mails: tjli@isa.ac.cn (T.L.); yinyulong@isa.ac.cn (Y.Y.)

Abstract: In various pathological conditions of tissue injury, oxidative stress is often associated with autophagy. However, the H_2O_2-induced oxidative stress initiates autophagy and its molecular mechanism is still obscure. Here we report that intragastric and peritoneal administration of H_2O_2 caused different degrees of oxidative stress and changed intestinal permeability, morphology, and barrier function in piglets. Western blotting studies revealed that H_2O_2 increased the autophagosome-related protein LC3-I and LC3-II abundance and the ration of LC3-II to LC3-I content after exposure to 10% H_2O_2 in the jejunum. Meanwhile, the data from beclin1 also indicated that H_2O_2 initiated autophagy in response to oxidative stress. In addition, H_2O_2 activates the NF-κB and Nrf2/keap1 signals, which may involve in H_2O_2-induced autophagy. In conclusion, administration of H_2O_2 caused intestinal oxidative stress and triggered autophagy response, which might be associated with NF-κB and Nrf2/keap1 signals.

Keywords: H_2O_2; oxidative stress; autophagy; NF-κB; Nrf2/keap1

Introduction

Oxidative stress is considered to be oxidative imbalance as excessive reactive oxygen species (ROS) overwhelms antioxidant defenses, including enzymes system and nonenzymatic antioxidants [1, 2]. Hydrogen peroxide (H_2O_2), a highly reactive oxygen species, has been shown to attribute to generation of other ROS, such as hydroxyl radicals, and the imbalanced oxidant/antioxidant status *in vivo* [3] and *in*

RSC Advances Accepted Manuscript

vitro [4]. The oxidative imbalance correlates with the modification of protein, lipid oxidation, and nucleic acid breaks, which may further impair cellular function and result in clinical deterioration [1, 5].

Autophagy, a process for cellular cleaning through a self-digestion of unnecessary intracellular proteins and dysfunctional organelles, is a well conserved mechanism from yeast to mammalian cells and also contributes to the maintenance of cellular homeostasis and of the energetic balance in cellular and tissue remodeling, and cellular defense against extracellular insults and pathogens [6]. Recently, autophagy has been demonstrated to play an important role in cell homeostasis by regulating the turnover of long-lived proteins and getting rid of damaged structures [7]. Many studies have shown that autophagy can be stimulated by various factors, including nutrient starvation [8] and infection [9]. Although, it was confirmed that oxidative stress also can promote autophagy in vitro[10], little is known about administration of H_2O_2-induced autophagy and the molecular mechanism between intestinal oxidative stress and the formation of autophagosomes *in vivo*. According to similarities to humans regarding morphology, function of organs, and metabolic rate, piglets were used as the in vivo model. We hypothesized that H_2O_2 administration could induce autophagy in piglets. Thus, the oxidative stress and autophagy were studied in pigs after administration of H_2O_2. The activation of NF-κB and Nrf2/keap1 signaling pathways was also analyzed after administration of H_2O_2. In this study, we found that administration of H_2O_2 causes intestinal oxidative stress and triggers autophagy, which may be associated with NF-κB and Nrf2/keap1 pathways.

Results

H_2O_2 suppressed the activity of antioxidant enzymes and caused oxidative stress

H_2O_2 is a highly reactive oxygen species. So, the primary focus was to monitor the oxidative parameters in the serum. The data showed that intragastric administration of H_2O_2 suppressed catalase with a dose-dependent decrease ($P < 0.05$) in the serum at 50 h (Table S1 in Supplementary material). While compared with intragastric administration, the activity of catalase in the per-10% H_2O_2 group significantly decreased ($P < 0.05$). Meanwhile, intragastric administration of 10% H_2O_2 significantly inhibited GSH-Px activity at 98 h and 170 h ($P < 0.05$). In addition, the oxidative stress parameters from the great curvature and jejunum (Table S2 in Supplementary material) further demonstrated that intragastric administration and peritoneal injection of H_2O_2 disrupted the oxidative balance.

H_2O_2 changed intestinal permeability, morphology, and tight junctions

Now our focus is to investigate the effect of H_2O_2 on intestinal function and integrity. The serum LPS level and DAO activity are two major biomarkers of intestinal injury [11], and the date showed that intragastric administration of 10% H_2O_2 decreased the activity of DAO ($P < 0.05$) in the serum at 50 h and 98 h (Fig.1). Meanwhile, the LPS levels were significantly higher after intragastric administration at 50 h and 98 h ($P < 0.05$) compared with control group (Fig.1), suggesting a positive correlation between the increased intestinal permeability and intragastric administration of H_2O_2. In addition, the macroscopic observations of intestinal morphology also demonstrated that H_2O_2 caused intestinal injury via the treatment of hematoxylin and eosin (H&E) staining (Fig.2). Although, the histological data seemed that intragastric administration of H_2O_2 increased jejunal and ileal villus height ($P < 0.05$)

RSC Advances Accepted Manuscript

(Table S3 in Supplementary material), the duodenal villus height, crypt depth, and the ratio of villus height to crypt depth in these three tissues were not affected after espouse to H_2O_2 ($P > 0.05$).

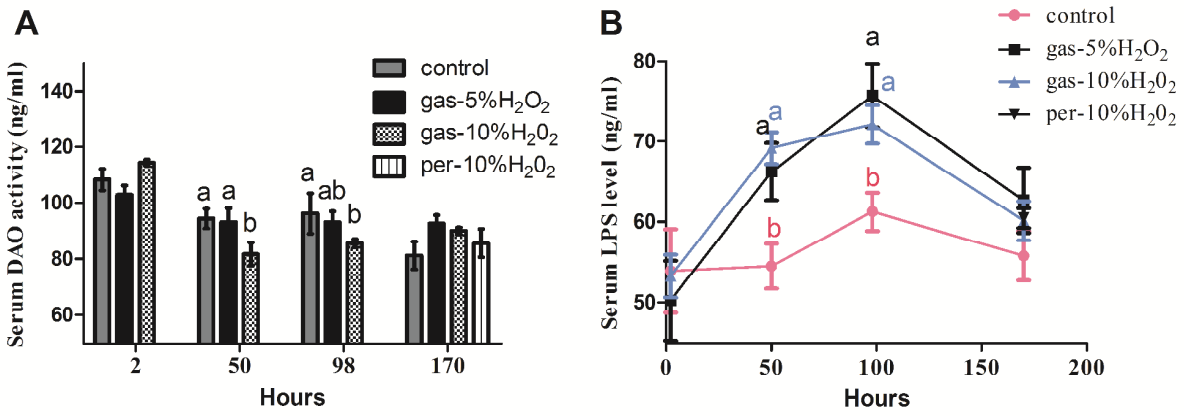

Fig.1 Diamine oxidase (DAO) activity (A) and lipopolysaccharides (LPS) level (B) in serum after exposure to H_2O_2 (n=6). DAO activity and LPS level in serum were measured at 2, 50, 98, and 170 h after administration of H_2O_2 and represented in terms of nanogram per milliliter (ng/mL). The details of methodology are described under Materials and methods. Data are presented as mean ± SE.

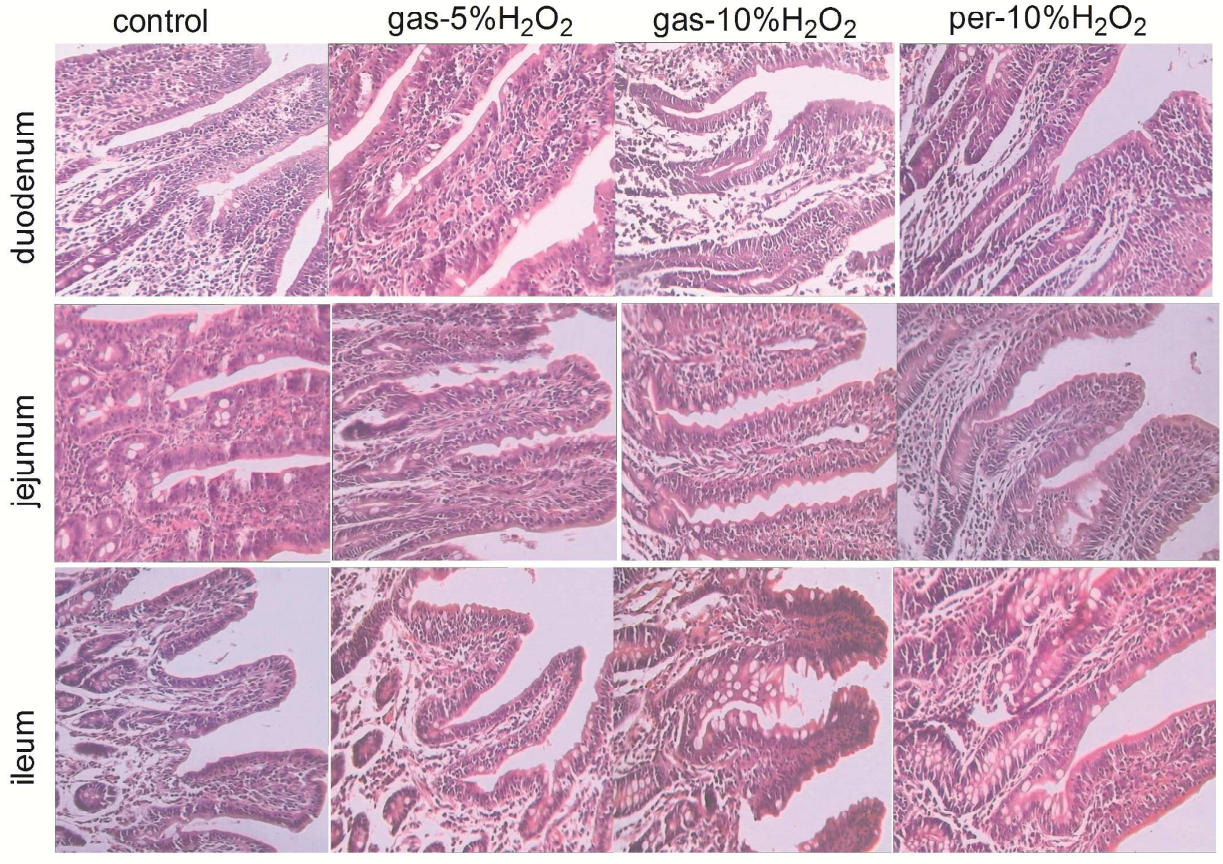

Fig.2 Histological evaluation of intestinal tissues (HE×250) after exposure to H_2O_2 (n=6). The details of histomorphometry described under Materials and methods. No histological

RSC Advances Accepted Manuscript

damage was observed in the control group and per-10% H_2O_2 group in the duodenum, jejunum, and ileum. Whereas the piglets administrated with H_2O_2 exhibited scattered villi and desequameted in these tissues.

Tight junctions, the first physical barrier against a variety of pathogens, act as maintaining the homeostasis in gastrointestinal tract [12]. As shown in fig. 3, both of intragastric administration and peritoneal injection of H_2O_2 significantly enhanced ($P < 0.05$) the abundances of claudin1, occludin, and ZO1 in the jejunum compared with the control group. Meanwhile, The PCR data showed that only intragastric administration of 5% H_2O_2 up-regulated the expression of ZO1 (about 1.33-fold) and there was no difference between other groups (Fig.3) in the jejunum.

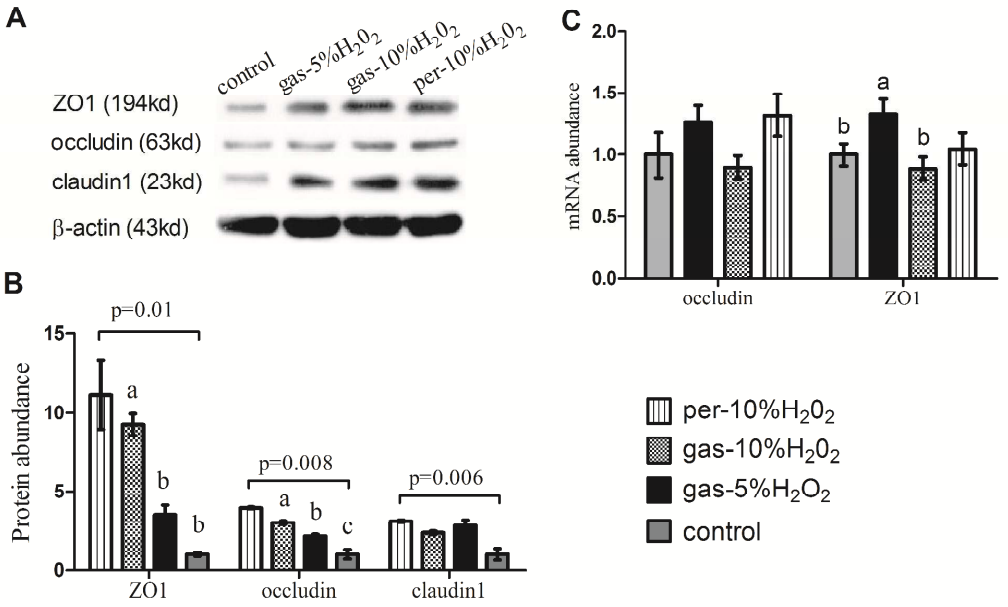

Fig.3 Jejunal tight junctions were measured via western blot (A and B) and RT-PCR (C) analyses at 7 d (170 h) after administration of H_2O_2 (n=6). The details of methodology are described under Materials and methods. Data are presented as mean ± SE.

H_2O_2 induce autophagy response in the jejunum

LC3 involves in the formation of autophagosomes and exists in two forms: an unprocessed 18kDa cytosolic protein (LC3-I) and a processed 16 kDa protein (LC3-II). LC3-II is membrane-bound and increases during autophagy by conversion from LC3-I, therefore the ratio of LC3-II to LC3-I is often used as an indicator of increased autophagy [13]. LC3I and LC3-II levels in the jejunum were measured, and the western blotting results (Fig.4) showed that administration of H_2O_2 increased both abundance of LC3-I and LC3-II ($P < 0.05$) and the ratio of LC3-II to LC3-I content increased after exposure to 10% H_2O_2, indicating that H_2O_2 induced the conversion of LC3-I to LC3-II. Meanwhile, we also investigated the abundance of beclin1. But we failed to notice any significant difference ($P > 0.05$) in the beclin1 expression via intragastric administration of H_2O_2. While the beclin1 level in the per-10% H_2O_2 was significantly increased ($P < 0.05$). Furthermore, the PCR data also demonstrated that H_2O_2

RSC Advances Accepted Manuscript

affected the autophagic gene expression as shown in table S5 in Supplementary material. These results confirmed that administration of H_2O_2 induces autophagy in the jejunum.

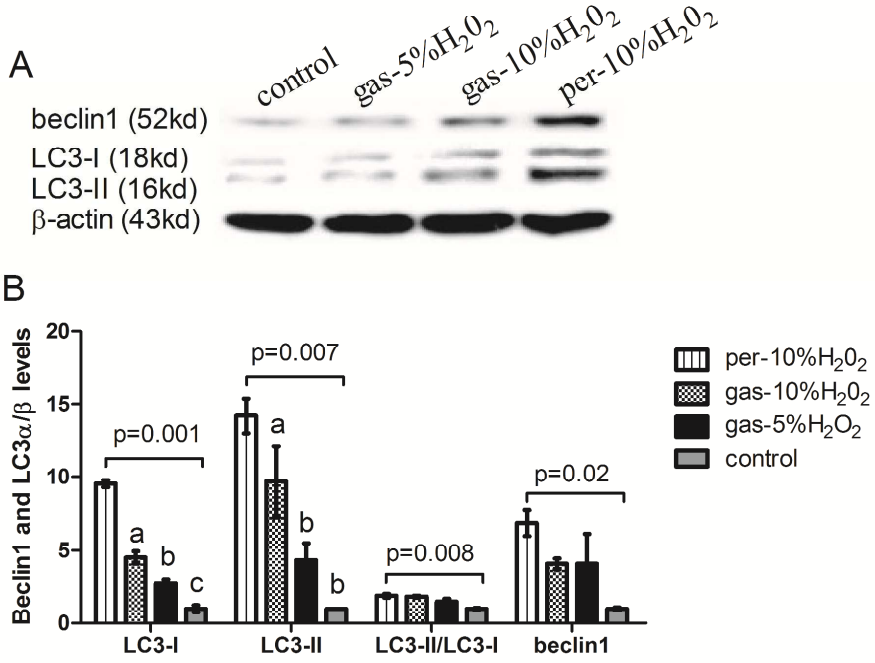

Fig.4 The jejunal autophagy-related proteins (LC3 and beclin1) at 7 d (170 h) after administration of H_2O_2 (n=6). The details of methodology are described under Materials and methods. Data are presented as mean ± SE.

Activation and nuclear translocation of NF-κB by H_2O_2

The nuclear factor kappa B (NF-κB) involves in cellular response to stimuli such as cytokine, ultraviolet irradiation, oxidized low-density lipoprotein, and microbes or viral antigens. After activation, NF-κB serves in cell growth, differentiation, development, apoptosis, and oxidative stress [1]. We preformed western blot to determine the abundances of IKKα/β, p-IKKα/β, nuclear NF-κB, and cytoplasmic NF-κB in the jejunum after exposure to H_2O_2 (Fig.5). An increase of p-IKKβ/IKKβ ($P < 0.05$) in the per-10% H_2O_2 group was evident compared with other groups (Fig.5), indicating an IKKβ phosphorylation. Then we observed that both nuclear and cytoplasmic NF-κB enhanced ($P < 0.05$) after exposure to 10% H_2O_2 and, interestingly, the abundances of NF-κB kept equal between nucleus and cytoplasm in the same group (Fig.5). So it was clear that H_2O_2 caused phosphorylation of IKKβ, and then increased accumulation of NF-κB and its translocation into nucleus to initiate gene expression.

RSC Advances Accepted Manuscript

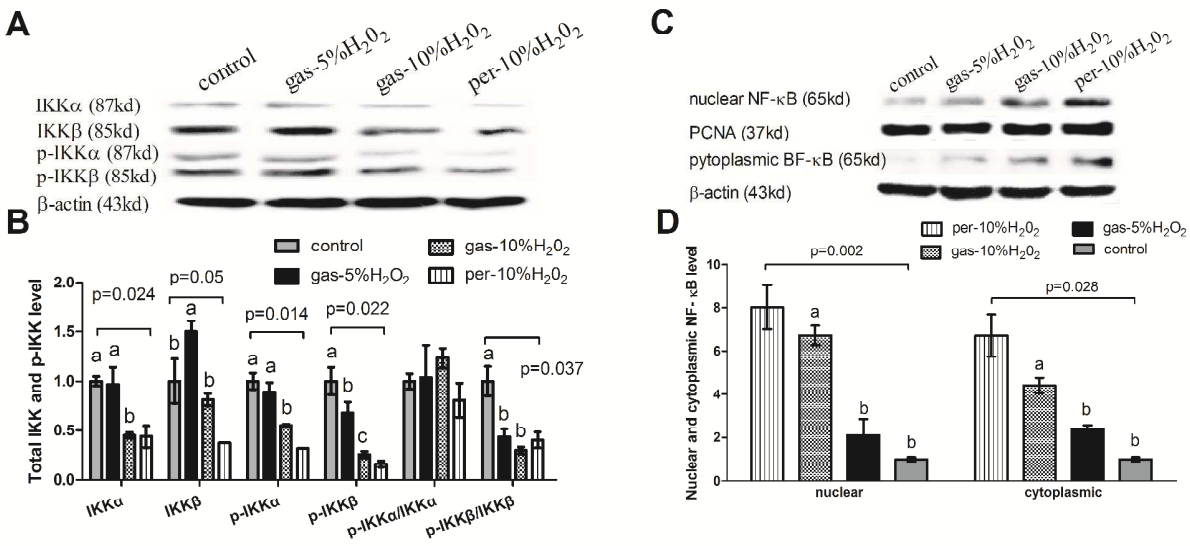

Fig.5 The jejunal NF-κB signaling pathway was measured via western blot at 7 d (170 h) after administration of H_2O_2 (n=6). The details of methodology are described under Materials and methods. Data are presented as mean ± SE.

Activation and nuclear translocation of Nrf2/Keap1 by H_2O_2

After observation of NF-κB signal alteration, we next investigated the Nrf2/Keap1 signaling pathway after exposure to H_2O_2. Nrf2/Keap1 is a major antioxidant transcription factor controlling a wide number of gens including quinine oxidoreductase 1, heme oxygenase-1, and glutathione-S-transferases [1]. We were interested to find out the involvement of Nrf2/Keap1 in the H_2O_2-induced autophagy. Activation of Nrf2/Keap1 is reliant on its upstream proteins Keap1. In an inactivated state, Nrf2 is sequestered in the cytosol via Keap1 and soon be degraded. Modification of Keap1 is necessary for the accumulation of free Nrf2 in the cytosplasm, which then translocate into nucleus [1]. While, interestingly, the results showed that administration of 10% H_2O_2 increased ($P < 0.05$) Keap1 abundance in the jejunum (Fig.6). Moreover, the cytoplasmic and nuclear Nrf2 abundances were also increased ($P < 0.05$) after exposure to H_2O_2 (Fig.6). These results indicated that H_2O_2 increased localization of Nrf2 inside the nucleus but not through the degradation of Keap1.

RSC Advances Accepted Manuscript

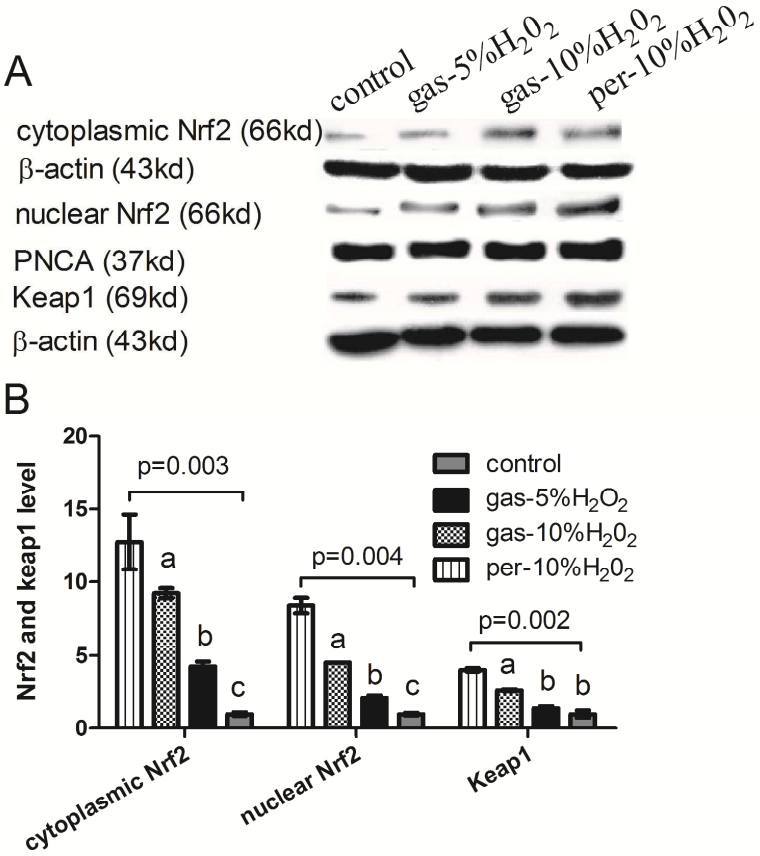

Fig.6 The jejunal Nrf2/Keap1 signaling pathway was measured via western blot at 7 d (170 h) after administration of H_2O_2 (n=6). The details of methodology are described under Materials and methods. Data are presented as mean ± SE.

Discussion

Epidemiological evidences have demonstrated that oxidative imbalance can lead to irreparable oxidative injury and cell death when it goes beyond control as oxidative stress affect almost all the cellular macromolecules [1]. In the current study, H_2O_2 was applied to induce oxidative stress as many previous data have suggested that H_2O_2 is highly reactive oxygen species and can be capable of diffusing throughout the mitochondria and across cell membranes and causing many types of cellular injury, so it seems to be a suitable agent for investigating reactive oxygen metabolite-induced gastrointestinal oxidative damage [1, 14].

H_2O_2 can be metabolized to several of ROS, including hydroxyle radicals, which is considered to be the most dangerous compound to the organism. The excessive and un-eliminated H_2O_2 and its metabolites can oxidize virtually all types of macromolecules: carbohydrates, nucleic acids, lipids and proteins [1]. The increased ROS overwhelms the antioxidant defenses and then lead to a state of oxidative stress, which may further impair body function and result in clinical deterioration. The present data indicated that H_2O_2 inhibited catalase, GSH-Px, and T-AOC activities and increased MDA level, suggesting a significant disruption in the oxidative balance after exposure to H_2O_2 treatment.

RSC Advances Accepted Manuscript

This is in agreement with several previous reports, which also demonstrated that H_2O_2 caused a substantial reduction in GSH content associated with reduction in GPx activity [3, 14].

Ingestion of H_2O_2 changed permeability and caused dysfunction in the intestine. Previous studies demonstrated that intragastric administration of 6% and 15% H_2O_2 produced hemorrhagic and necrotic mucosal lesions in the fundic area in rats [3, 14]. Although, we failed to observe the lesions in the gastrointestinal tract after exposure to H_2O_2, the macroscopic observations of intestinal morphology showed that the villus was scattered and desquamated seriously in the duodenum, jejunum, and ileum after intragastric administration of H_2O_2. In addition, H_2O_2-induced oxidative stress is known to attribute to the increased permeability [15]. Similarly, the present study also showed an increase in permeability after intragastric administration of H_2O_2 indicated by the decreased DAO activity and increased LPS level in serum. The activity of serum DAO is associated with the maturation and integrity of small intestinal mucosa [16]. DAO is particularly abundant in enterocytes at the tip of small intestinal villi, from where it is released into the peripheral circulation and then inactivated in the liver. Numerous compelling evidences have demonstrated that the decreased serum DAO activity is in parallel with the intestinal mucosal injury [11, 16]. Meanwhile, the high level of serum LPS is also considered to be the consequence of the increased intestinal permeability [17]. Therefore, it is clear that H_2O_2 increased the intestinal permeability in piglets and, logically, the increased permeability partially corresponded to down-regulation of tight junction. However, the current data exhibited an increase in the expression of tight junction, including claudin1, occludin, and ZO1 after administration of H_2O_2. The mechanism may be that permeability alteration induced by H_2O_2 correlates with changes of the localization of the tight junction rather than the expression of the tight junction [18].

H_2O_2 induces autophagy in the jejunum. Autophagy is a physiologic mechanism in which cells degrade unnecessary or dysfunctional cellular organelles through the lysosomal machinery [6]. During autophagy, LC3-I is lapidated and converted to LC3-II, which is translocated to the autophagosomal membrane. Therefore, the ratio of LC3-II to LC3-I content and accumulation of LC3 were widely used as markers of autophagy [13]. Consistent with prior reports [19], our results also showed that administration of H_2O_2 promoted the conversion of LC3-II from LC3-I, indicating the autophagosome formation in the jejunum. Although, excessive and dysregulated autophagy has recently been considered to be deleterious in the pathogenesis of various diseases [20], autophagy in this study may protect tissues against cell deaths under oxidative stress induced by H_2O_2 in piglets due to higher susceptibility of the oxidized proteins to be taken up by autophagosome, subsequently degraded by lysosome, which contributes to the efficient removal of oxidized proteins and reduces the further oxidative damage by these oxidants [21]. In addition, peritoneal injection of H_2O_2 significantly increased autophagy related-protein beclin1, suggesting that beclin1 also plays a role in H_2O_2-induced autophagy [22]. Furthermore, several proteins also have been identified to involve in oxidative stress-induced autophagy. For example, Tang *et al.* has reported that autophagy-related protein 9 (Atg9) plays a dual role in the regulation of ROS-induced autophagy, which acts as a negative feedback regulator of c-Jun N-terminal kinase (JNK) activity in mammalian cells [23]. Meanwhile, Tang *et al.* further demonstrated that Atg1-mediated myosin II activation is crucial for the movement of the Atg9 transmembrane protein between the Golgi and the forming autophagosome, which provides a membrane source for the formation of autophagosomes [24].

NF-κB, whose activation required a distinct IKK subunit, can be up-regulated by two major different pathways: the classical pathway involving IKKβ and the alternative or non-canonical

RSC Advances Accepted Manuscript

pathway is IKKα-dependent [25]. However, to date, these pathways which play a role in H_2O_2 induced-autophagy remain unknown. Our research is the first study to observe that H_2O_2 significantly increased the ratio of p-IKKβ/IKKβ and the activation of IKK/NF-κB signaling axis depends on the classical pathway. The activated IKK phosphorylates IκBα (an inhibitory protein) on Ser-32, Ser-36, and Tyr-42, or other tyrosine residues, causing subsequent ubiquitination and degradation of IκBα and accumulation of NF-κB proteins in the cytoplasm. Free NF-κB dimers translocate into the nucleus to regulate the expression of multiple target genes [1]. Many reports have shown that the activated NF-κB signaling pathway plays a critical role in autophagy [25, 26]. Su *et al.* (2012) reported that p65, a downstream protein of NF-κB signaling pathway, mediated the expression of LC3 [27], which is corresponding to autophagosome formation. In addition, the high abundant expression of beclin1 is in parallel to p65 expression and inhibition of NF-κB significantly attenuated beclin1 expression and autophagy [26]. Beclin1 is the first mammalian protein described to upregulate autophagy via formation of autophagosomes and the initiation of autophagy [26]. Thus, NF-κB has been considered to be an important regulatory mechanism in autophagy response. Djavaheri-Mergny *et al.* has indicated that stimulation of autophagy may be a potential way bypassing the resistance of cancer cells to anti-cancer agents that activate NF-κB [28]. Furthermore, NF-κB also is known to be a major proinflammatory transcription factor controlling a wide number of genes, including cytokines [29]. So we also investigated the expressions of several cytokines after administration of H_2O_2. The results showed that the activated NF-κB signal may affect the cytokines (IL-6, IL-8, IL-17 and TNF-α) gene expression (Table S6 in Supplementary material). Taking together, NF-κB plays a vital role in mediating antioxidant and proinflamatory gene expression and autophagy in response to H_2O_2 challenge.

Although the Nrf2 (nuclear factor erythroid 2-related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) signaling pathway also has been investigated in oxidative stress and autophagy by previous studies [30], the mechanism is still obscure. Nrf2 is a basic "cap and collar" leucine zipper transcription factor, which regulates environmental stress response by regulating the expression of genes for antioxidants and detoxification enzymes. The Nrf2-directed environmental stress response protects cells against variety of stressors, including environmental pollutants such as electrophiles and oxidizing agents, immunotoxicants, and inflammation [31]. Keap1, a cysteine-rich protein that acts as a substrate adaptor for the ubiquitination of Nrf2 by the Cul3-Rbx1 E3 ubiquitin ligase complex, targets Nrf2 for proteasomal degradation and maintains the transcription factor at a low level under non-stress conditions [1]. Under oxidative stress and excessive ROS, the oxidation or conjugation of key cysteine residues in the Keap1 would increase, then the modifications of the Keap1 generally weak its activity as an E3 ligase adaptor. The net effect of such Keap1 modification is disrupted, thus Nrf2 is no longer degraded in the cytosol and translocate into the nucleus and induce transcription of its target genes [1]. Therefore, the degradation of Keap1 is logically associated with up-regulation of Nrf2 as other reports described [30]. However, to the best authors' knowledge, this is the first study to show that overexpression of Keap1 also increased localization of Nrf2 inside the nucleus in H_2O_2-induced autophagy. Recent studies have shown that some other proteins, such as p21 and p26, also contribute to the activation of Nrf2 [30, 32]. The cyclin-dependent kinase inhibitor p21 can disrupt the interaction between Nrf2 and Keap1 and trigger the activation of Nrf2 signal since p21 competes with Keap1 for binding to the Nrf2 and thereby protects Nrf2 from ubiquitination [33]. Meanwhile, p62 was identified as another protein to disrupt the Keap1-Nrf2 interaction [34], but the mechanism is due to its competition with Nrf2 for binding to Keap1. Furthermore, p62 is a multifaceted adaptor protein and it can interact

RSC Advances Accepted Manuscript

with LC3 to regulate autophagic removal of protein aggregates and damaged intracellular organelles [35]. Li *et al.* reported that Nrf2 drives a novel antioxidant independent autophagic clearance for β-cell protection in the setting of diabetes [36]. However, there still need further study to illustrate the mechanism that how oxidative stress activates Nrf2/Keap1 signaling pathway and mediates autophagy.

Materials and methods

Animal surgery and experimental design

This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving animal subjects were approved by the animal welfare committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (No.13-07). Twenty-four healthy piglets of similar bodyweight (11.29 ± 0.32 kg) (Landrace× Large White) (ZhengHong Co., China) were had a general anesthesia (Zoletil 50, Virbac Co., France) and then operated to install a silicone coated latex T-shape catheter (Zhan Jiang Star Enterprism Co., China) in the helicobacter. After surgery, all piglets recovered a week, then randomly divided into four groups (n=6): a control group in which piglets received an intragastric administration via the T-shape catheter and a peritoneal injection of 10mL/10kg PBS buffer, separately; a gas-5% H_2O_2 group in which piglets were given an intragastric administration of 5% H_2O_2 and a peritoneal injection of PBS buffer with similar dosage; a gas-10% H_2O_2 group in which piglets received an intragastric administration of 10% H_2O_2 and a peritoneal injection of PBS buffer with similar dosage; and a per-10% H_2O_2 group in which piglets received an intragastric administration of PBS buffer and a peritoneal injection of 10% H_2O_2 with similar dosage. Administration dosage of H_2O_2 in the present study was according to our previous experiment (unpublished data). Blood was sampled from a jugular vein at 0 (2 h), 2 (50 h), 4 (98 h), and 7 d (170 h) before feeding. All piglets were allowed free access to water and fed twice at 8:00 AM and 4:00 PM throughout the experimental period. All piglets were killed after 7 days according to our previous data (unpublished). Samples were taken from the greater curvature, helicobacter, duodenum, jejunum, and ileum. One of the gut samples (3cm) was kept in 10% neutral buffered formalin for histomorphometry analysis, and the other (approximately 2g) was immediately frozen in liquid nitrogen and stored at -70℃ for subsequent gene expression and western blotting analyses.

Measurement of plasma and tissue oxidative stress parameters

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, total antioxidant capability (T-AOC), malondialdeyhde (MDA), $OH^{\bullet}$, H_2O_2 from the intestine and serum were measured using spectrophotometric kits in accordance with the manufacturer's instructions (Nanjing Jiangcheng Biotechnology Institute, China).

Diamine oxidase activity and LPS level in the serum

Serum diamine oxidase (DAO) activity lipopolysaccharide (LPS) were determined according to our previous report [37].

Histomorphometry determination

RSC Advances Accepted Manuscript

Intestinal samples (3cm) were kept in 4% neutral buffered 10% formalin for H&E staining. Villus height and crypt depth were measured using an image-analysis system [37].

cDNA synthesis and quantification mRNA by real-time PCR analysis

Extraction of total RNA and its reverse transcription were performed according to our previous reports [2, 5]. Primers were designed with Primer 5.0 according to the gene sequence of pig (http://www.ncbi.nlm.nih.gov/pubmed/) to produce an amplification product (Table S7 in Supplementary material). β-actin was used as a housekeeping gene to normalize target gene transcript levels. Real-time PCR was performed according to our previous study [37]. Relative expression was normalized and expressed as a ratio to the expression in control group [2, 37].

Western bolt analysis

Jejunal proteins were extracted with nuclear and cytoplasmic extraction reagents in accordance with the manufacturer's instructions (Thermo Fisher Scientific Inc., USA). The method for western bolt was described in our previous reports [2, 5]. The antibodies used in this study as followed: IKKα/β, p-IKKα/β, keap1, light chain 3 (LC3) (Santa Cruz Biotechnology, Inc., CA, USA), beclin1, occludin, claudin1, ZO1, Nrf2, and NF-κB (Abcam, Inc., USA). Mouse β–actin antibody (Sigma) was used for total and cytoplasmic protein loading control. Rabbit PCNA antibody (Sigma) was used for nuclear protein loading control. The expression ratio of target proteins was normalized against β-actin.

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 software. The effects of intragastric administration of H_2O_2 (5% and 10%) were performed by using the one-way analysis of variance (ANOVA) to test homogeneity of variances via Levene's test and followed with Tukey's multiple comparison test. Comparison between control and per-10% H_2O_2 group was performed by using Student's T test. Data are expressed as the mean ± standard error of the mean. Values in the same row with different superscripts are significant ($P < 0.05$), while values with same superscripts are not significant different ($P > 0.05$).

Conclusions

Our experiments proved that administration of H_2O_2 suppressed the activity of antioxidant enzymes and caused oxidative stress. Meanwhile, the intestinal permeability, morphology, and barrier function also changed after exposure to H_2O_2 in piglets. All these imbalances might induce autophagy via activation of NF-κB and Nrf2/Keap1 signaling pathways (Fig.7). Further studies should be conducted to completely determine the mechanism in H_2O_2 induced autophagy.

RSC Advances Accepted Manuscript

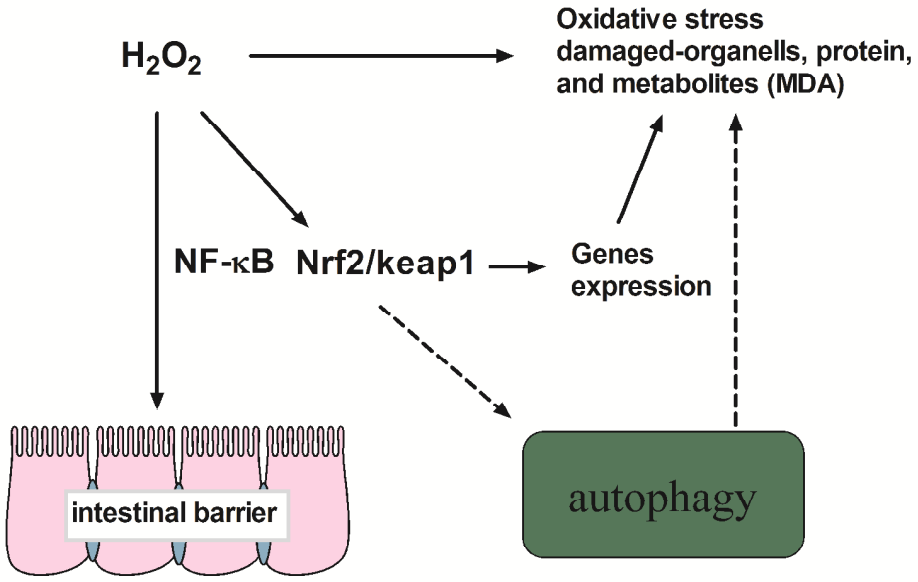

Fig.7 H_2O_2 induced oxidative stress triggers autophagy, which may be associated with NF-κB and Nrf2/keap1 signaling pathways.

Acknowledgement

This study was supported by the National Natural Science Foundation of China (NO. 31272463) and Hunan Provincial Natural Science Foundation of China (NO. 12JJ2014).

Author Contributions:

T.L. and Y.Y. conceived and designed the experiments; J.Y., J.D., and Z.C. performed the experiments; J.Y. and W.R. analyzed the data; J.Y. wrote the paper.

Competing interests

All authors have no conflict of interest.

Supplementary material

Table S1: Oxidative stress parameters in serum after exposure to H_2O_2;

Table S2: Oxidative stress parameters in the greater curvature (A) and jejunum (B) after exposure to H_2O_2;

Table S3: The intestinal villus height and crypt depth after exposure to H_2O_2;

Table S4: Gene expression of occluding and ZO-1 in the pylorus, great curvature, duodenum, jejunum, and ileum after exposure under H_2O_2 at 7 d (170 h);

RSC Advances Accepted Manuscript

Table S5: Gene expression of LC3 and beclin1 in the pylorus, great curvature, duodenum, jejunum, and ileum after exposure under H_2O_2 at 7 d (170 h).

Table S6: Pro-inflammatory cytokines (IL-6, IL-8, IL-17 and TNF-α) gene expression in the pylorus, great curvature, duodenum, jejunum, and ileum were detected with RT-PCR after exposure under H_2O_2 at 7 d (170 h).

Table S7: PCR primer sequences: the forward primers (F) and the reverse primers (R) used on this study are tabulated along with their accession No. and product length.

Reference

1. J. Yin, W. K. Ren, X. S. Wu, G. Yang, J. Wang, T. J. Li, J. N. Ding, L. C. Cai and D. D. Su, *J Food Agric Environ*, 2013, **11**, 132-139.
2. J. Yin, M. M. Wu, H. Xiao, W. K. Ren, J. L. Duan, G. Yang, T. J. Li and Y. L. Yin, *J Anim Sci*, 2013.
3. A. M. Mohamadin, O. M. Ashour, N. A. El-Sherbeny, A. M. Alahdal, G. M. Morsy and A. B. Abdel-Naim, *Clin Exp Pharmacol Physiol*, 2009, **36**, 367-372.
4. E. E. Essick, R. M. Wilson, D. R. Pimentel, M. Shimano, S. Baid, N. Ouchi and F. Sam, *PLoS One*, 2013, **8**, e68697.
5. J. Yin, W. Ren, G. Liu, J. Duan, G. Yang, L. Wu, T. Li and Y. Yin, *Free Radic Res*, 2013, **47**, 1027-1035.
6. S. Saito and A. Nakashima, *Placenta*, 2013, **34 Suppl**, S79-84.
7. W. L. Li, S. P. Yu, D. Chen, S. S. Yu, Y. J. Jiang, T. Genetta and L. Wei, *Neuroscience*, 2013, **244**, 16-30.
8. A. Kuma, M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa and N. Mizushima, *Nature*, 2004, **432**, 1032-1036.
9. V. Deretic, M. Delgado, I. Vergne, S. Master, S. De Haro, M. Ponpuak and S. Singh, *Current topics in microbiology and immunology*, 2009, **335**, 169-188.
10. C. Luo, Y. Li, H. Wang, Y. Cui, Z. Feng, H. Li, Y. Li, Y. Wang, K. Wurtz, P. Weber, J. Long and J. Liu, *Current cancer drug targets*, 2013.
11. T. Namikawa, I. Fukudome, H. Kitagawa, T. Okabayashi, M. Kobayashi and K. Hanazaki, *Oncology-Basel*, 2012, **82**, 147-152.
12. T. Suzuki, *Cell Mol Life Sci*, 2013, **70**, 631-659.
13. X. Deng, F. Zhang, W. Rui, F. Long, L. Wang, Z. Feng, D. Chen and W. Ding, *Toxicol In Vitro*, 2013, **27**, 1762-1770.
14. K. Sakurai and K. Yamasaki, *Jpn J Pharmacol*, 1994, **64**, 229-234.
15. X. P. Zhou, D. Yuan, M. X. Wang and P. N. He, *Am J Physiol-Heart C*, 2013, **304**, H82-H93.
16. I. Fukudome, M. Kobayashi, K. Dabanaka, H. Maeda, K. Okamoto, T. Okabayashi, R. Baba, N. Kumagai, K. Oba, M. Fujita and K. Hanazaki, *Medical molecular morphology*, 2013.
17. S. Guo, R. Al-Sadi, H. M. Said and T. Y. Ma, *Am J Pathol*, 2013, **182**, 375-387.
18. S. Fischer, M. Wiesnet, D. Renz and W. Schaper, *European journal of cell biology*, 2005, **84**, 687-697.
19. M. B. Azad, Y. Chen and S. B. Gibson, *Antioxid Redox Signal*, 2009, **11**, 777-790.
20. L. Murrow and J. Debnath, *Annu Rev Pathol-Mech*, 2013, **8**, 105-137.
21. R. Kiffin, U. Bandyopadhyay and A. M. Cuervo, *Antioxid Redox Sign*, 2006, **8**, 152-162.
22. E. E. Essick, R. M. Wilson, D. R. Pimentel, M. Shimano, S. Baid, N. Ouchi and F. Sam, *Plos One*, 2013, **8**.
23. H. W. Tang, H. M. Liao, W. H. Peng, H. R. Lin, C. H. Chen and G. C. Chen, *Developmental cell*, 2013, **27**, 489-503.
24. H. W. Tang and G. C. Chen, *Autophagy*, 2011, **7**, 778-779.
25. V. Cianfanelli and F. Cecconi, *Cell Cycle*, 2012, **11**, 436-437.
26. M. Zeng, X. Wei, Z. Wu, W. Li, B. Li, Y. Zhen, J. Chen, P. Wang and Y. Fei, *Biochem Biophys Res Commun*, 2013, **436**, 180-185.
27. C. C. Su, S. Y. Chien, S. J. Kuo, Y. L. Chen, C. Y. Cheng and D. R. Chen, *Mol Med Rep*, 2012, **5**, 1019-1022.
28. M. Djavaheri-Mergny, M. Amelotti, J. Mathieu, F. Besancon, C. Bauvy, S. Souquere, G. Pierron and P. Codogno, *J Biol Chem*, 2006, **281**, 30373-30382.
29. D. Liang, F. Li, Y. Fu, Y. Cao, X. Song, T. Wang, W. Wang, M. Guo, E. Zhou, D. Li, Z. Yang and N. Zhang, *Inflammation*, 2013.

RSC Advances Accepted Manuscript

30. S. H. Bae, S. H. Sung, S. Y. Oh, J. M. Lim, S. K. Lee, Y. N. Park, H. E. Lee, D. Kang and S. G. Rhee, *Cell Metab*, 2013, **17**, 73-84.
31. B. N. Das, Y. W. Kim and Y. S. Keum, *Oxidative Medicine and Cellular Longevity*, 2013.
32. L. Zhu, E. C. Barret, Y. Xu, Z. Liu, A. Manoharan and Y. Chen, *PLoS One*, 2013, **8**, e55695.
33. W. M. Chen, Z. Sun, X. J. Wang, T. Jiang, Z. P. Huang, D. Y. Fang and D. D. Zhang, *Molecular cell*, 2009, **34**, 663-673.
34. A. Lau, X. J. Wang, F. Zhao, N. F. Villeneuve, T. Wu, T. Jiang, Z. Sun, E. White and D. D. Zhang, *Mol Cell Biol*, 2010, **30**, 3275-3285.
35. S. Pankiv, T. H. Clausen, T. Lamark, A. Brech, J. A. Bruun, H. Outzen, A. Overvatn, G. Bjorkoy and T. Johansen, *J Biol Chem*, 2007, **282**, 24131-24145.
36. W. Li, W. Wu, H. Song, F. Wang, H. Li, L. Chen, Y. Lai, J. S. Janicki, K. W. Ward, C. J. Meyer, X. L. Wang, D. Tang and T. Cui, *Febs Lett*, 2014, **588**, 2115-2124.
37. J. Yin, W. Ren, J. Duan, L. Wu, S. Chen, T. Li, Y. Yin and G. Wu, *Amino Acids*, 2013.

RSC Advances Accepted Manuscript