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Ferulic Acid in Chinese Herbal Medicine: Regulating Autophagy for Oxidative Damage Reduction

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Abstract: Ferulic Acid (FA) is the primary functional component in herbal plants and traditional fermented foods. To comprehend the mechanism of action of ferulic acid, in terms of its regulation of autophagy, antioxidants and the inhibition of inflammatory pathways, the study was conducted. Oxidatively damaged human-derived hepatocytes were treated with FA and the processing concentration of FA is from 50-800 μM. Autophagy, oxidative and inflammatory critical proteins were assessed through western blotting and qRTpcr with analysis of variance. The group treated with FA exhibited an increase in downstream Heme Oxygenase-1 (HO-1) and nuclear factor erythroid-2-related factor 2 (Nrf2) in the nucleus and a decrease in Kelch-like EC-associated protein 1 (Keap1) and Nrf2 in the cytosol, while inhibiting key inflammatory proteins, Cyclooxygenase-2 (COX-2) in comparison with oxidative stress group. Prior to this, autophagy-positive associated protein microtubule-associated protein 1 Light Chain 3 beta (LC3) were increased and protein sequestosome 1 (P62) were reduced. In summary, previous studies have only clarified that FA is a functional component, without clarifying its mechanism. FA as a precursor found in Chinese herbs and other phenols, demonstrates the capacity to reduce oxidative damage through autophagy, establishing an experimental foundation for the beneficial functions of Chinese herbal medicines and fermented products containing ferulic acid and providing a theoretical basis for future products.

Keywords: Ferulic Acid, Human Hepayic Cell Line (LO_2 Cells), Autophagy, Inflammation, Oxidative Stress

Introduction

Oxidative homeostasis is crucial for maintaining normal physiological function and its disruption is associated with various diseases (Wang *et al*., 2020). Nowadays, it is becoming increasingly important to alleviate oxidative stress damage to a certain extent through traditional Chinese herbs or foods, but the underlying mechanism is still unclear. Primary causes of oxidative stress include both endogenous and exogenous stimuli, such as environmental pollutants and sun radiation (Belch *et al*., 1991). The accumulation of oxidants results in DNA damage and protein misfolding, leading to cellular and tissue injury and disease (Hendriks *et al*., 2016). Elevated free radical accumulation within the body can trigger inflammation and damnify cells and tissues, causing oxidative damage (Auer *et al*., 1993). Improving antioxidant capacity can directly alleviate oxidative stress. Autophagy serves as the principal intracellular clearance pathway for oxygen radicals and damaged organelles (Tang *et al*., 2010). It plays a vital part in cellular self-protection by responding to external cues and degrading and recycling oxygen-free radicals, cytoplasmic aggregates, damaged organelles and invading pathogens (Mizushima *et al*., 2008; 2010). Following their secondary utilization, this process provides energy

and generates various autophagic lysosomes in mitochondria and other organelles to support regular physiological processes (Schroder and Tschopp, 2010; Saitoh and Akira, 2010). Inflammation is closely associated with diseases (Cicuéndez *et al.*, 2021). Autophagy, acting as a primitive immune system, can regulate metabolism and, to some extent, mitigate the harm caused by inflammatory agents (Groulx *et al.*, 2012; Ulivi *et al.*, 2011).

FA exhibits diverse pharmacological effects, notably in terms of its antioxidant and anti-inflammatory (Shaukat *et al.*, 2022; Wu *et al.*, 2021). Usually used for drugs containing ferulic acid, but its mechanism of action is not clear. This research was mainly aimed at clarifying how ferulic acid can be an antioxidant. FA, named after the plant ferula officinalis from which it was initially isolated, is a cinnamic acid derivative (Mattila and Kumpulainen, 2002). This current study primarily focuses on the efficacy of its derivative, ethyl ferulate, with some attention to FA itself (Aljawish *et al.*, 2012). FA naturally occurs in the trans structure and plays a significant role in traditional Chinese treatments like cortex eucommiae (Zhou *et al.*, 2017). Its presence serves as a crucial determinant of the quality of these medicinal herbs (Mancuso and Santangelo, 2014). In addition to Chinese herbs, which contain substantial levels of FA, common fermented foods, cereal products and coffee also feature high levels of this compound. Its derivatives and subsequent metabolites play essential roles as both flavor and health components in fermented foods like vinegar, baijiu and yellow wine, among others (Bezerra *et al.*, 2017). The specific mechanism of FA remains uncertain, even though it plays a significant role as a major component of Chinese herbal medicine, a crucial functional ingredient in various fermented foods and a precursor to numerous health factors.

In this research, we explored the impact of FA on cellular autophagy, the oxidative pathway and inflammation in normal human hepatocytes. The marker proteins for cellular autophagy are LC3 and P62, while the marker for the antioxidant pathway is Nrf2 entering the nucleus from the cytoplasm and regulating HO-1. We focused on observing the effects of ferulic acid on these key proteins under oxidative stress conditions. We outlined its specific mechanism of action, which will serve as the foundation for the development of future herbal products and fermented foods.

Materials and Methods

Chemicals and Reagents

Human Hepayic cell Line LO_2 (HL-7702, human), laboratory stored in -80°C refrigerator and passaged. FA 99% purity, F103701, dissolved in DMSO, Shanghai Aladdin Biochemical Science and Technology Co. Ltd; C8H18N6-2HCl (AAPH), purity ≥98%, Suzhou Yako Science and Technology Co. Ltd; cultivate cells: RPMI-1640 culture medium, 1% Penicillin double antibody, Gibco Co. Ltd; fetal bovine serum, 0.25% trypsin. Geminig; Cell Counting Kit-8 kit (CCK-8), Dojindo Co. Ltd; cell counting sheet, nucleoprotein and cytoplasmic protein extraction kit (BB-3112-50T), Bebo Biotechnology Co. Ltd; PBS buffer (P1020), Beijing Solepol Technology Co. Ltd; Western blot: Primary antibodies: Nucleoprotein internal control (Lamin B, 12987-1AP), Nrf2, 16396-1-AP, Keap1, 10503-2-AP, HO-1, 66743-1-Ig, prostaglandin endoperoxide synthase 2 (COX-2, 66351-1-Ig), NF-κB, 10745-1-AP, proteintech Co. Ltd; LC3, PM036, P62, PM045, MBL Co. Ltd; Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, 5174S). CST, USA; secondary antibody: Rabbit (7074S), CST Co. Ltd; BCA reagent kit (PC0020), 5× Protein supersampling buffer (P1040), Beijing solepol technology Co. Ltd; marker (1610374), Bio-Rad; ECL exposure solution (RPN2232), cytiva Co. Ltd; PVDF membrane (IPVH00010), Merck, USA; Marker (1610374), BIO-RAD Co. Ltd. gene sequence design primers, LC3 primers (5'-GAGAAAGCAGCTTCTCTGTTCTGG-3', 5'-CGTTCACCAAGGGAG-3'); P62 primers (5'-TGCCCAGACGACTTGTG-3', 5'-AGTGTCCGTTTTCACCTCC-3'); GAPDH primers (5'-TCCCTGTGAACGGGAAG-3', 5'-GGGGAGTGCGCTGT-3')., Sangon Biotech Co. Ltd.

Cell Culture

The cells were cultured by RPMI-1640 medium. The cell culture was maintained in 5% CO_2 at 37°C. Subsequently, the cells were subjected to treatment with FA (50, 100, 200, 300, 400, 600 and 800 μM) for 24 h and then 400 μM AAPH for an additional 3 h.

Cell Viability Assay

The CCK-8 kit produced debris with an orange-yellow coloration, which corresponded to the number of viable cells. Each well of a 96-well plate received cell suspensions containing 1×10^4 cells with 100 μL. After a standard 24 h incubation in a cell culture incubator and washing, the cells were treated with various concentrations of FA (50, 100, 200, 300, 400, 600 and 800 μM) for either 24 or 48 h (with three replicates for each concentration). Following another PBS wash, 100 μL of a mixed culture solution (CCK8 solution: 1640 in a 1:10 ratio) was added. It was then re-treated 3 h in the absence of light and enzyme marker measurements were recorded.

Extraction of Nuclear and Cytoplasmic Proteins

Cells were plated at 1.6×10^5 cells per well in 6-well plates. Then a 24 h incubation in a cell culture incubator, the cells were exposed to varying concentrations of FA (100, 200, 300 and 400 μM) for 24 h. Subsequently, 400 μM

AAPH was introduced and the cells were treated for 3 h. Protein extraction was performed following the kit instructions and protein samples were then prepared, boiled, centrifuged and stored at -80°C.

Western Blotting

For each well, 7 µL of nuclear and cytoplasmic protein samples were used. The aim was to consider the antioxidant protein Nrf2 in the nucleus and cytoplasm. Electrophoresis was conducted at 20 mA for each gel and halted when the blue band descended by 0.5 cm below the plate. The membrane was transferred using the "wet transfer" method, creating a layered "sandwich." Before this, the PVDF membrane was soaked in methanol. It was placed in the transfer tank, which was kept in an ice bath.

The membrane was transferred at 120 V for 60 min and then removed using forceps. Subsequently, the membrane was sealed with TBST containing 5% skimmed milk powder for an hour. The membranes were then cut at regular intervals based on markers and overnight at 4°C on a shaker with antibody (1:1000). After the incubation, the membrane was thoroughly rinsed with TBST three times, each time for 5 min. The secondary antibody (1:5000) was incubated for an additional hour. Once this was completed, the membrane was rinsed with TBST three times, each time for 5 min and then developed using chemi scope 6200 touches with chemiluminescence. The grey value was subsequently analyzed using image J.

Statistical Analysis

All data are presented as mean ± standard deviation from a minimum of three replicates for each sample. Statistical analysis was carried out using SPSS 23 and significant differences were determined using analysis of variance and the Bonferroni post hoc test. A significance level of $p<0.05$ was considered statistically significant.

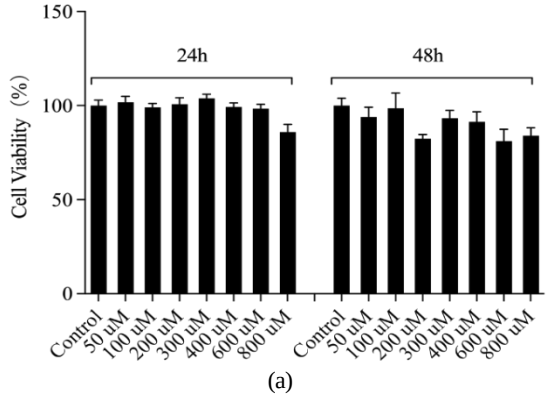
Results

Impact of FA on LO_2 Cell Viability

Cell viability reflects the effect of various substances on overall cell health and a lower cell survival rate indicates that the substance causes damage to the cell. LO_2 cells were treated by FA for varying time intervals (24 and 48 h) to assess their impact on cell survival and proliferation. It suggested that FA did not significantly impact the proliferation of LO_2 cells, with a survival rate ranging from 81.2-101.8% when FAs concentration was in 0-800 µM. Among the various concentrations of FA tested, those within the range of 50-400 µM displayed the highest level of survival, similar to the control (0 µM) (Fig. 1A), with no significant difference. Furthermore, the trend observed with the 24 h treatment was consistent with that of the 48 h treatment. As a result, the experiment was limited to 24 h and concentrations ranging from 0-400 µM of FA were chosen for subsequent experiments.

Impact of FA on AAPH-Induced Oxidative Pathway Protein Expression in LO_2 Cells

The impact of FA on protein expression of key cellular antioxidant proteins, including Nrf2 and HO-1, was analyzed via Western blot. Nrf2 plays a vital role in the antioxidant pathway by primarily regulating antioxidant components like superoxide dismutase, glutathione and other essential substances (Sathibabu Uddandrao *et al*., 2018). It also functions as a significant transcriptional regulator. In a state of oxidative equilibrium, Nrf2 remains inactive, bound to keap1 and unable to perform its antioxidative function (Bitar, 2012). However, when subjected to internal or external stimuli, Nrf2 undergoes phosphorylation, detaches from Keap1 and enters the nucleus to form a heterodimeric complex (Saito, 2013). The binds to antioxidants, thus controlling the transportation of downstream antioxidative proteins, which include HO-1, Glutathione (GSH) and NAD(P)H: Quinone Oxidoreductase 1 (NQO1). This regulation also impacts the transport of Superoxide Dismutase (SOD) and related proteins (Hayes and Mcmahon, 2009; Kobayashi *et al.*, 2006).



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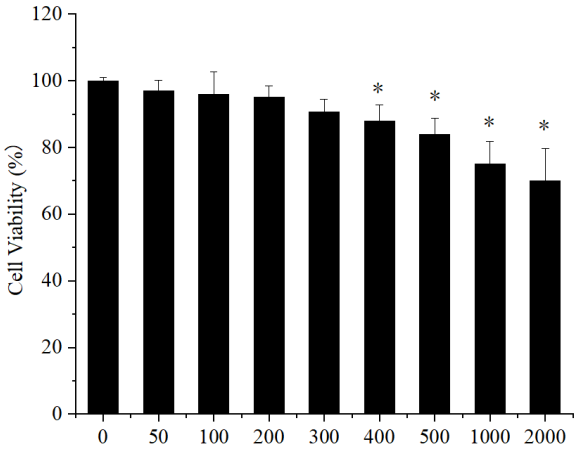

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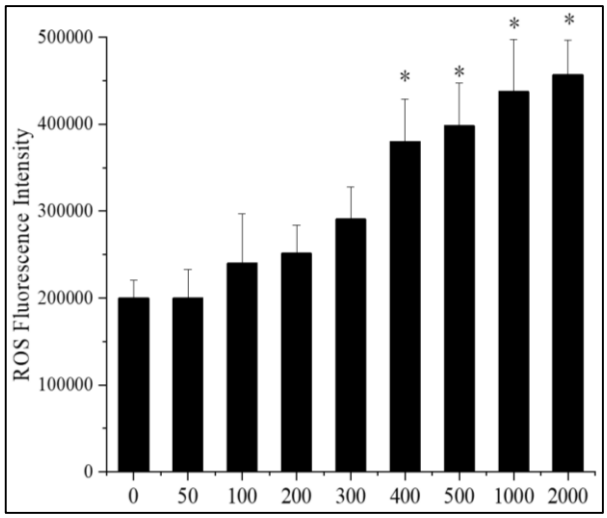

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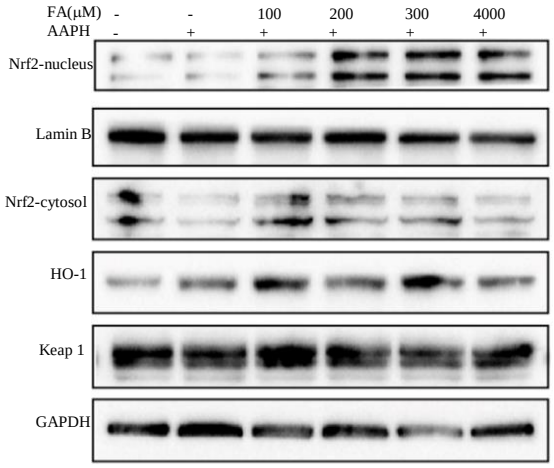

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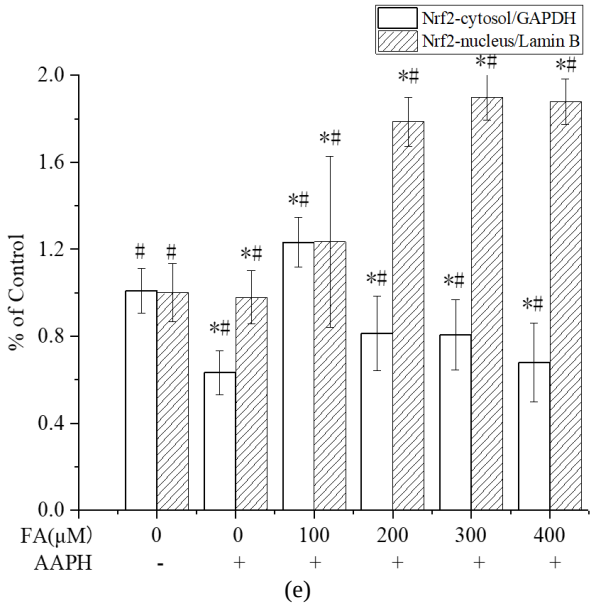

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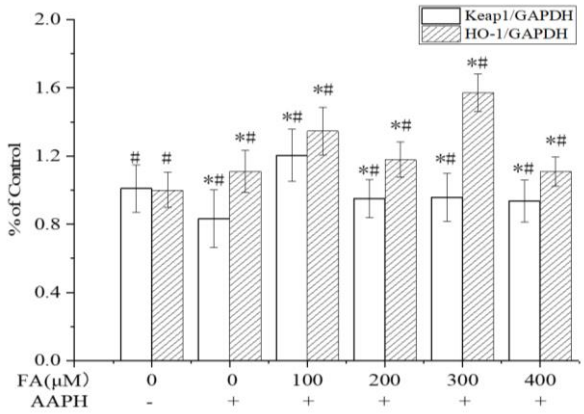


(f)

Fig. 1: (A) LO_2 cells were treated with different concentrations (0-800 μM) of FA for 24h and 48 h; (B) LO_2 cells were treated with 0-2000 μM AAPH for 3 h; (C) The ROS generation in AAPH-treated LO_2 cells; (D) FA upregulated Nrf2-Antioxidant Response Element (ARE) pathway in AAPH-treated LO_2 Cells. The expression levels of Nrf2, Keap1 and HO-1 in cell nucleus and cytoplasm were detected by western blotting analysis; (E) Quantification of Nrf2 in cell nucleus and cytoplasm expression; (F) Quantification of Keap1 and HO-1 in cell expression. Data are reported as mean ± S.D. (n = 3) for each group. *p<0.05 vs. control group, #p<0.05 vs· AAPH-treated group, n.s. means no significant difference

The findings in Fig. 1b-d reveal that treatment with FA led to an abate in protein Nrf2 levels in the nucleus. Simultaneously, the Nrf2 protein level in the cytoplasm progressively decreased, while the housekeeping protein Lamin B in the nucleus remained relatively stable. These results imply that higher concentrations of FA facilitate Nrf2s movement from cytoplasm to nucleus, enhancing its functionality. Inside the nucleus, the expression of HO-1, a crucial downstream antioxidant protein regulated by Nrf2, continued to rise in conjunction with the increase in Nrf2 levels. As the concentration of FA increased, Keap1 decreased and underwent further degradation through autophagy and ubiquitination. This suggests that FA, particularly in the concentration range of 100-400 μM, exerts its antioxidant effect by promoting the split of Nrf2 from Keap1 and enhancing downstream HO-1. The semi-quantitative results (Figs. 1e-f) closely align with the gray values obtained from the immunoblots.

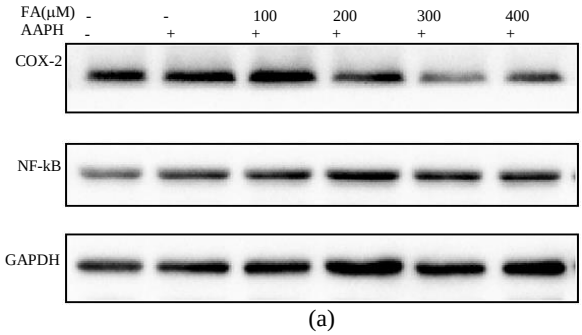
Impact of FA on AAPH-Induced Inflammatory Pathway Protein Expression in LO_2 Cells

Oxidative stress and inflammation are often interconnected (Mcgarry *et al.*, 2018). The results presented in Figs. 2a-b indicates that in the AAPH-treated group, key proteins in the inflammatory pathway, namely COX-2 and NF-κB, were elevated, leading to an increase in inflammatory factors. The expression of these proteins in different FA-treated groups showed varying degrees of

reduction. Notably, a significant reduction in COX-2 expression was observed, suggesting that FA primarily exerts its anti-inflammatory effect by modulating the COX-2 pathway. However, the impact on the NF-κB pathway was less evident. Semi-quantitative results confirmed that the AAPH group exhibited the highest expression of critical inflammatory proteins. Nevertheless, various concentrations of FA indicated a certain degree of inflammation inhibition.

Impact of FA on Autophagy

As depicted in Figs. 2c-d, the AAPH group exhibited a significant response to oxidative stress and inflammatory factors but did not notably stimulate autophagic flow. This could be explained by the organism's ability to self-remediate through alternative pathways to restore a healthy state. However, the upregulation of LC3, a critical autophagy protein, alongside the downregulation of P62 following treatment with varying concentrations of FA, induced cellular autophagy. Simultaneously, this process led to attenuation of oxidation and inflammation. The semi-quantitative results from the respective protein blots were consistent with these findings. At the mRNA level of autophagy-related molecules, LC3 exhibited an upward trend in varying concentrations of FA-treated groups, while P62 showed the opposite trend at the mRNA level (Fig. 2e). These trend patterns aligned with the protein level expressions, indicating that different concentrations of FA can modulate cellular autophagy to some extent.



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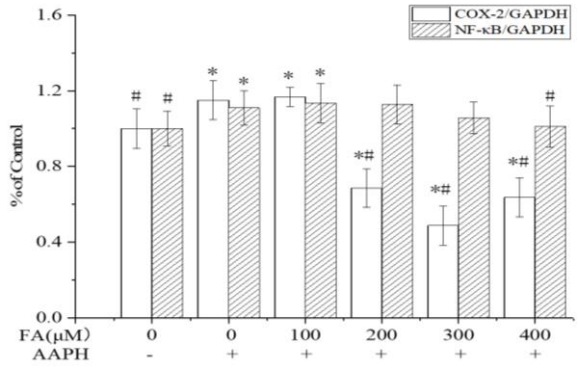

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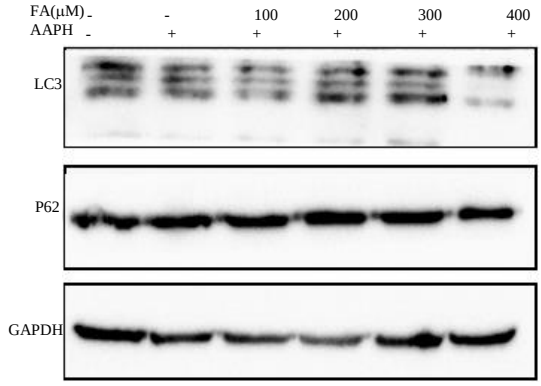

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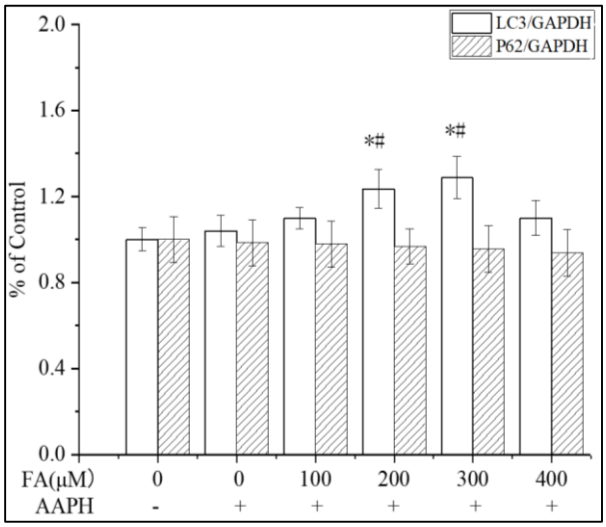

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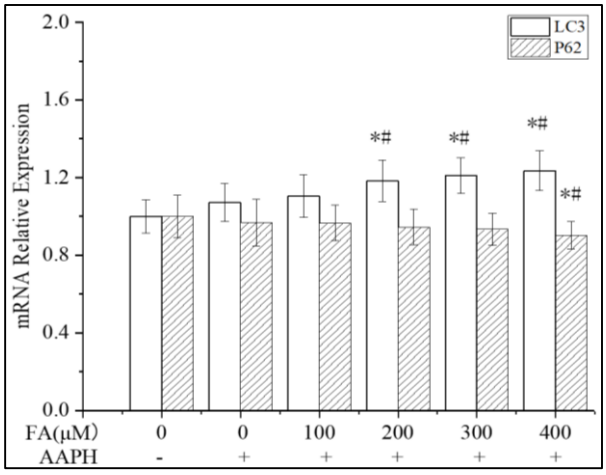

(e)

Fig. 2: (A) The expression levels of COX-2 and NF-κB were detected by western blotting analysis; (B) Quantification of COX-2 and NF-κB; (C) The expression levels of LC3 and P62 were detected by western blotting analysis; (D) Quantification of LC3 and P62; (E) The mRNA expression levels of LC3 and P62

Discussion

Oxidative stress occurs when there is a disparity between the effects of antioxidants and the detrimental influence of excessive ROS (Furukawa *et al.*, 2004; Wiel *et al.*, 2019). The excessive activation of ROS leads to the expression of inflammation-related factors, such as COX-2 and NF-κB (Khan *et al.*, 2012; Yang *et al.*, 2011). In response to counteract the oxidant detrimental, the body triggers a complex response mechanism to prevent and alleviate cellular damage while sustaining normal cellular growth (Parida and Das, 2005). Autophagy could able to clear ROS, improve antioxidant capacity and inhibit inflammation. The stronger the antioxidant capacity, the less ROS there is. The Nrf2-Keap1 serves as a critical defense mechanism against oxidation (Zhou *et al.*, 2017). Under normal circumstances, Nrf2 is situated cytoplasm of the cell as part of a Keap1/Nrf2 complex. To initiate the mitigation process, Nrf2 recognizes protomer ARE. Its purpose is to address oxidative stress, maintain oxidative balance and activate the cell's inherent antioxidant defenses.

Moreover, it triggers the activation of genes, including HO-1, in downstream expression. Therefore, by modulating the Nrf2/Keap1/ARE pathway, it is possible to mitigate damage and enhance antioxidant capacity (David *et al.*, 2017). In this study, an oxidative damage model was induced in LO_2 cells using the oxidative stress inducer AAPH. It was demonstrated that the concentration range of 100-400 μM of FA's activity in hepatocytes was attributed to HO-1. This induction is governed by Keap1-dependent post-translational phosphorylation of ERK, dependent on Nrf2 activation (Kim and Jang, 2014; Xu *et al.*, 2006).

Oxidative stress stimulates autophagy, which helps mitigate the adverse effects of stress. Autophagy is a programmed cellular process that entails the degradation of a majority of cytoplasmic contents using autophagic lysosomes. LC3, a marker of autophagy, plays a vital role in the formation of autophagy vesicles while eliminating mitochondria to prevent excessive ROS production. On the other hand, P62 primarily functions as a receptor for protein aggregates, undergoing ubiquitylation and degradation and plays a pivotal role in monitoring autophagic flux. In comparison to the control, the FA group was able to promote cellular autophagy to some extent, both at the protein and mRNA levels.

Conclusion

In summary, FA demonstrates a protective effect on cells damaged by oxidative stress by inducing cellular autophagy, increasing levels of autophagy marker proteins and facilitating Nrf2 into the nucleus. This activation subsequently promotes the downstream protein HO-1. Currently, a cellular-level analysis of the mechanism governing FA's regulation of cellular autophagy and antioxidant activity has been carried out. Animal models will be used to validate the specific quantitative and effective relationship, providing theoretical data for the development of products related to traditional fermented foods and Chinese herbs.

Acknowledgment

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Author's Contributions

Zhiyuan LV and Qiaozhen Zhao: Contributed equally to this study: Performed the experiment and wrote the manuscript.

Shanbin Chen: Made the conceptualization, methodology, writing-original draft preparation, written-review and edited.

Wenmei Zhao: Performed the experiment and methodology.

Heng Zhong: Performed the experiment.

Ruiqi Luo, Wei Jiang, Wei Liu and Mengchao He: Performed formal analysis.

Xinglin Han and Deliang Wang: Performed data curation, supervision.

Mengmeng Zhang, Xinyu XU and Xiaomeng Zhang: Provided resources and performed supervision.

Ethics

All authors have read and agreed to the published version of the manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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