RESEARCH ARTICLE

Beverage containing ginger and snow pear inhibits smoke-induced pyroptosis in rat lungs and protects small intestine

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Tobacco smoke is the leading risk factor for lung cancer, accounting for over 80% of lung cancer deaths. Reducing the damage of cigarette smoke to the lungs through dietary therapy is currently a research hotspot. It has been reported that cigarette smoke extract induced inflammation and contributed to pyroptosis through the ROS/NLRP3/caspase-1 pathway in human bronchial epithelial cells. To investigate the effect of ginger and snow pear beverage on smoke-induced pyroptosis, ginger, snow pear, loquat, honey, and skimmed milk powder were used to prepare a lung moistening drink with excellent palatability. Male rats were divided into the drink group and control group and exposed to cigarette smoke for 60 days. Blood biochemical indices and organ coefficients were measured, and lung and small intestine tissues were excised for HE staining. The mRNA expression of pyroptosis in the lung tissue was evaluated by fluorescence quantitative polymerase chain reaction (qPCR). The results showed that globulin and urea nitrogen levels were significantly higher in control (C) group than that in beverage (B) group, while the opposite effect was observed for the albumin/globulin ratio and cholesterol level. The changes in the weight of the lung tissue were obvious and significantly higher in group B than that in group C. The histological structure of the lung tissue in group C was abnormal with evidence of significant pathological changes in the alveolar wall and capillary bronchial tube wall along with a few inflammatory cells. The pathological changes in group B showed significantly improved. The small intestinal villus length, intestinal crypt depth, goblet cell number, and V/C value were significantly higher in group B. In addition, the drink significantly reduced the mRNA expression of NLRP3, caspase1, GSDMD, IL-18, and IL-1β genes in the lung tissue. The results indicated that the tested drink exerted protective effects on the lungs and small intestine, which inhibited the release of inflammatory factors from the cellular pyroptosis signaling pathway, alleviated the occurrence of pyroptosis, protected the lung tissue, and reduced the cigarette smoke-induced damage and lesions.

Keywords: cigarette; smoke; ginger; snow pear; pyroptosis; inflammation; pathological damage.

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Introduction

Smoking is known to cause respiratory, digestive, endocrinal, and other systemic diseases. Cigarette smoke contains a large number of carcinogens, with nicotine being the main active ingredient. The lungs, which have a huge surface area and can be in full contact with cigarette smoke, are the most severely damaged organ from cigarette smoking. Some studies have shown that smoking can lead to chronic lung disease and make individuals susceptible to acute

lung injury and infections as well as dysfunctions of alveolar macrophages [1]. Therefore, it is of great importance to advocate smoking cessation and develop healthy food products that nourish and moisten the lungs to alleviate the harmful effects of cigarette smoking.

Ginger, snow pear, and honey have been known to moisten the lungs and exert excellent protective effects against a variety of respiratory diseases [2-5]. Ginger, as a natural product that can acutely relax airway smooth muscle (ASM) [2], has long been reported to possess anti-inflammatory properties. Yocum *et al*. demonstrated that chronic administration of whole ginger extract or 6-shogaol, a bioactive component of ginger, augmented the Treg polarization of naive CD4 cells *in vitro* and mitigated house dust mite antigen-mediated lung inflammation in mice *in vivo*. Such decrease in inflammation was associated with reduced airway responsiveness *in vivo* [2]. In addition, curcumin, as one of the main active ingredients in ginger, has antioxidant, anti-inflammatory, and immunomodulatory effects [3]. Combinations of honey and *Nigella sativa* (NS) showed significant improvement in all pulmonary functions, including forced expiratory volume, forced vital capacity, and peak expiratory flow rate in both moderate and severe, uncontrolled persistent asthma compared with baseline. Asthma control test scores also improved significantly in patients using combinations of honey and NS compared with baseline [4]. Chronic obstructive pulmonary disease (COPD) refers to a lung disorder associated with symptoms of dyspnea, cough, and sputum production. Traditionally, Yijin-tang (YJT), a mixture of ginger, Chinese liquorice, and tangerine peel, has been prescribed for the treatment of respiratory system diseases caused by dampness phlegm. It has been reported that YJT significantly suppressed inflammatory cell counts and reduced IL-1, IL-6, and TNF-α levels in bronchoalveolar lavage fluid (BALF) and lung tissue. In addition, YJT not only decreased airway wall thickness, average alveolar intercept, and lung fibrosis, but also suppressed the expression of matrix metallopeptidase (MMP)-7, MMP-9, TGF-β, and collagen deposition. Moreover, YJT suppressed phosphorylation of NF-κB as well as expression of cyclooxygenase-2 (COX-2) and induced nitric oxide synthase (iNOS) [5].

Lung cancer is a leading cause of cancer death in the United States and globally with the majority of lung cancer cases attributing to cigarette smoking. Exposure to secondhand smoke has also been identified as a risk factor for lung cancer for three decades. Therefore, it is very important to protect lung health from the harm of cigarette smoke. Meanwhile, smoking can also bring various other respiratory diseases. Pyroptosis, also known as inflammatory cell necrosis, is a type of programmed cell death mediated by gasdermin, which is characterized by continuous cellular distension until membranolysis that results in the release of cellular contents and activation of an intense inflammatory response. Pyroptosis is an important natural immune response of the body and plays a pivotal role in the fight against infection. Studies have shown that cigarette smoke has a significant activating effect on cellular pyroptosis in COPD patients [6-9].

This study developed a ginger-snow pear drink with excellent palatability using these main ingredients and supplementary ingredients. By feeding the prepared drink to rats that inhaled cigarette smoke for a long period, the multiple indicators were tested to investigate the lung-moistening effect of proposed drink and the underlying mechanisms of action. The study could lay a theoretical foundation for the study of development and application of healthy food.

Materials and methods

Drink preparation

Skim milk powder was added to water at a 1:9 ratio and then mixed with loquat honey at a 3:1 ratio. The mixture was sterilized, cooled to room temperature, and inoculated with 1×10^5 *Lactobacillus acidophilus* for 24 h fermentation. Snow pears were cleaned, and the peel (core)

was removed. The fruits were cut into 1 cm small pieces, soaked into 0.2% ascorbic acid for 5 mins to protect the color, and pulped with 0.2% ascorbic acid at a 1:1 ratio. The pulp was filtered using six layers of gauze. Ginger was boiled with 4% sodium hydroxide for 5 mins, cleaned and peeled, and then soaked in 0.2% hydrogen sulfite for 2 h. Then the ginger was cleaned and cut into 1-2 mm thin slices, boiled for 5 mins, pulped in water at a 1:1 ratio, and filtered through six layers of gauze. All the ingredients, namely, yogurt, ginger juice, snow pear, and sugar were mixed at a ratio of 35%:8%:50%:7%. The final product was obtained after demulsification, high pressure (60 MPa) homogenization, blending, stabilization, mixing, acid conditioning, sterilization, cooling, filling, and was stored at 4°C.

Experimental grouping and treatment

Twenty 60-day-old healthy male SD rats were purchased from the Animal Core Facility of Zhengzhou University (Zhengzhou, Henan, China) (SCXK 2021-0009). All animal studies were performed according to the guidelines of the Institutional Animal Care and Use Committee of Zhengzhou University. All rats were randomly divided into drink group (group B) and control group (group C) with 10 rats in each group. Rats from both groups were placed in a 39.375 L plastic smoking box (45 × 35 × 25 cm^3) and allowed to inhale smoke from three simultaneously lit Hong Qiqu brand cigarettes (Anyang, Henan, China) at each time, twice a day at 9:00 am and 17:00 pm for 60 consecutive days. During this process, rats from group B had free access to the proposed drink while those in group C drank boiled tap water as water sources.

Measurement of organ coefficients

At the end of the experiments, all rats were euthanized by decapitation after isoflurane anesthesia and dissected. Five vital organs including heart, liver, spleen, lung, and kidney were excised and weighed to calculate the organ coefficients.

Measurement of serum indicators

Rat blood samples from each group were collected, left undisturbed, and centrifuged at 3,500 rpm, for 10 mins. The obtained serum was separated and stored at -20℃ for future analysis. Thirteen serum biochemical indices including albumin, total protein, globulin, albumin-to-globulin ratio, calcium, glucose, urea nitrogen, inorganic phosphorus, cholesterol, alanine aminotransferase, total bilirubin, creatinine, and urea nitrogen/creatinine ratio were tested by outsourcing samples to Servicebio Biotechnology Co., Ltd, Wuhan, Hubei, China. Each sample was tested thrice.

Tissue HE staining

The lung and small intestine tissues were excised and fixed in 10% neutral formaldehyde fixative. The samples were paraffin-embedded, sectioned, and stained using HE staining kit. Any pathological damage to the lung and small intestine was observed under ordinary light microscopy at 100× scale and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). Five intact villi were selected from each section and their length (μm) and crypt depth (μm) were measured and averaged. The mean villus to crypt ratio (villus length/crypt depth) and the number of chorioepithelium goblet cells were calculated.

Fluorescence quantitative polymerase chain reaction (qPCR)

The gene encoding β-actin was selected as the internal reference gene. The publicly available mRNA sequences of β-actin, caspase-1, NLRP3, GSDMD, IL-18, and IL-1β were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank). The primers were designed according to the principles of real-time fluorescence quantitative PCR primer design using Primer premier 5.0 (PREMIER Biosoft International, San Francisco, CA, USA) and Oligo 6.0 software (OLIGO, Colorado Springs, CO, USA) (Table 1), and sent to Bioengineering (Shanghai) Co., Ltd. (Shanghai, China) for synthesis. Total RNA was extracted from lung tissues using TRIzol (Takara Biomedical Technology Co., Ltd., Beijing, China) method and the concentration was measured using

Table 1. Primer sequences.

Genes	Primer Sequences (5'-3')	Products Length (bp)	Tm (℃)
β-actin (V01217.1)	F: AGAAGCTGTGCTATGTTGCTCTA R: AGACAGCACTGTGTTGGCATA	260	55
IL-1β (NM_008361.4)	F: ATCTCGCAGCAGCACATCAA R: ACGGGAAAGACACAGGTAGC	94	55
Caspase1 (NM_009807.2)	F: CGAGGGTTGGAGCTCAAGTT R: TCCTTGTTTCTCTCCACGGC	341	55
NLRP3 (XM_006246457.4)	F: GGTCAGCTGCTGTCTCACAT R: CCCATGTCTCCAAGGGCATT	282	55
GSDMD (AB103383.1)	F: GCGTGTGACTCAGAAGACCT R: ACCTCGGTCACCACAAACAG	120	55
IL-18 (NM_008360.2)	F: TCAGACAACTTTGGCCGACT R: TCAGTCTGGTCTGGGGTTCA	145	55

NanoDrop™ One/OneC (Thermo Scientific™, Shanghai, China). Total RNA was immediately reverse transcribed according to the instructions of the fluorescence quantitative reverse transcription kit. The resulting cDNA was stored at -20℃. Fluorescence quantitative PCR was performed in a 25 μL reaction system comprising 12.5 μL of SYBR Premix Ex Taq Ⅱ (Tli RnaseH Plus) (2×), 0.7 μL of PCR Forward Primer (10 mol/L), 0.7 μL of PCR Reverse Primer (10 mol/L), 2.0 μL of cDNA template, and 9.1 μL double distilled water (ddH_2O). The reaction was programmed as 95℃ for 30 s followed by 40 cycles of 95℃ for 5 s, Tm for 20 s, 72℃ for 30 s, while the Tm was set from 65.0℃ to 95.0℃ with a temperature gradient of 0.5℃ for 5 s. The relative expression of mRNA was compared using the relative quantification $2^{-\triangle\triangle Ct}$ method.

Data processing

The experimental data were analyzed using SPSS 21.0 statistical software (IBM, Armonk, New York, USA). The results were expressed as mean ± standard deviation. The least significant difference (LSD) method in one-way analysis of variance (ANOVA) was used for multiple comparisons between groups. The graphs were plotted using GraphPad Prism 6.0 (GraphPad Software, Boston, MA, USA). A value of $P < 0.05$ indicated statistically significant difference, while $P < 0.01$ indicated highly significant difference.

Results

Organ coefficient

After weighing the heart, liver, spleen, lungs, and kidneys of rats from each group, the organ coefficients were calculated (Table 2). The results showed that only the weight of the lungs was significantly different between the two groups ($P < 0.01$). No significant difference was observed in the weights of other organs between the two groups. Thus, the test drink could play a major protective role in the lungs after prolonged smoke stimulation.

Measurement of serum indicators

The results of 13 serum biochemical indices showed that globulin and cholesterol levels were significantly higher in group C than that in group B ($P < 0.05$ and $P < 0.01$, respectively). The Alb/Glb ratio was significantly lower in group C than that in group B ($P < 0.05$). The levels of urea nitrogen, total bilirubin, and creatinine were significantly higher in group B than that in group C ($P < 0.01$) (Table 3).

Pathological changes in the lung tissue and small intestine

Table 2. Organ coefficients of rats.

Groups	Heart (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)
B Group	0.53±0.009	3.84±0.031	0.23±0.009	0.66±0.006**	0.92±0.015
C Group	0.56±0.012	3.76±0.009	0.21± 0.006	0.54± 0.007	0.91±0.006

Note: ** indicates a significant difference between the B group and C group ($P < 0.01$).

Table 3. Results of biochemical index test.

Item	B Group	C Group
ALB	51.73±0.23	51.60±0.15
TP	80.90+0.32	81.97±0.33
GLO	28.73±0.33*	30.83±0.55
A/G	1.80±0.00*	1.67±0.03
Ca	2.94±0.05	2.76±0.05
Glu	4.83±0.05	4.94±0.37
BUN	9.16±0.05**	8.44±0.06
P	3.38±0.05	3.37±0.05
CHOL	3.78±0.03**	4.28±0.03
ALT	90.33±0.88	91.00±2.52
TBIL	4.71±0.08**	2.54±0.06
SCR	61.00±1.15**	54.33±0.88
BUN ／ Scr	37.33±0.33	37.67±0.88

Note: * indicates a significant difference between the B group and C group ($P < 0.05$). ** indicates a significant difference between the B group and C group ($P < 0.01$).

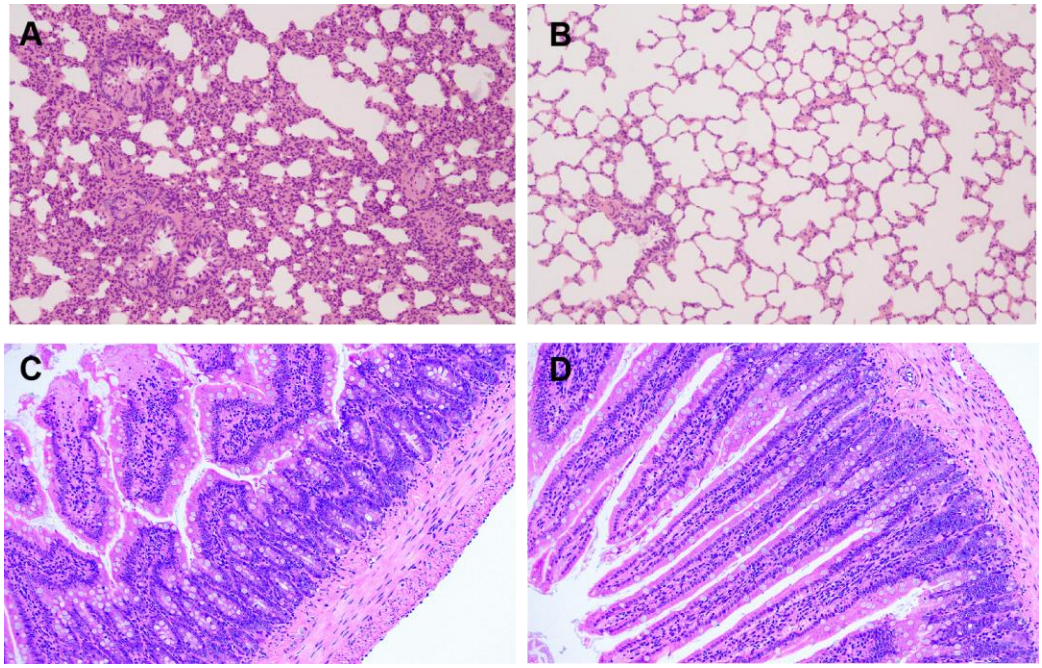

Figure 1. Histopathologic slides of lung (**A** and **B**) and small intestine (**C** and **D**). **A** and **C:** C groups. **B** and **D:** B groups.

Table 4. Small intestine index test results.

Groups	Villus length (μm)	Crypt depth (μm)	Villous epithelial goblet cells	Velvety ratio
B Group	691.49±3.82**	195.0±6.48**	28.60±2.01*	3.69*
C Group	504.62±29.13	125.48±8.35	18.20±2.44	3.55

Note: * indicates a significant difference between the B group and C group ($P < 0.05$). ** indicates a significant difference between the B group and C group ($P < 0.01$).

The staining results showed that the lung tissue structure of group C rats was abnormal with the evidence of the thickened alveolar wall, unclear structure, and some alveoli atrophy (Figure 1A). The bronchial epithelial cells were neatly and tightly arranged. The walls of bronchioles were partially ruptured, consistent with bronchial congestion, a small amount of hemorrhage in the bronchial cavity, and a few inflammatory cells in the bronchus. Group B lung samples showed normal lung tissue structure, normal alveolar wall thickness, normal alveolar size, regular arrangement of bronchial epithelial cells, individual inflammatory cells, and protein mucus (Figure 1B). The length of small intestinal villi and the depth of crypt were significantly higher in group B than that in group C ($P < 0.01$). The V/C value in group B was also significantly higher than that in group C ($P < 0.05$) (Table 4). The samples from group B had more complete intestinal villus structure, regular arrangement, clearer outline of intestinal epithelial cells, and distinct staining than the samples from group C (Figure 1D). However, in group C, the intestinal villi were sparse and severely broken with disorganized arrangement, poorer structural integrity of small intestinal epithelium, and altered intestinal morphology (Figure 1C).

Fluorescence quantitative PCR

The results of fluorescence quantitative PCR revealed the significant downregulation in the mRNA expression of NLRP3, caspase1, GSDMD, IL-18, and IL-1β in group B as compared with that in group C ($P < 0.01$) (Figure 2). Thus, the test drink significantly alleviated the occurrence of cell pyroptosis in the lung tissue and reduced the inflammatory response.

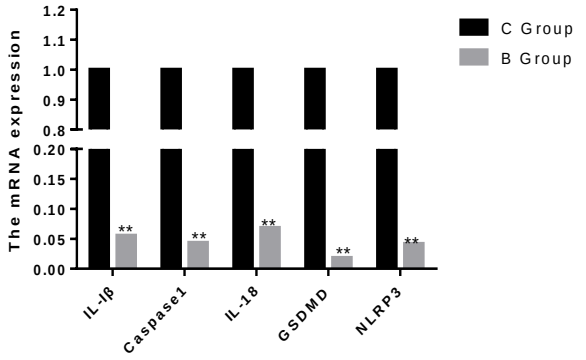

Figure 2. Expression level of pyroptosis key genes in rat lung tissue. ** indicates a significant difference between the B group and C group ($P < 0.01$).

Discussion

Smoking, including cigarette and e-cigarette smoking, has been reported to induce chronic lung, vascular, and tumor-related diseases and can shorten life expectancy by at least 10 years [10]. Hence, the importance of protecting the lung health cannot be overstated. Cigarette smoke is an aerosol comprising solids and droplets in the gas phase, and contains more than 4,500 different substances with toxic, mutagenic and carcinogenic effects. These substances include nicotine, tar, ammonia, carbon monoxide, formaldehyde, acrolein, acetone, polycyclic aromatic hydrocarbons, hydroxyquinone, nitrogen oxide, and cadmium. The particulate matter inhaled from cigarette smoke is deposited in different parts of the respiratory system; larger particles get deposited in the upper respiratory tract, and smaller particles settle in the alveoli. Studies have shown that cigarette smoke can cause oxidative stress, leading to chronic low-grade inflammation [1]. The development of complementary therapeutic nutraceuticals has, therefore, become one of the

popular means to reduce the damage caused by cigarette smoking.

At present, the development and production of new lung-moistening drinks is becoming very common. From the perspective of food therapy, our team applied ingredients with excellent lung-moistening effects, such as ginger, snow pear, and loquat honey, and adjusted their ratios to produce a drink with lung-nourishing and lung-moistening effects. The results showed that the drink significantly improved the organ coefficients of the lungs and had a significant protective effect on the lung tissue, but no significant effect was observed on the heart, liver, spleen, and kidney. It also reduced globulin content, attenuated immune response, increased urea nitrogen content, lowered cholesterol, reduced mRNA expression of NLRP3, caspase1, GSDMD, IL-18, and IL-1β (key genes involved in cell pyroptosis), and alleviated the inflammatory response in rats. Pathological tissue observation showed that the lung structure was abnormal in rats inhaling cigarette smoke, as evident from thickened alveolar walls and a few inflammatory cells visible in the bronchi. On the other hand, the lung tissue structure of rats fed with the ginger-snow pear drink was normal, consistent with thinner alveolar walls and fewer inflammatory cells. Therefore, the inhalation of cigarette smoke causes serious damage to the lungs in the respiratory system. The small intestine is the main digestion and absorption site in animals. After inhaling cigarette smoke, the intestinal villi of rats became sparse, severely broken with a large number of shedding, and disorganized. The epithelial structural integrity of the small intestine was poor, and the intestinal morphology changed. In contrast, the villi and crypt depth of the small intestine of the rats drinking the ginger-snow pear drink greatly improved. The villus length and crypt depth can increase the surface area of the small intestine and enhance its absorption capacity, which is closely related to the health degree of the organism. These results show that the ginger-snow pear drink can not only alleviate the lung damage caused by cigarette smoke but also exert protective effects on the digestive system. Some studies have shown that ginger can be used to treat gastrointestinal motility disorders [11]. Ginger or propolis can improve methotrexate-induced ileum damage by increasing the number of goblet cells and the length of the brush border of the ileum [12]. Honey also exhibits a protective effect on the small intestine. During intestinal healing and adaptation after large intestinal resection, honey may help in weight gain, intestinal mucosal growth, and adaptation, as evident from an increase in the residual intestinal villus height, villus weight, crypt depth, and villus density; what's more, honey was more effective when combined with other drugs [13]. In summary, ginger and honey in ginger-snow pear drink may play the primary and secondary roles, respectively. Ginger-snow pear drink can reduce lung damage and inflammatory response caused by cigarette smoke, inhibit the occurrence of cellular pyroptosis, and alleviate pathological damage to the small intestine.

Considering human bronchial epithelial cell pyroptosis, cigarette smoke extract was shown to increase the release of lactate dehydrogenase (LDH), interleukin (IL)-1β and IL-18 and upregulate the transcription and translation of caspase-1 and NLR family pyrin domain containing 3 (NLRP3) in cells [9]. A previous report showed that 10% cigarette smoke extract increased the mitochondrial oxidative stress in bronchial and alveolar epithelial cells, upregulated inflammatory gene expression, downregulated antioxidant gene expression, and increased the expression of caspase-1 and NLRP3 [14]. The results of our study are consistent with those described in previous reports. 6-gingerol is a major component of ginger with anti-inflammatory and antioxidant effects. Previous study has shown that 6-gingerol inhibited the expression of pyroptosis-related proteins, including NLRP3, IL-18, IL-1β, and caspase-1 [15-17]. Ginger also exhibits significant protective functions against lung diseases. Long-term administration of ginger extract or ginger bioactive components reduced house dust mite

antigen-mediated lung inflammation in mice [2]. Curcumin induces apoptosis of small-cell lung cancer cells [18] and inhibits the enzymatic activity of the intracellular structural domain of the epidermal growth factor receptor (EGFR) involved in the growth of lung cancer [19], thus providing a significant protective effect against lung cancer [3]. Ginger has been shown to be effective against pulmonary symptoms associated with COVID-19 pneumonia in various preclinical and clinical trials [20]. A mixture of ginger, licorice, and orange peel is thought to reduce inflammation of the respiratory tract caused by cigarette smoke [5]. Many studies have shown that honey can moisten the lungs and reduce cough and asthma. Abbas AS et al. found that honey showed relatively high efficacy in asthma patients when combined with other medications [4]. Honey treatment also protected the midbrain and lungs from paraquat-induced toxic damage in rats [21]. The results of the present study are similar to some of the previous reports, but there are no published studies on the use of ginger, snow pear, and loquat honey to prepare a beverage that prevents the lung damage and cell pyroptosis induced by cigarette smoke. In this study, we used various modern biological techniques to elucidate that the ginger-snow pear drink can improve cigarette smoke-induced damage and lesions from multiple aspects, which may not only provide a theoretical basis for the production and promotion of therapeutic drinks but also serve as a solution to the health problems caused by smoking.

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