Effect of grape seed proanthocyanidin extract in peripheral blood mononuclear cells of severe asthmatic patients

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Abstract

Backgroud To explore the Effect of grape seed proanthocyanidin extract（GSPE） in peripheral blood mononuclear cells of severe asthmatic patients. Methods 40 severe asthmatic patients were randomly and averagely divided into 2 groups: DXM group (n=20) and GSPE+DXM group (n=20), and 20 healthy volunteers as control gorup. Heparinized peripheral venous blood from each subject was collected in a sterile vacuum tube. The levels of interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 were detected by ELISA. The protein and mRNA expressions of Nuclear factor erythroid 2-related factor 2 (Nrf2), glutamatecysteine ligase modifier subunit (GCLM) inducible nitric oxide synthase (iNOS) and inactivation of histone deacetylase-2 (HDAC2) were detected by Western blot and qRT-PCR respectively. Glutathion(GSH) was measured by using Glutathione Fluorometric Detection Kit, and Nrf2-ARE binding ability was measured by using TransAM Nrf2 Transcription Factor ELISA Kit. Results The results showed that the levels of IL-8 and MCP-1 in normal control group were lower than those in severe asthma group ($P < 0.05$). Treatment with GSPE+DXM reduced the levels of IL-8 and MCP-1 significantly when compared with DXM only ($P < 0.05$).The mRNA expression of iNOS in DXM group was significantly higher than that in control group. However, after adding GSPE treatment, the expression dramatically decreased ($P < 0.001$). On the contrary, lower mRNA expressions of Nrf2, GCLM and HDAC2 were found in DXM group than in control group ($P < 0.001$). Accordingly, when treated with GSPE, these expressions elevated and reached a statistical significance ($P < 0.05$). Consistently, the results from western blot analysis confirmed the role of GSPE on the protein expressions of iNOS, Nrf2, GCLM and HDAC2 in PBMCs of patients with severe asthma ($P < 0.001$). The PBMCs from patients with severe asthma exhibited lower Nrf2-ARE binding ability and produced less GSH than normal controls. GSPE treatment effectively augmented the Nrf2-ARE binding ability and the expression of GSH, which demonstrated the biological antioxidant potential of GSPE ($P < 0.001$). Conclusion GSPE can alleviate glucocorticoid resistance by regulating Nrf2-iNOS-HDAC2 signaling pathway in severe asthma, suggesting that GSPE have potential clinical application prospects in the treatment of severe asthma.

Background

Bronchial asthma is a common respiratory disease characterized by airway inflammation and airway hyperresponsiveness. However, the pathogenesis of asthma has not been fully clarified. About 5-10% of asthmatic patients are diagnosed as severe asthma[1], which is defined as asthma that requires high dose inhaled corticosteroids plus a second controller or treatment with systemic corticosteroids to control, or which remains uncontrolled despite this therapy[2]. Hospital visits and hospitalizations of these severe asthmatic patients have led to a significant increase in mortality, resulting in a heavy personal and socio-economic burden. 50% of annual public health spending on asthma comes from people with severe asthma[3].

At present, the main treatment medicines of asthma include glucocorticoid, β_2 receptor agonist, muscarinic antagonists, theophylline, leukotriene receptor antagonists, immunosuppressant, and others.

However, it is found that glucocorticoid resistance exists in patients with severe asthma, which is the main reason for the difficulty in treatment of severe asthma. The mechanism of glucocorticoid resistance is complicated and still unclear. Glucocorticoid resistance is mainly caused by inactivation of histone deacetylase-2 (HDAC2), which is critical for the inhibition of the glucocorticoid receptor which mediates the antiinflammatory effect of corticosteroids[4]. Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the cellular antioxidant response by upregulating genes encoding many phase II detoxifying or antioxidant enzymes, which are involved in protecting against oxidative and electrophilic stresses[5]. Under oxidative and nitrative stress, increased inducible nitric oxide synthase (iNOS) and decreased antioxidant enzymes precipitate the nitrosylation modification of HDAC2, resulting in impaired function and deacetylase enzymatic activity of HDAC2[6]. Oxidative and nitrative stress is augmented in severe asthma with increased iNOS, and the Nrf2 and HDAC2 functions are impaired in these patients. Thus we hypothesized that Nrf2-iNOS-HDAC2 pathway had a critical role in regulating glucocorticoid sensitivity of asthma.

Grape seed proanthocyanidin extract (GSPE), a class of flavonoid compounds formed by catechins, epicatechins, catechins and epicatechins[7], has beneficial features such as anti-inflammation, anti-oxidation, antineoplastic and antibiosis[8, 9]. Our previous animal experiments indicated that GSPE could inhibit airway inflammation and airway hyperresponsiveness by down-regulating iNOS[7], and could reduce airway remodeling, and restore sensitivity to glucocorticoid treatment by targeting Nrf2-iNOS-HDAC2 axis[10]. Low expression of Nrf2 caused upregulation of iNOS and downregulation of GSH, leading to dysfunction of HDAC2 and glucocorticoid resistance[10]. The aim of present study was to confirm the involvement of Nrf2-iNOS-HDAC2 axis in the mechanism of glucocorticoid resistance and further discuss the role of GSPE on the peripheral blood mononuclear cells (PBMCs) derived from patients with severe asthma.

Methods

Patients & samples

Forty adult patients with severe asthma and twenty control subjects were recruited from the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University. Patients with severe asthma were diagnosed based on the ERS/ATS guidelines[2]. The inclusion criteria were as follows: (1) $FEV_1 \geq 40\%$ predicted, and $FEV_1 \geq 55\%$ predicted after bronchodilator; (2) less than 6 times of acute exacerbation of asthma in the last 6 months; (3) no hospitalization for asthma in the last 6 months; (4) no tracheal intubation due to asthma in the last 1 year; (5) oral administration of prednisolone less than 20mg per day; (6) non-smoking patients. Participants with cardiac insufficiency, abnormal liver function, pulmonary embolism, coinfection, tuberculosis and blood system diseases were not included. At the period of drawing blood, all enrolled patients were in a stable clinical condition without acute attack of asthma. Severe asthmatic patients were randomly divided into 2 groups: DXM group (n=20) and GSPE+DXM group (n=20). Twenty healthy volunteers had no history of asthma, no other allergic and immune system

diseases, and were non-smokers. The detailed information of all subjects was displayed in Table 1. Heparinized peripheral venous blood from each subject was collected in a sterile vacuum tube between 7:00 and 9:00 AM.

Cell separation and culture

PBMCs were enriched by Ficoll-Hypaque (TBD, Tianjin, China) gradient centrifugation within 4 hours. The cell viability determined by trypan blue exclusion assay was greater than 95%. PBMCs were cultured in RPMI 1640 (Gibco, Invitrogen, UK) containing 10% foetal bovine serum and 1% penicillin, streptomycin. The diluted PBMCs were distributed in 1ml aliquots into 24-well plates (1.0×10^5/well) and incubated at 37°C with 5%CO_2. GSPE (50μg/ml) were added to the plates of GSPE+DXM group. After 16 hours, dexamethasone (DXM, 1μM) was added to the culture medium. Following 1 hour incubation to allow adherence, the cell culture supernatants and cell pellets were collected and stored at -80°C for further detection. The PBMCs of control group were cultured alone without any reagents.

Enzyme linked immunosorbent assay (ELISA)

The levels of interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 were detected by ELISA kit (Biolegend, USA) according to the manufacturer's instructions. The independent experiment was repeated 3 times.

Quantitative real-time PCR (qRT-PCR)

Total RNA from human PBMCs was extracted using Qiagen RNeasy Kit (Qiagen, Valencia, CA, USA). The cDNA was synthesized by reverse transcription using random primers and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). qRT-PCR was carried out on ABI 7000 Taqman system (Applied Biosystems, Foster City, CA, USA). Nrf2, GCLM, iNOS and HDAC2 primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). And β-actin was used as housekeeping gene. The sequences of primers of each target gene were shown in Table 2.

Protein extraction and Western blot

Total protein extracts from PBMCs were harvested using Qproteome Mammalian Protein Prep Kit (Qiagen, German). Protein concentrations were measured using the BCA protein assay (Beyotime, Shanghai, China). We loaded 15ug samples per lane on 10% SDS-PAGE gel for separation, and then transferred them to a PVDF membrane and incubated in blocking solution (5% skimmed milk in Tris-buffered saline) for 2h at room temperature. After washing three times with TBS and Tween (0.1% Tween-20, 100mM Tris-HCl, and 150mM NaCl, pH7.5), the membrane was incubated with the first antibodies of Nrf2, GCLM, iNOS and HDAC2 (1:1000 dilution) at 4°C overnight with gentle shaking. After complete elution, the membrane was incubated with HRP-conjugated secondary antibodies (1:5000 dilution, 2.5% BSA, PBS, 0.1% Tween-20) for 1h at room temperature. The proteins were quantified using ECL Plus Substrate (Amersham Biosciences) with β-actin as the gatekeeper protein.

Determination of GSH and Nrf2-antioxidant response element (ARE) binding ability

GSH was measured by using Glutathione Fluorometric Detection Kit (Biovision, Mountain View, CA, USA), and Nrf2-ARE binding ability was measured by using TransAM Nrf2 Transcription Factor ELISA Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions.

Statistical analysis

Differences among each group were accessed by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison tests. Data were expressed as the mean ± standard error of the mean (SEM). A *P* value <0.05 was considered statistically significant. All calculations were carried out by using SPSS 17.0 software.

Results

Characteristics of asthmatic patients

As shown in Table 1, there were no statistically significant differences in age, gender, BMI, FEV_1/FVC%, and FEV_1(% of predicted) between DXM and GSPE+DXM group.

GSPE reduces IL-8 and MCP-1 levels in PBMCs of patients with severe asthma

The results showed that the levels of IL-8 and MCP-1 in normal control group were lower than those in severe asthma group (P<0.05). Treatment with GSPE+DXM reduced the levels of IL-8 and MCP-1 significantly when compared with DXM only (P<0.05, Figure 1).

GSPE affects the expressions of iNOS, Nrf2, HDAC2 and GCLM in PBMCs of patients with severe asthma

The mRNA expression of iNOS in DXM group was significantly higher than that in control group. However, after adding GSPE treatment, the expression dramatically decreased (P<0.001, Figure 2). On the contrary, lower mRNA expressions of Nrf2, GCLM and HDAC2 were found in DXM group than in control group (P<0.001, Figure 2). Accordingly, when treated with GSPE, these expressions elevated and reached a statistical significance (P<0.05, Figure 2). Consistently, the results from western blot analysis confirmed the role of GSPE on the protein expressions of iNOS, Nrf2, GCLM and HDAC2 in PBMCs of patients with severe asthma (P<0.001, Figure 3).

GSPE increases the Nrf2-ARE binding ability and expression of GSH in PBMCs of patients with severe asthma

The PBMCs from patients with severe asthma exhibited lower Nrf2-ARE binding ability and produced less GSH than normal controls. GSPE treatment effectively augmented the Nrf2-ARE binding ability and the expression of GSH, which demonstrated the biological antioxidant potential of GSPE (P<0.001, Figure4 and 5).

Discussion

The majority of asthma patients can be effectively treated with currently available medication, such as inhaled corticosteroids (ICS) and long acting β_2-agonist (LABA)[11]. However, severe asthma, a subset of asthma, is characterized by being difficult to achieve disease control despite high-dose ICS plus LABA or oral corticosteroids. Till now, few effective therapeutical strategies for severe asthma can be available, and the underlying mechanisms of severe asthma remain unclear. In present study, we showed that the PBMCs from patients with severe asthma expressed higher levels of IL-8 and MCP-1 *in vitro* than normal controls, and GSPE could inhibit these expressions. Additionally, GSPE decreased the expression of iNOS, and increased the expressions of Nrf2, HDAC2 and GCLM in the PBMCs from patients with severe asthma, not only in mRNA but also in protein. Such effect might be attributed to the biological antioxidant potential of GSPE, since GSPE increased the Nrf2-ARE binding ability and expression of GSH.

Glucocorticoid plays an important role in inhibiting inflammatory response by activating anti-inflammatory genes and inhibiting pro-inflammatory genes[12]. Activated cytosolic glucocorticoid receptor binds to the coactivator of pro-inflammatory transcription factors, and then directly inhibits the activity of histone acetyltransferase, especially recruits HDAC2, to make histone deacetylation and chromatin remodeling, thus inhibiting the activation of inflammatory genes[12]. HDAC2 deficiency is a major cause of glucocorticoid resistance[4, 13]. The defect of HDAC2 expression and activity may be due to the increase of oxidative stress[4]. Oxidative stress can lead to changes in the core histone acetylation/deacetylation balance, resulting in increased inflammatory response and hormone insensitivity. GSH is an antioxidant tripeptide that plays a key role in maintaining the intracellular redox state. GSH-dependent enzymes include GSH reductase and GSH peroxidase[14]. The rate-limiting step in GSH synthesis is the formation of γ-GC by Glutamate-cysteine ligase (GCL)[15]. GCL is a kind of two-subunit enzyme composed of modifier (GCLM) and catalytic subunit (GCLC)[14, 15]. Nrf2 is an important determinant of the expression of GCLM and GCLC[16, 17]. Some studies have shown that defective function of Nrf2 leads to increases of iNOS and GSH expression[5, 10]. High expression of iNOS increases the production of NO and promotes the nitrosation of HDAC2[7, 10], while low expression of GSH decreases the denitrifying HDAC2. Our results showed that the expression of iNOS increased and the expression of Nrf2, HDAC2, GSH and GCLM decreased in severe asthma group (DXM group and GSPE+DXM group), confirming that Nrf2-iNOS-HDAC2 signaling pathway played an important role in the mechanism of steroid resistance in asthma.

IL-8 and MCP-1 are chemokines closely related to asthma. IL-8, secreted from T lymphocytes, epithelial cells, airway smooth muscle cells and macrophages, participates in airway inflammation by inducing migration and activation of neutrophils and aggregation of eosinophils and basophils[18]. Glucocorticoid can inhibit inflammatory factors and chemotaxis of chemokine to inflammatory cells to reduce airway inflammation and airway hyperresponsiveness. It has been found that the secretion of IL-8 in bronchial tissue of asthma patients is closely related to the severity of asthma[19, 20]. Many studies have shown that MCP is associated with asthma[21], and the expression of MCP-1 receptor is increased in asthma

patients, which affects airway inflammation and airway remodeling in asthma[22]. In this study, we found that the expression of IL-8 and MCP-1 was increased in severe asthma group, and GSPE+DXM treatment decreased it more efficiently than DXM treatment, which verified the role of IL-8 and MCP-1 in asthma and the synergistic effect of GSPE on glucocorticoid.

GSPE are internationally recognized as the most effective natural antioxidants for free radicals in the human body[23]. It can clean off the free radicals and reduce the membrane lipid peroxidation[24] and has a wide range of anti-oxidation, anti-tumor and other pharmacological effects[8]. Our previous studies have shown that GSPE can reduce airway inflammation and airway hyperresponsiveness by reducing the expression of iNOS, and restore sensitivity to glucocorticoid treatment by regulating Nrf2-iNOS-HDAC2 axis in asthmatic mice [7, 10, 25]. In present study, our results showed that the expression of iNOS increased and the expression of Nrf2, HDAC2, GSH and GCLM decreased in severe asthma group, suggesting that Nrf2-iNOS-HDAC2 signaling pathway played an important role in the mechanism of severe asthma. Furthermore, our results manifested that GSPE had the reverse effect on the above chemical factors and enzymes, and the potential on increasing Nrf2-ARE binding ability of GSPE. Therefore, we have reason to believe that GSPE can alleviate glucocorticoid resistance by targeting Nrf2-iNOS-HDAC2 axis.

Conclusions

GSPE can alleviate glucocorticoid resistance by regulating Nrf2-iNOS-HDAC2 signaling pathway in severe asthma, suggesting that GSPE have potential clinical application prospects in the treatment of severe asthma.

Abbreviations

ARE: antioxidant response element

BMI: body mass index

DXM: dexamethasone

EOS: eosinophils

FEV_1%: percent of predicted forced expiratory volume in 1 second

FEV_1/FVC%: FEV_1 as percentage of forced vital capacity

GCL: Glutamate-cysteine ligase

GCLM: glutamatecysteine ligase modifier subunit

GSH: glutathion

GSPE: grape seed proanthocyanidin extract

HDAC2: inactivation of histone deacetylase-2

ICS: inhaled corticosteroids

iNOS: inducible nitric oxide synthase

IL-8: interleukin -8

LABA: long acting β_2-agonist

MCP-1: monocyte chemotactic protein-1

Nrf2: Nuclear factor erythroid 2-related factor 2

PBMCs: peripheral blood mononuclear cells

qRT-PCR: Quantitative real-time PCR

Declarations

Ethics approval and consent to participate

This study was approved by the ethics institute of Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University and the signed informed consent was obtained from all participants.

Consent for publication

Written informed consent was obtained from the patient for publication of Table 1.

Availability of data and materail

All data generated or analyzed during this study are included in this article and its supplementary information files.

Competing interests

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Not applicable

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Tables

Table 1. Subjects characteristics

	Normal control (n=20)	Severe asthma (DXM group, n=20)	Severe asthma (GSPE+DXM group, n=20)
Age (years)	39.6±12.72	33.11±8.64	35.56±7.29
BMI	20.81±1.73	21.92±2.13	21.71±2.13
Gender (male/female)	8/12	11/9	10/10
Smoker/Non-smoker	0/20	0/20	0/20
EOS (%)	0.77±0.54	7.55±3.79	6.74±2.93
FEV_1%	117.87±6.63	43.66±4.84	43.91±4.45
FEV_1/FVC%	89.69±1.74	45.65±4.65	47.31±4.76

BMI: body mass index; EOS: eosinophils; FEV_1%: percent of predicted forced expiratory volume in 1 second; $FEV_1/FVC\%$: FEV_1 as percentage of forced vital capacity. Data presented as mean ± SEM.

Table 2. Sequences of primers used in qRT-PCR

Primer name	Forword primer	Reverse primer
β-Actin	TGTGATGGGTGTGAACCACG	CAGTGAGCTTCCCGTTCACC
HDAC2	TGTGCTTGCCATCCTCGAAT	GGTCATCACGCGATCTGTTG
Nrf2	AACAGAACGGCCCTAAAGCA	GGGATTCACGCATAGGAGCA
iNOS	GAGCCACAGTCCTCTTTGCT	CAACCTTGGTGTTGAAGGCG
GCLM	AAGTTAACCTGGCCTCCTGC	CGATGACCGAGTACCTCAGC

Figures

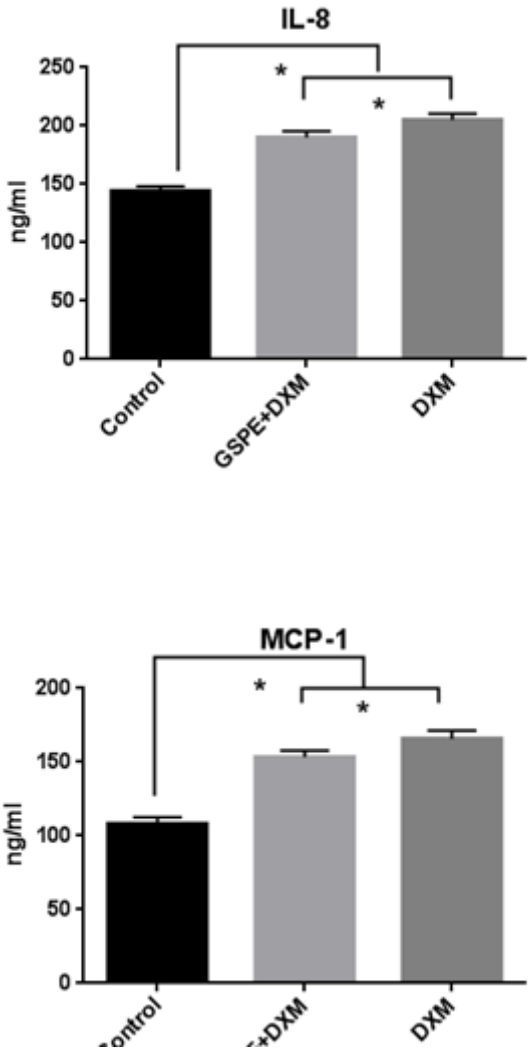


Figure 1

Effect of GSPE on the expression of IL-8 and MCP-1 *P<0.05.

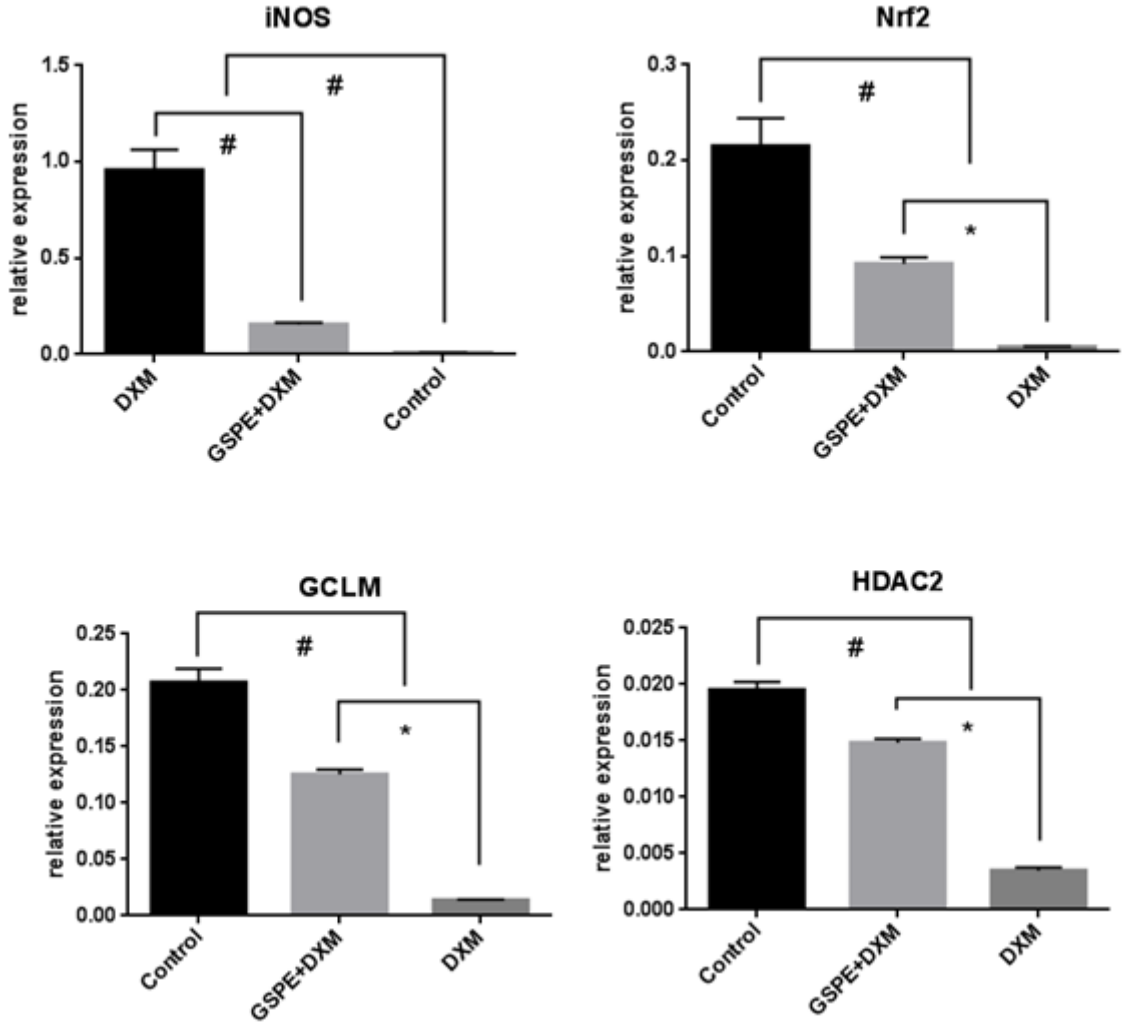
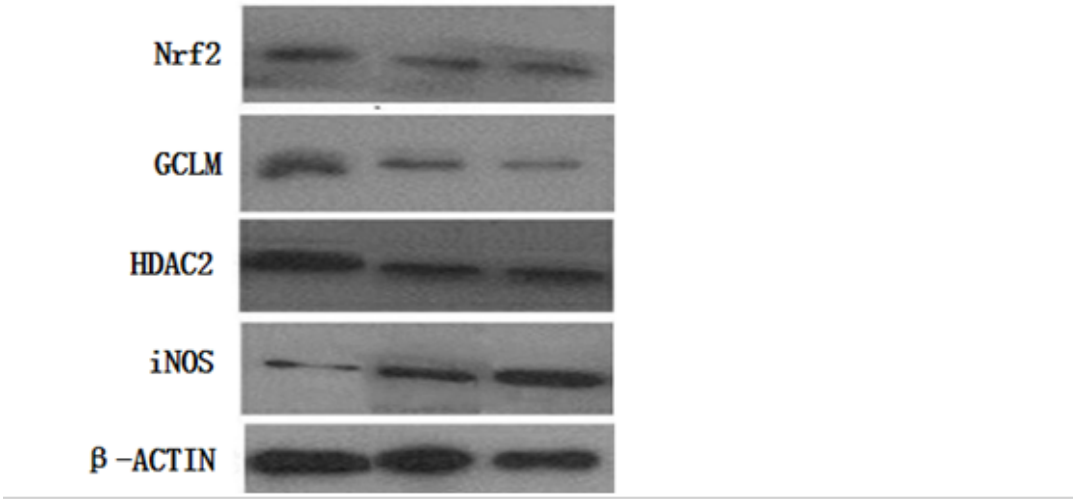


Figure 2

Effect of GSPE on the mRNA expression of iNOS, Nrf2, GCLM, and HDAC2 #P<0.001, *P<0.05.



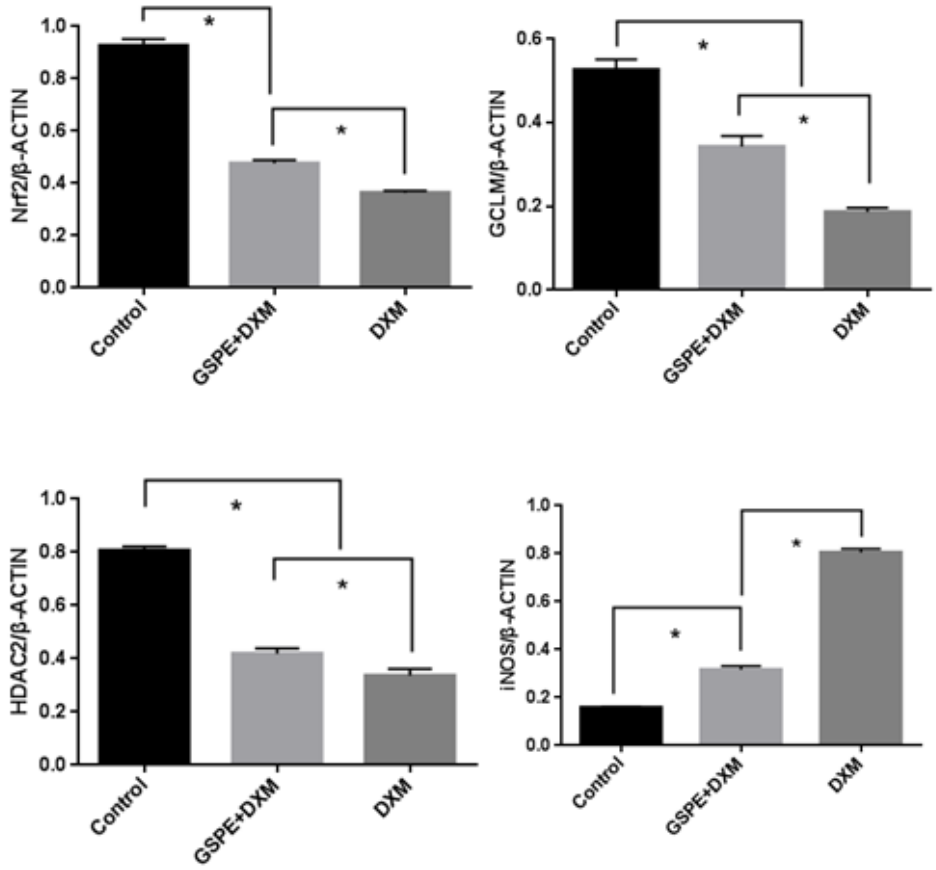


Figure 3

Effect of GSPE on the protein expression of iNOS, Nrf2, GCLM and HDAC2 *P<0.001.

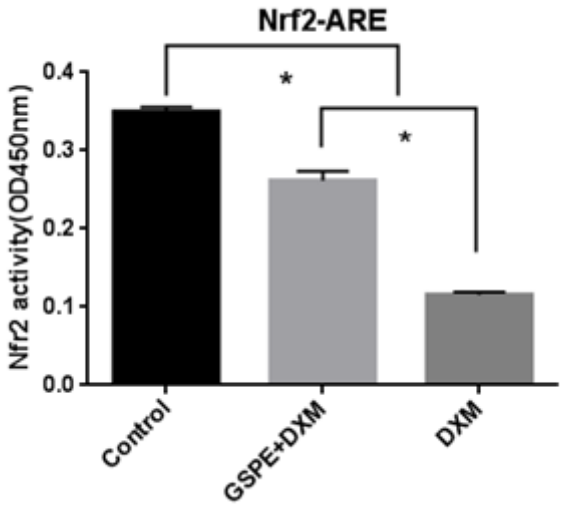


Figure 4

Nrf2-ARE binding ability *P<0.001.

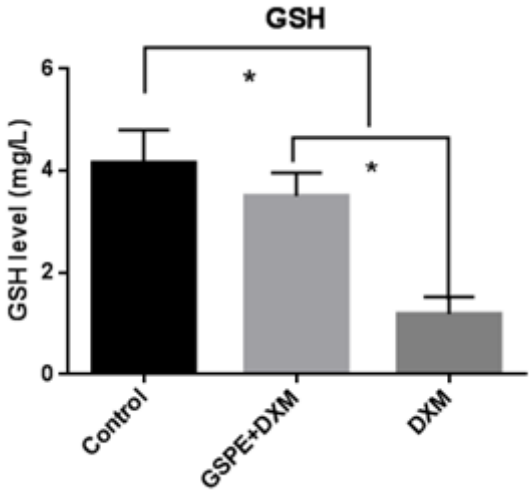


Figure 5

Effect of GSPE on the expression of GSH *P<0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- rtPCR.xls
- renamed785c8.png
- ELISA.xls
- nrf2are.xls