Original Article
Protective effects of calycosin weakens coronary heart disease-induced inflammation in a pig model

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Abstract: To evaluated the effect of calycosin weakens coronary heart disease-induced inflammation in pig model and explore its possible mechanism. Adult pig was used to structure coronary heart disease (CHD) model group and gave with 1, 2 and 4 mg/kg of calycosin for 28 days. In CHD model, calycosin inhibited left ventricular ejection fraction and systolic internal diameter. Importantly, the creatine kinase (CK), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), cardiac troponin (cTnT), ventricular ejection fraction (LVEF) and systolic internal diameter (LVIDs) levels of CHD model group was reversed by treatment with calycosin. Moreover, inflammation reactions of CHD model pig were suppressed by treatment with calycosin through NF-κB protein expression. In addition, administration of calycosin activated the protein expression of FXR and STAT3, and promoted phosphorylation of Akt protein expressions CHD model pig. Our findings suggest that protective effects of calycosin weaken CHD-induced inflammation in pig model via NF-κB, FXR, STAT3, and Akt signaling pathway.

Keywords: Calycosin, coronary heart disease, inflammation, NF-κB, FXR, STAT3, Akt

Introduction

Coronary heart disease (CHD) is a pathophysiologic process influencing large and mid arteries. For long time, people hold that atherosclerosis is the result of accumulation of cholesterol on arterial wall [1]. However, with a series of studies conducted, peoples' ideas to the role of inflammation are becoming more and more definite [2]. Major ingredients of atherosclerotic plaques are various immune cells. And effector molecules released by immune cells quickens the development of plaques. CHD is the representation of activation of inflammation in plaques [3]. Consequently, atherosclerosis can be deemed as a kind of inflammatory disease, resulting from interactions of immune factors and metabolic risk factors. It manifests as the occurrence and progression of CHD on vascular wall [4].

IL-6 is a kind of multi-functional circulatory cytokines, whose biological activities are induction of P cells to produce antibodies and promotion the formation of cytotoxic T cells [5].

After heart surgery, IL-6 would increase and reach peak value after 4-6 hours [6]. IL-6 has functions of pro-inflammation and anti-inflammation [6]. It is an important reaction medium in acute traumatic and repair process [7]. It can activate neutrophile granulocyte and delay the phagocytosis of aging and dysfunctional neutrophil by phagocytes [8]. Thus, inflammatory mediator would be worsened. IL-6 can weaken actions of TNF-α and IL-1 by promotion of the release of TNFRs and IL-1R. A large number of releases by IL-6 are a signal of risk for patients [9].

Isolated from Astragalus Mongholicus Bunge, total flavonoids of Astragali Radix (TFA) is main active ingredient with functions of antioxidance and scavenging free radical [10]. Studies found that TFA has biological effects as anti-damage, anti-mutation, anti-tumor and inhibition of atherosis [11]. Calycosin is a major ingredient isolated from TFA, which can be used as immunopotentiator or modifier with actions of anti-bacteria, anti-tumor, anti-aging and anti-oxidance [12]. The purpose of this study was to investi-
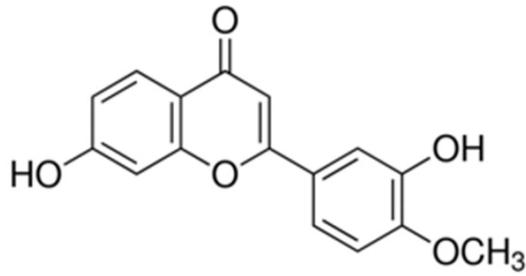
Figure 1. The chemical structure of calycosin.

gate the possible effects of calycosin weakens CHD-induced inflammation in pig model and the underlying mechanisms.

Materials and methods

Experimental animals

Male Chinese miniature pig (20-30 kg) were acquired from institute of laboratory animal science, Jining Medical University (Jining, China) and fed a standard laboratory diet and water ad libitum, and housed in a climate-controlled Zooloretto at 22-24°C, 60-70% humidness with 12-h day and night cycles. 2 kg of cholesterol, 0.5 kg of cholate and 10 kg of lard oil were added into 100 kg ordinary feed. All experiment pigs were randomly assigned to control group (n = 6), CHD model group (Vehicle n = 10) and CHD model + calycosin treated group (n = 10). The CHD model and CHD model + calycosin treated group were fed with high-fat diet for 2 weeks. 30 mg/kg of sodium barbital was injected in experiment pig from ear vein. Common carotid artery from pig anaesthetized was separated and ligatured far-end heart was imbedding 6F arterial sheath tube, 200 U/kg was injected from the arterial sheath tube side. Left anterior descending branch was merged by guide wire and then sacculus was merged into left anterior descending branch and pressured air pressure (10~12 ATM) for 30 s and this process was repeated 3 times following maintain balloon pressure air gap (1~1.5 ATM). Arteria carotis communis was ligatured after removal of the catheter and balloon. In CHD model + calycosin treated group, the experiment pig was administered 4 mg/kg of calycosin for 28 days. Calycosin (≥98%, HPLC) was purchase from Sigma-Aldrich Co. (CA, USA).

Determinations of CK, CK-MB, LDH, cTnT, TNF-α and IL-6 levels

Serum samples from very group were obtained after calycosin treated group. The CK, CK-MB, LDH and cTnT levels were measured using respective commercial kits (Sangong Biotech, Shanghai, China) following the manufacturer's instruction. The TNF-α and IL-6 levels were measured using respective ELISA kits (Elabscience, Wuhan, China) following the manufacturer's instruction.

Determinations of left ventricular ejection fraction (LVEF) and systolic internal diameter (LVIDs)

S5-1 linear probe (iE33 xMatrix Ultrasound, Philips Healthcare, Andover, MA, USA) was performed and used to analyze LVEF and LVIDs levels from every group.

Total RNA was extracted from the tissue samples of very group using TRIzol® (Takara Bio, Inc., Dalian, China), according to the manufacturer's instructions

Total RNA were measured by reading the absorbance at an optical density (OD) of 260/280 nm. CDNA was compounded from total RNA using a PrimeScript RT reagent kit, according to manufacturer's instructions (Takara Biotechnology, Co., Ltd.). ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, USA) was used to conducted qRT-PCR. The conditions for amplifying were as following: 95°C for 45 min, 40 cycles of 95°C for 15 sec and 60°C for 35 sec.

Western blotting

Proteins were extracted from the tissue samples of very group using a protein extraction reagent (Beyotime, Shanghai, China). The supernate was obtained after centrifugation at 12000 g × 10 min at 4°C and then protein concentration was measured using the BCA Protein Assay kit (Beyotime, Shanghai, China). An equal quantity of total protein (50 μg) was separated on an 8% SDS-PAGE gel (Beyotime Institute of Biotechnology) and transferred to a PVDF membrane (0.22 mm; EMD Millipore, Billerica, MA, USA). The membrane was then blocked with 5%
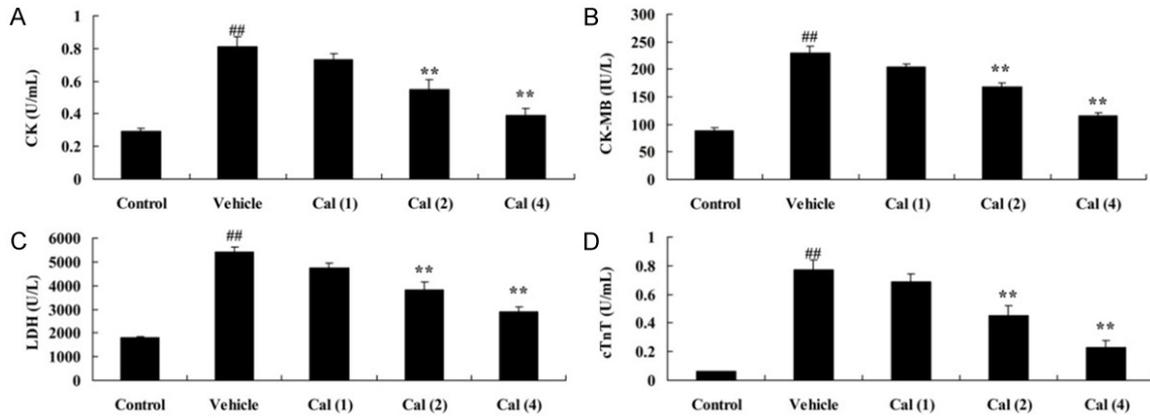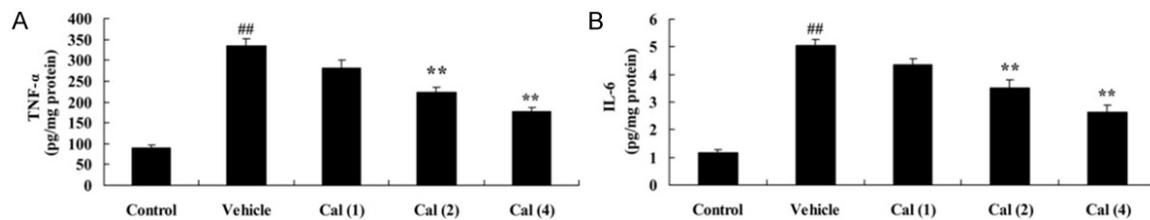
Figure 2. Protective effects of calycosin weaken the levels of CK, CK-MB, LDH, cTnT in CHD of pig model. Protective effects of calycosin weakens the levels of CK (A), CK-MB (B), LDH (C), cTnT (D) in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##P<0.01 compared with control group, **P<0.01 compared with vehicle group.
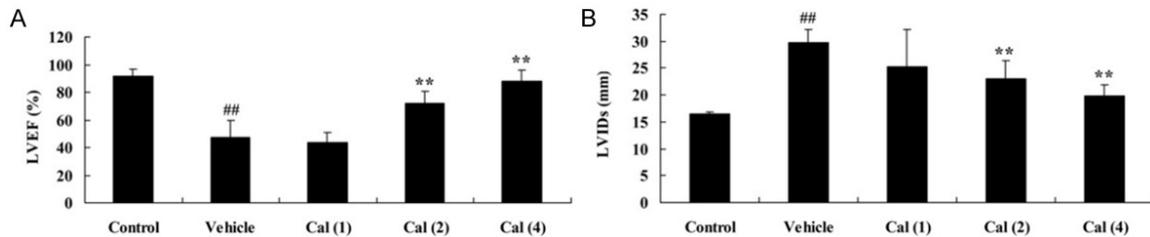
Figure 3. Protective effects of calycosin affect the levels of LVEF and LVIDs in CHD of pig model. Protective effects of calycosin affect the levels of LVEF (A) and LVIDs (B) in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##P<0.01 compared with control group, **P<0.01 compared with vehicle group.

Figure 4. Protective effects of calycosin weaken the levels of inflammation in CHD of pig model. Protective effects of calycosin weaken TNF-α (A) and IL-6 (B) levels in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##P<0.01 compared with control group, **P<0.01 compared with vehicle group.

non-fat milk and incubated with anti-FXR (1:5000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), phosphorylation-signal transducers and activators of transcription 3 (p-STAT3) (1:5000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), NF-κB (1:5000, Santa Cruz Bio-technology, Inc., Dallas, TX, USA) and p-Akt (1:5000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) by incubation at 37°C for 60 min with horseradish peroxidase-conjugated antibody (1:5000 dilution; ZhongShan, Beijing, China). Then, the membrane was stained with ECL Plus (Beyotime Institute of Biotechnology).
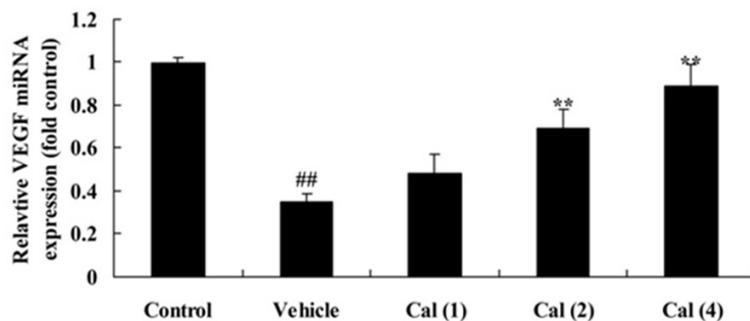
Statistical analysis

All the values were represented as the means ± standard error. Data were analyzed by SPSS
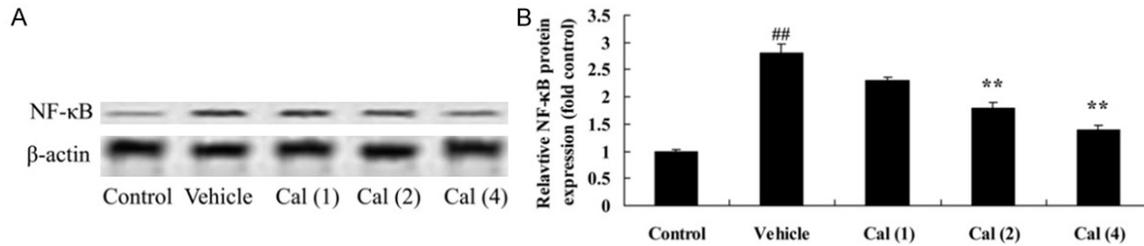
Figure 5. Protective effects of calycosin affect the level of NF-κB in CHD of pig model. Protective effects of calycosin affect the level of NF-κB protein expression by western blotting assays (A) and statistical analysis of NF-κB protein expression (B) in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##p<0.01 compared with control group, **p<0.01 compared with vehicle group.

Figure 6. Protective effects of calycosin weaken the levels of VEGF miRNA expression in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##P<0.01 compared with control group, **P<0.01 compared with vehicle group.
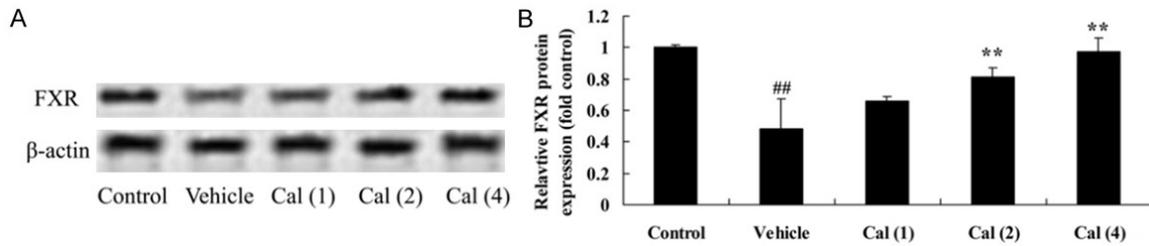
17.0 software and were analyzed by ANOVA and post hoc Bonferroni test. A two-tailed *P* value of less than 0.05 was considered statistically significant.

Results

Protective effects of calycosin weaken the levels of CK, CK-MB, LDH and cTnT in CHD of pig model

The chemical structure of calycosin was displayed in **Figure 1**. We investigated whether the protective effects of calycosin weaken the levels of CK, CK-MB, LDH and cTnT in CHD of pig model. After treatment with calycosin for 28 days, the levels of CK, CK-MB, LDH and cTnT were higher than those of control group (**Figure 2**); meanwhile, treatment with calycosin (2 and 4 kg) significantly reduced the levels of CK, CK-MB, LDH and cTnT in CHD pig (**Figure 2**) in dose-dependent manner.

Protective effects of calycosin affect the levels of LVEF and LVIDs in CHD of pig model

To elucidate the protective effects of calycosin affects the levels of LVEF and LVIDs in CHD of pig model, LVEF and LVIDs were measured in very group. We obviously found that the LVEF level in CHD of pig model was lower than that of control group (**Figure 3A**). However, treatment with calycosin significantly increased the LVEF level in CHD pig (**Figure 3A**). Meanwhile, the LVIDs level in CHD of pig model was higher than that of control group (**Figure 3B**). Calycosin treatment significantly decreased the LVIDs level in CHD pig (**Figure 3B**).

Protective effects of calycosin weaken the levels of inflammation in CHD of pig model

On the 28th day, TNF-α and IL-6 levels were significantly increased in CHD of pig model, compared to the control group (**Figure 4**). Promisingly, calycosin treatment (2 and 4 kg) significantly weakened the TNF-α and IL-6 levels in CHD pig (**Figure 4**).

Protective effects of calycosin affect the level of NF-κB protein expression in CHD of pig model

In this study, an increase in the level of NF-κB protein expression of CHD model group, compared with the control group (**Figure 5**). With the 2 or 4 mg/kg of calycosin, the NF-κB pro-

Figure 7. Protective effects of calycosin weaken the levels of FXR protein expression in CHD of pig model. Protective effects of calycosin affect the level of FXR protein expression by western blotting assays (A) and statistical analysis of FXR protein expression (B) in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##p<0.01 compared with control group, **p<0.01 compared with vehicle group.
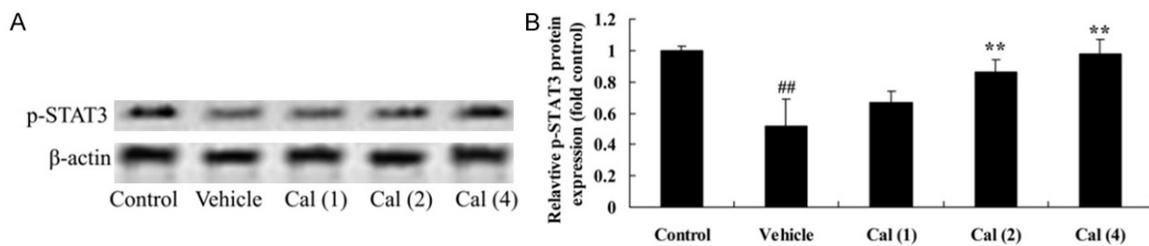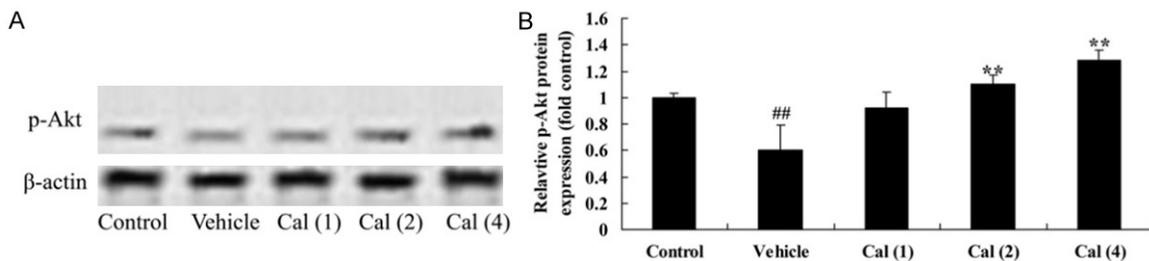

Figure 8. Protective effects of calycosin weaken the levels of STAT3 protein expression in CHD of pig model. Protective effects of calycosin affect the level of p-STAT3 protein expression by western blotting assays (A) and statistical analysis of p-STAT3 protein expression (B) in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##p<0.01 compared with control group, **p<0.01 compared with vehicle group.

Figure 9. Protective effects of calycosin weaken the levels of p-Akt protein expression in CHD of pig model. Protective effects of calycosin affect the level of p-Akt protein expression by western blotting assays (A) and statistical analysis of p-Akt protein expression (B) in CHD of pig model. Control, control group; Vehicle, CHD model group; Cal (1), 1 mg/kg calycosin; Cal (2), 2 mg/kg calycosin; Cal (4), 4 mg/kg calycosin. ##p<0.01 compared with control group, **p<0.01 compared with vehicle group.

tein expression was decreased in dose-dependent manner, which was significantly lower compared CHD model group (**Figure 5**).

Protective effects of calycosin weaken the levels of VEGF miRNA expression in CHD of pig model

As shown in **Figure 6**, VEGF miRNA expression in CHD of pig model was higher than that of control group. Meanwhile, treatment with 2 or 4

mg/kg of calycosin significantly weakened VEGF miRNA expression of CHD pig in dose-dependent manner (**Figure 6**).

Protective effects of calycosin activate the levels of FXR protein expression in CHD of pig model

Compared with the sham control group, even lower expression of FXR protein in CHD of pig model group was observed (**Figure 7**). As

expected, FXR protein was significantly promoted by 2 or 4 mg/kg of calycosin in dose-dependent manner (**Figure 7**).

Protective effects of calycosin activate the levels of STAT3 protein expression in CHD of pig model

As shown in **Figure 8**, a statistically significant lower level of p-STAT3 protein expression in CHD of pig model group, compared with the sham control group. However, treatment with 2 or 4 mg/kg of calycosin significantly activated the higher level of p-STAT3 protein expression in CHD pig (**Figure 8**).

Protective effects of calycosin activate the levels of p-Akt protein expression in CHD of pig model

Compared with the model control group, we found that the p-Akt protein expression was significantly weakened in CHD of pig model (**Figure 9**). However, 2 or 4 mg/kg of calycosin treatment dose-dependently significantly increased the p-Akt protein expression in CHD pig (**Figure 9**).

Discussion

With a series of studies conducted, peoples' ideas to the role of inflammation are becoming more and more definite. Major ingredients of atherosclerotic plaques are various immune cells [13]. And effector molecules released by immune cells quickens the development of plaques. Acute coronary syndrome is the representation of activation of inflammation in plaques [14]. Consequently, atherosclerosis can be deemed as a kind of inflammatory disease, resulting from interactions of immune factors and metabolic risk factors. It manifests as the occurrence and progression of CHD on vascular wall [15]. In our study, we found that the protective effects of calycosin weakened the levels of CK, CK-MB, LDH and cTnT, increased the LVEF level and inhibited the LVIDs level in CHD pig. In addition, Junqing G et al. reported that calycosin treatment improved left ventricular ejection fraction in rat models with myocardial infarction [10].

NF-κB is an important nuclear transcription factor in cell nucleus. It participates in gene regulation of immune response, stress response, apoptosis and inflammation [16]. Its activation,

expression and the relationship with renin-angiotensin system (RAS) has attracted extensive attention in recent years [17]. NF-κB is an essential regulatory factor for resynthesis of Ang's precursor-angiotensinogen [18]. The nuclear transfer and transcriptional activity of NF-κB can be activated by epinephrine, ET, Ang II and multiple stimulating factors [18]. Our results indicate that the protective effects of calycosin significantly weakened the TNF-α and IL-6 levels and the level of NF-κB protein expression, and suppressed the VEGF miRNA expression of CHD pig. Tang et al. indicated that calycosin promotes angiogenesis involving estrogen receptor through VEGF signaling pathway in zebrafish and HUVEC [12]. Xu et al. suggested that calycosin protects local inflammation and reduce AGEs-induced macrophage migration [11].

STAT3 regulates biological behaviors of tumor and immune cells by mediating extra-cellular signals of inflammatory mediators [19]. STAT3 was found in studies on IL-6 signal pathway and was firstly cloned from mouse hepatocyte cDNA [20]. STAT3 is inactivated in non-simulated normal cells. Family members of IL-6 (IL-6, IL-11, OSM and LIF) can quickly activate STAT3 signal pathway through its gp130 [21]. Studies discovered that anti-inflammation functions of FXR inhibit activities of NF-κB and/or AP1 as well as down-regulate expressions of inflammatory factors and adhesion molecules [22, 23]. FXR agonist can inhibit the activation of STAT5 and STAT3 induced by IL6 [22]. These inhibitive effects of FXR may be realized by up-regulation of expressions of CISH and SOCS3 [23]. Thus, amplification of inflammatory response is inhibited and anti-inflammation effects are realized. In this study, calycosin significantly increased FXR and p-STAT3 protein expression, activated the increased the p-Akt protein expression in CHD pig. Chen et al. indicated that the protective effects of calycosin against liver injury via activation of FXR and STAT3 in mice [12].

In conclusion, the protective effects of calycosin weakens the levels of CK, CK-MB, LDH and cTnT, promoted the LVEF level and inhibited the LVIDs level in CHD pig. Meanwhile, the next investigation disclosed that the protective effects of calycosin on CHD were linked with inhibition of VEGF, suppression of FXR/STAT3 and activation of Akt passageway.

Acknowledgements

Disclosure of conflict of interest

None.

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