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Compound Danshen Dropping Pills Enhanced Erythrocyte Oxygen Delivery Capacity via Modulating ROS-Dependent Mechanism based on Target-Network Analysis

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Abstract:

Hypoxia is the main survival challenge that human beings encounter in high altitudes, it is also the leading cause of Acute Mountain Sickness (AMS). Studies have shown that hypoxia induces a large number of reactive oxygen species (ROS) in AMS patients, and the surge of ROS leads to the reduction of oxygen delivery capacity of erythrocyte, senescence and inflammatory impairment of erythrocyte and vascular endothelial cells to a certain extent. Through depicting a target-pathway network, our study indicates that Compound Danshen Dropping Pills (CDDP), which is one of the best-known traditional Chinese medicine used for the treatment of myocardial ischemic diseases, can improve red blood cell oxygen delivery capacity in AMS patients, alleviate tissue and organ damage, relieve a series of clinical symptoms caused by hypoxia through ROS clearance and related mechanisms. We further elucidate the active ingredients of CDDP targeting ROS related pathway by target-ingredient correspondence analysis. Tanshinone IIA, catechol and some other compounds of CDDP were identified to have certain targeting effect on ROS and ROS dependent pathways. This study provides new understandings of CDDP in clinical application on AMS.

Key words:

ROS, CDDP, erythrocyte oxygen delivery capacity, AMS

Introduction:

Hypoxia is the main survival challenge that human beings must confront in plateau areas. The altitude of plateau area is relatively high, with high altitude areas ranging from 1500 meters to 3500 meters above sea level, extremely high altitude areas ranging from 3500 meters to 5500 meters above sea level, and extreme altitude areas ranging from 5500 meters above sea level[1]. The air pressure decreases with the elevation, and so does the oxygen pressure [2]. A series symptom of AMS will occur when people rise up to an altitude of more than 2500 meters above sea level and the hypoxic pressure exceeds the adaptive capacity of human body, including headache, dizziness, palpitation, nausea, vomiting, fatigue, numbness of limbs, convulsions, etc. More seriously, patients may suffer life-threatening high altitude cerebral edema or high altitude pulmonary edema [3]. The latter two serious cases usually occur in the first three days after reaching an altitude of 3000 meters. Although the two symptoms have different pathophysiological mechanisms, in patients, they both show heart rate acceleration[4], and a sharp decline of oxygen saturation of hemoglobin in arterial blood [5]. Evidence shows AMS is rarely occurred at altitudes below 2500 meters, but rise up to 75% incidence at altitudes above 3000 meters. It is evident that the incidence of AMS increases with the elevation[6]. Hypoxia is the main cause of AMS occurrence [7, 8].

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Studies show that the production of ROS and other oxidative stress biomarkers increase a lot in AMS patients because of hypoxia [9, 10]. The accumulated ROS leads to the reduction of red blood cell oxygen delivery capacity, as well as aging and inflammatory damage of erythrocyte and vascular endothelial cells, which seriously jeopardize oxygen transport capacity of arterial blood and cause a series of pathophysiological reactions such as tissue hypoxic damage consequently. Under the plateau condition of low oxygen and low pressure, the oxidative stress reaction in cells is significantly intensified [11]. ROS generated by oxidative stress of erythrocyte itself and surrounding cells cannot be completely neutralized by the antioxidant system. Unneutralized ROS oxidize ferrous hemoglobin and ferric hemoglobin to methyl hemoglobin and tetravalent iron (ferrum) hemoglobin[12], respectively, depriving the oxygen delivery capacity of hemoglobin. Further reacting with ROS degrades heme and produces free iron. Heme degradation products adhere to red blood cell membrane, causing its senescence. ROS also promotes the production of inflammatory factors and destroys the membrane phospholipid bilayer of erythrocyte and vascular endothelial cells, resulting in declined erythrocyte deformability and vascular endothelial cells injury. [13-15]Hence, cumulative ROS in erythrocyte leads to heme degradation, hemoglobin reduction, as well as impairment in deformability and mobility of red blood cells, which ultimately contributes to the dysfunction of erythrocyte and vascular endothelial cells, incapacity of oxygen delivery by red blood cell (fig. 1).

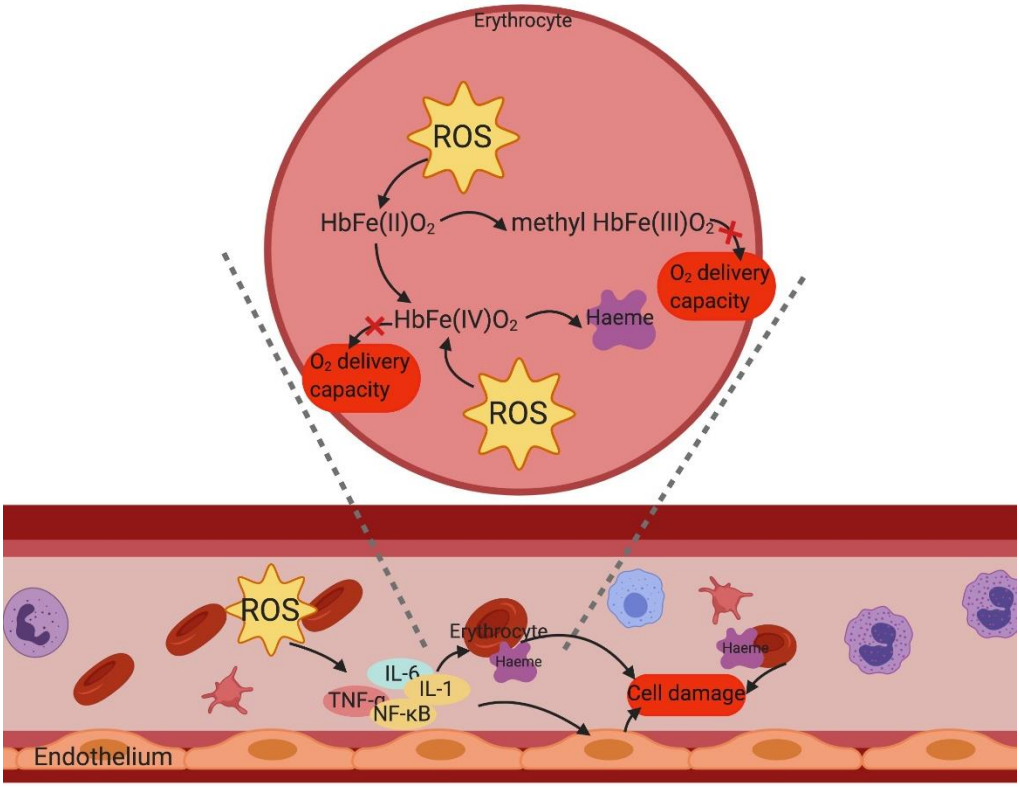

Fig. 1. ROS induces oxygen delivery capacity loss and erythrocyte impairment.

In this study, we found that CDDP improves oxygen delivery capacity of red blood cell, ameliorates hypoxic damage in tissues and organs, alleviates a series of clinical symptoms caused by hypoxia in AMS patients through ROS scavenging and related molecular mechanisms' regulating. CDDP is a modern and industrialized oral-taking plant medicine. Its active substances are Saviae Miltiorrhizae Radix, Notoginseng Radix and Borneol, among which Borneol can promote the absorption of the active ingredients of Saviae Miltiorrhizae Radix and Notoginseng Radix. In recent years, a number of research results show that CDDP can prevent or relieve AMS related symptoms, including nausea, vomiting, headache, dizziness, fatigue and sleep disorders

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(https://clinicaltrials.gov/ct2/show/NCT03552263) and related clinical symptoms caused by hypoxia in heart and other tissues. Clinical data shows that pretreatment of CDDP remarkably reduced the incidence of myocardial ischemia and high-altitude pulmonary edema in healthy volunteers who entered the altitude of 5000 meters. The symptoms of headache, dizziness, chest tightness, dry mouth and insomnia in AMS-prone patients can also be alleviated significantly after taking CDDP ($P<0.01$) [16-18]. In this study, we mainly focused on the antioxidant mechanism of CDDP. In-depth network analysis of the data collected from CDDP related literatures and data performed experimentally indicated that a significant underlying mechanism linking CDDP's role in AMS was reducing ROS accumulation caused by oxidative stress, improving oxygen delivery capacity of erythrocyte and further ameliorating tissue and organ hypoxic damage. This study provides a new understanding of CDDP in clinical application on AMS, aiming to relieve the corresponding clinical symptoms of the patients.

Methods

Target Set of CDDP

Literature source

We have collected CDDP related documents published in CNKI and PubMed databases respectively, among which CNKI's search term is "Danshen Dropping Pills". As of November 25, 2019, a total of 3,719 related articles have been obtained. PubMed's search terms are "Compound Danshen Dripping Pills", "Fufang Danshen Diwan", "T89", "Dantonic" and "Cardiotonic Pills" and 57 articles have been obtained by November 25, 2019.

After sorting out the retrieved documents, effective information was screened and collected through manual reading. Two researchers with doctorates in biology summarized the contents of the literature respectively, and the third researcher will correct the inconsistent contents. In the process of reading the literature, the research articles with CDDP as experimental sample were selected to record the experiment methods and results of CDDP, including animal models, drug administration, as well as protein/ genes modulated by CDDP, which were defined as the targets of CDDP.

Experiment source

The protein kinase family is one of the largest enzyme families, and the human kinase spectrum contains more than 500 kinases. Its dysfunction plays an important causal role in many human diseases, including cancer, central nervous system diseases, cardiovascular diseases, etc. The full KP panel (KmATP), Kinase Profiler (Eurofins scientific, Inc.), developed by Eurofins, contains 413 kinase targets. In this study, the direct target of 25 μg/ml CDDP was screened by using the kinase spectrum, and the kinase with activity < 80% was defined as the target of CDDP.

Then the targets of CDDP collected from the above two sources were incorporated together as the CDDP target set for subsequent analysis.

Enrichment Analysis of CDDP Acting Target Pathway

The CDDP target set was analyzed for pathway enrichment through the enrichment function of Pathway Maps in MetaCore Database Analysis Platform (https://portal.genego.com/).The database contains manually proofread data with high accuracy and high reliability, including interaction relations of 1.7million and 1600+ pathway maps, most of which have clear regulation-directions, and can perform complex complete pathways and interaction network analysis (PPI) on the data set,

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providing a new perspective for the interpretation of disease pathogenesis and drug action mechanism.

Regulatory Effects of CDDP Acting Targets on ROS-Related Pathways

We analyzed the signal pathway closely related to ROS in order to explain the mechanism related to CDDP's enhancement of erythrocyte oxygen delivery capacity through ROS-related pathways. Mapping the target set of CDDP on these pathways to check the distribution of targets on ROS-related pathways. Three pathways closely related to ROS were selected for in-depth analysis and integration: oxidative stress _ ROS-induced cellular signaling (p value = 9.114e-22), role of adipose tissue hypoxia in obesity and type 2 diabetes（P value=6.679E-15）, interaction of deficient alpha-MSH signaling with TNF-alpha in melanoma（P value=3.305E-11）.

Target-Ingredient Correspondence Analysis

To further find out which ingredients of CDDP act on the targets of ROS pathway, we first sorted out the important ingredients of CDDP formula, including blood absorbing ingredients, bioactive equivalent combinatorial components (BECCs), metabolites and other important ingredients, 40 ingredients in total [19-24]. Then three strategies were used to predict the specific ingredients from CDDP as potential regulators of ROS pathway.

1. The targets with known activity data of the above 40 ingredients were obtained from ChEMBL Database (https://www.ebi.ac.uk/chembl/) [25], PubChem (https://pubchem.ncbi.nlm.nih.gov/) [26, 27], BingdingDB (http://www.bindingdb.org/bind/index.jsp) [26, 27],

2. In silico target-ingredient relationship prediction is critical, especially for the ingredients which have no targets with known activity data. Hence, we adopted the Multi-Voting SEA[28] model to predict the targets of the above 40 ingredients. Multi-Voting SEA model was built by four different molecular fingerprints (MACCS, Morgan, Topological and Atom pair) and was achieved with precision range from 71 to 90.6%.

3. The molecular docking technology was used to predict the docking of 40 ingredients with the targets with 3D protein structure. The docking software was Autodock 4.2[29].

The information of target-ingredient relationship from the above three data sources was used as the basis for active ingredients prediction for ROS pathway related critical genes.

Results and discussion

Target Set of CDDP

First of all, we collected and integrated the targets of CDDP, which were divided into literature data set and experimental data set (Table 1): literature data set was from retrieval of CDDP related articles published in CNKI and PubMed databases, manual reading and screening of effective information, a list including 79 targets of CDDP was obtained (additional file 1); The source of the experimental data sets was 413 commercial kinase profiles (Full KP Panel [Km ATP], KinaseProfiler) (Eurofins Scientific, Inc.) developed by Eurofins, and 69 kinase targets of CDDP were identified (additional file 2).

Table 1. Targets of CDDP

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Sources of CDDP Targets	Number of targets
Literature source targets	79
Full-KP Panel targets	69

The Molecular Mechanism of CDDP Improves Oxygen Delivery Capacity of Red Blood Cell

In high altitudes, healthy residents are exposed to low pressure and hypoxia, and their heart rate gradually increases. Oxidative stress biomarkers such as ROS in the body dramatically increase within 24 hours[9], leading to oxidative damage of proteins and DNA to some extent [10]. Moreover, accumulating ROS will oxidize ferrous hemoglobin and ferric hemoglobin, which play key roles in oxygen delivery in erythrocyte, into methyl hemoglobin and tetravalent iron (ferrum) hemoglobin [12], respectively, disabling them to carry oxygen. Moreover, erythrocytes become more prone to be phagocytized by white blood cells when erythrocyte membrane is adhered by the heme degradation products, generated by ROS reacting with hemoglobin. In addition, ROS also promotes the production of inflammatory factors and impairs the membrane phospholipid bilayer of erythrocyte and vascular endothelial cells, resulting in the reduction of red blood cell deformability and the damage of vascular endothelial cells (fig. 1) [13-15].Therefore, ROS severely disrupted the oxygen delivery capacity of arterial blood, causing a series of hypoxia based pathophysiological reactions in AMS patients [11].

Accumulating evidence from bench to beside demonstrated convincingly that CDDP exerted comprehensive effects of resisting high altitude hypoxia induced impairments, promoting hypoxia tolerance, improving physiological conditions and protecting cardiovascular tissues. CDDP has been applied for prevention and treatment of acute and chronic high altitude diseases such as AMS, high altitude cerebral edema, high altitude pulmonary edema, high altitude myocardial ischemia and achieved satisfactory therapeutic efficacy[16-18, 30-32]. Literatures reported that salvia miltiorrhiza and its active ingredients (such as salvianolic acid), which are potent oxygen free radical scavengers, can improve the activity of superoxide dismutase (SOD), promote oxygen delivery capacity of blood cells, and neutralize the damage of heart, brain, kidney, lung and other important organs caused by high altitude hypoxia effectively[31]. Pathway enrichment by CDDP target set was introduced in MetaCore Database Analysis Platform (https://portal.genego.com/), deciphering the significant role CDDP plays in ROS-related pathways, since ROS-related pathway (oxidative stress _ ROS-induced cellular signaling (P value = 9.114e-22)) ranks number one (fig. 2). The full results of pathway enrichment are shown in additional file 3. Further manual curated analysis interpreted the common molecular mechanism of CDDP and ROS-related pathways (fig. 3 A, see additional file 4 for figure legend):

1. CDDP up-regulates ROS-eliminating related genes -GPX1 and CAT(fig. 3 A&B①): Glutathione peroxidase (GPX1) catalyzes glutathione to reduce organic hydroperoxide and hydrogen peroxide (H_2O_2), thus protecting cells from oxidative stress [33, 34]; Catalase (CAT) is a key anti-oxidative stress antioxidant which exists in the peroxisome of almost all aerobic cells. Catalase converts active oxygen hydrogen peroxide into water and oxygen, thus attenuating the toxic effect of hydrogen peroxide [34].CDDP can up-regulate the expression of glutathione peroxidase 1(GPX1) [35] and catalase[36], indicating CDDP can effectively eliminate ROS and relieve pathological reactions caused by hypoxia stress.

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2. CDDP inhibits TNFα expression[37] and reduces ROS production (fig. 3A&B ②): tumor necrosis factor α (TNF-α) triggers ROS production in mitochondria of cells around erythrocyte. Previous studies reported that TNFα induced aging damage of erythrocyte[38, 39]. CDDP inhibits TNFα expression, suggesting that CDDP suppresses ROS production and improves oxygen delivery capacity of erythrocyte.

3. CDDP represses the expression of inflammation-related factors, which may seriously jeopardize erythrocyte and vascular endothelium (fig. 3A&B ③): CDDP has been demonstrated to decrease the expression of leukocyte adhesion protein (ICAM1) [40]. ICAM1 is a leukocyte adhesion protein, and its expression and activation in vascular endothelial cells mediate the adhesion of circulating monocytes/leukocytes to the surface of endothelial cells and increase the damage of vascular endothelium [41, 42]. CDDP exhibits protective effect on vascular endothelium from leukocyte damage and improves blood fluidity as consequence. In addition, CDDP up-regulates the expression of NFKB inhibitor alpha(NFKB1A) in cells [43], and down-regulates the expression of MMP9 [44], NFKB[45], TNFα[37], IL-1[46], IL-6[37] and some other inflammatory factors, abrogating the damage of erythrocyte and vascular endothelium caused by ROS in hypoxic environment[47-51].

All the analysis results mentioned above indicates that CDDP effectively improve the oxygen delivery capacity of erythrocyte by inhibiting ROS, which is believed as a fatal molecule jeopardizing cells and tissues that increased in AMS patients (fig. 3), suggesting that hypoxia and corresponding clinical symptoms in AMS patients could be relieved by CDDP. Furthermore, CDDP has been demonstrated to reduce myocardial oxygen consumption and blood viscosity, to improve microcirculation and erythrocyte deformability, to inhibit platelet activation and thrombosis, to resist lipid peroxidation and myocardial ischemia, to relax coronary artery and other cardiac protective effects [52]. Besides regulating ROS-related pathway targets mentioned above, CDDP also stimulates nitric oxide (NO) synthesis through other pathways such as promoting the expression of nitric oxide synthase (NOS) protein in cells [53, 54]. Elevated nitric oxide can also relieve cardiac tissue hypoxia through stabilizing blood pressure, relaxing vascular smooth muscle cells, inducing vasodilation and reduce cardiac preload [55].

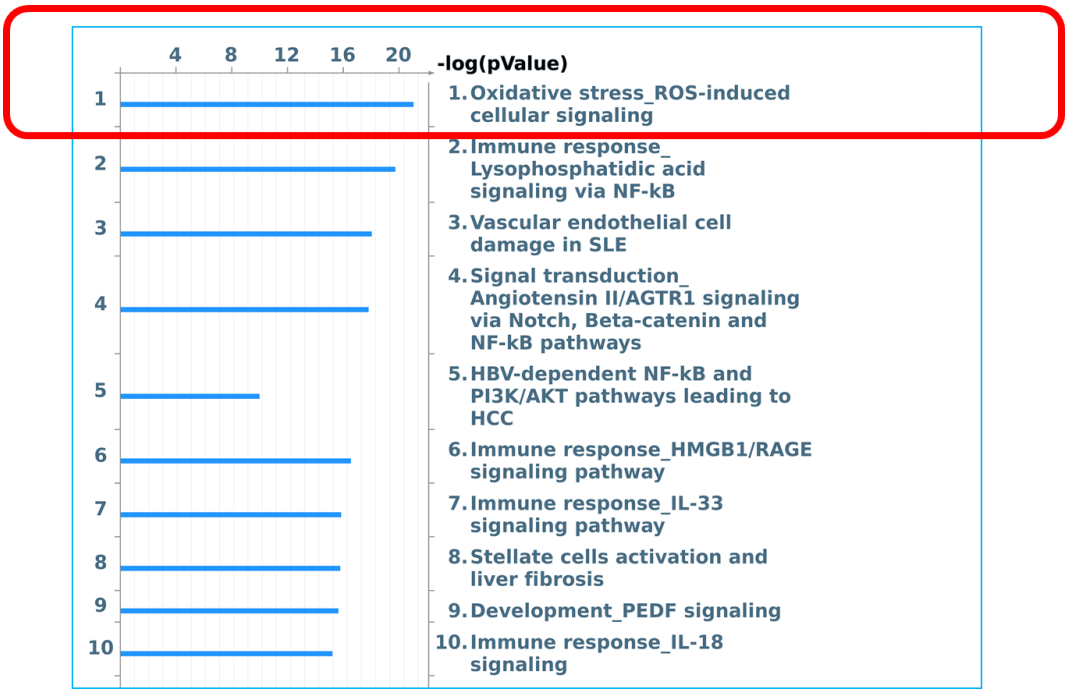

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Fig.2. top 10 pathways enriched by CDDP's target set

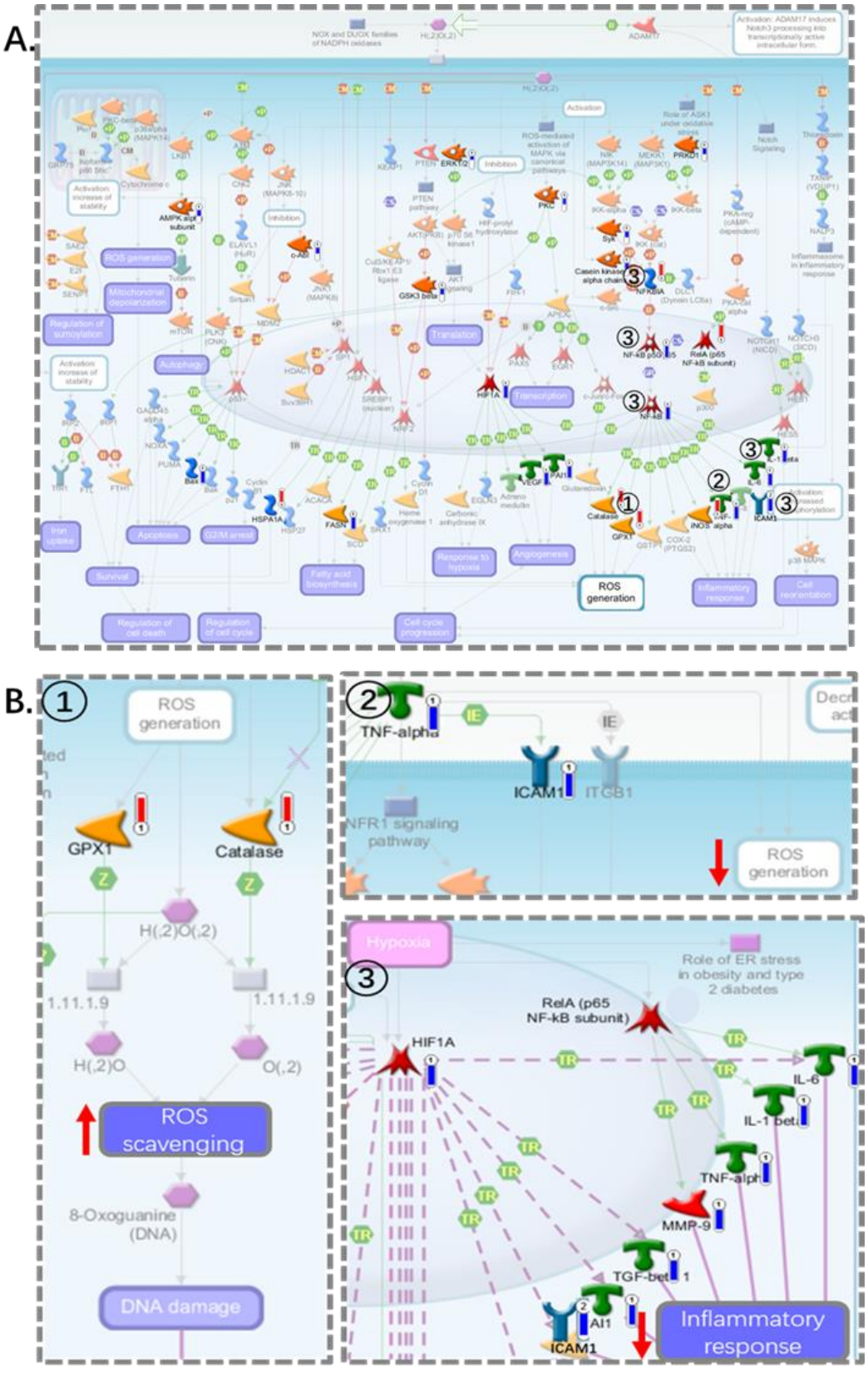

Fig.3. Molecular mechanism of CDDP improves erythrocyte oxygen delivery capacity by inhibiting ROS and anti-inflammatory effect

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A. Overall pathway diagram of oxidative stress _ ROS-induced cellular signaling, in which CDDP target is highlighted (red bar indicates up-regulation, blue bar indicates down-regulation, and pathways ①-③ correspond to ①-③ in B). B ①.CDDP increases GPX1 and CAT expression, and effectively scavenges ROS; ② CDDP down-regulates TNFα expression and inhibits ROS generation;③ CDDP exerts effect of up-regulating anti-inflammatory factor (NFKB1A) and down-regulating pro-inflammatory factors (ICAM1, MMP9, NFKB, TNFα, IL-1, IL-6), attenuating the damage of Erythrocyte and vascular endothelial cells.

Analysis of potential ingredients from CDDP modulate ROS pathway

We further carried out ROS pathway-oriented target-ingredient correspondence analysis of CDDP. We sorted out 40 important ingredients and then used three different analysis strategies (see Additional file 5 for analysis results) to predict the important ingredients of CDDP that have regulatory effect on ROS pathway (Table 2). The results suggest that Tanshinone I and catechol, the active ingredients from salvia miltiorrhiza in CDDP, target TNF α, a major ROS inducer. Quercetin from Panax notoginseng targets Catalase (CAT), a key anti-oxidative stress antioxidant to eliminate ROS. Many other active ingredients from Salvia miltiorrhiza, such as Cryptotanshinone, Tanshinone IIA, Protocatechuic aldehyde, etc., also targets inflammatory factors downstream of ROS, such as IL1B, MMP, NFKB1, etc. The results of target-ingredient correspondence analysis provided more information to show various inhibitory effects of CDDP on ROS.

At present, representative drugs for AMS treatment include acetazolamide, dexamethasone, aminophylline, etc. Acetazolamide is the first-choice drug for AMS prevention, and is the only drug approved by FDA for this indication. Researches show that acetazolamide can improve hypoxia tolerance of mice and increase arterial oxygen saturation[56, 57]. However, patients have to suffer by many side effects of acetazolamide, such as numbness of hands, feet and face, discomfort of upper abdomen, nausea, etc., which become more obvious with the duration of taking acetazolamide[58-60]. Hormonal drugs and aminophylline also have side effects such as insomnia, indigestion/epigastric discomfort, irritability, etc.[61-63]. Traditional Chinese medicines usually possess less side effects, such as rhodiola rosea, also exhibits improving the antioxidant system and reducing the production of free radicals[64, 65]. Many studies show that CDDP is well tolerated and has similar efficacy with rhodiola rosea for AMS treatment[17, 18, 30, 52]. Additionally, CDDP possesses a variety of cardiac protective effects such as reducing myocardial oxygen consumption, improving microcirculation and preventing myocardial ischemia while resisting oxidation and eliminating ROS[52], which offers a strong rational to promote the therapeutic strategy of using CDDP for people under the harsh environment of high altitude hypoxia.

Table 2. Ingredients of CDDP modulating ROS Pathway

Compound Name	Plant	Target
Tanshinone I	Salvia miltiorrhiza	TNFα、NFKBIA、IL1B
Cryptotanshinone	Salvia miltiorrhiza	IL1B
Tanshinone IIA	Salvia miltiorrhiza	IL1B、IL1B、NOS2
Protocatechuic aldehyde	Salvia miltiorrhiza	IL1B

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Caffeic acid	Salvia miltiorrhiza	IL1B
protocatechuic acid	Salvia miltiorrhiza	IL1B
catechol	Salvia miltiorrhiza	IL1B、TNFα、NFKBIA
vanillic acid	Salvia miltiorrhiza	IL1B
Quercetin	pseudo-ginseng	NFKBIA、NFKB1、MMP、CAT、NFKBIA

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