

# The Protective Effect of Compound Danshen Dripping Pills on Oxidative Stress after Retinal Ischemia/Reperfusion Injury in Rats

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## Abstract

**Objective:** To investigate the effect of Compound Danshen Dripping Pills (CDDP) on oxidative stress after ischemia/reperfusion (I/R) injury in the rat retina. **Methods:** Adult male SD rats were randomly divided into 3 groups: sham (group A), I/R (group B), and I/R plus CDDP (group C). Retinal ischemia/reperfusion injury (RIRI) was introduced by increasing the intraocular pressure (IOP) to 110 mmHg for 60 min via cannulation into the anterior chamber. Right after the insult, CDDP was administered intragastrically (450 mg/kg/d) for 7 days. The levels of malondialdehyde (MDA), the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) in the retinal tissues were determined on d1 and d7 after the ischemic insult. **Results:** Following ischemia, the MDA levels in group B and group C were significantly higher than those in group A ( $p < 0.01$ ). CDDP significantly lowered MDA levels in group C when compared with group B ( $p < 0.01$ ). The activities of SOD, GSH-Px and CAT were higher in group A than in group B and group C ( $p < 0.01$ ). CDDP could increase the activities of SOD, GSH-Px and CAT remarkably in group C when compared with group B ( $p < 0.01$ ). **Conclusion:** CDDP can protect the retina from I/R injury through reducing oxidative stress, and thus may be a promising method for the treatment of ischemic retinal disorders.

## Keywords

Compound Danshen Dripping Pills (CDDP), Retinal Ischemia/Reperfusion Injury (RIRI), Oxidative Stress, Rats

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## 1. Introduction

Ischemia/reperfusion (I/R) occurs in many clinical conditions and can seriously impair the affected organ or tissue.

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sues [1]. Retinal ischemia/reperfusion injury (RIRI) is a common cause of visual impairment and blindness. Central retinal artery occlusion (CRAO), central retinal vein occlusion (CRVO), branch retinal artery occlusion (BRAO), branch retinal vein occlusion (BRVO), acute glaucoma, diabetic retinopathy, and age-related macular degeneration (AMD) are all associated with retinal ischemia [2]. The retina is highly dependent on its oxygen supply, while restoration of blood flow after ischemia paradoxically initiates more serious tissue injury and cell death, leading to an irreversible impairment of retinal function [2]. Therefore, the management of RIRI is crucial.

Compound Danshen Dripping Pills (CDDP) is a Chinese patent medicine, developed on the basis of Traditional Chinese Medicine (TCM) theory and modern preparation technologies. It is widely used in the prevention and treatment of coronary arteriosclerosis, angina pectoris, hyperlipidemia, and other cardiovascular diseases in China [3]. This new drug compound mainly consists of salvia miltiorrhiza and panax notoginseng [4]. In the past few decades, CDDP has been demonstrated to have antioxidant, anti-inflammatory properties, and it can also protect endothelial function, inhibit platelet adhesion as well as improve microcirculation [5]-[8]. Because of its characters of multi-components, multi-effects, and multi-targets, CDDP went through phase II clinical research in USA in 2010 [9]. Although many studies have provided evidences that CDDP is I/R protective in cardiovascular disorders, its potential protective effects in ameliorating retina ischemic disorders have received little attention. Thus, we hypothesized that CDDP could have some therapeutic efficacy on RIRI. In our previous study, we have already demonstrated that CDDP can exert protective effects on the retina under ischemic conditions through examining resultant ERG and histological findings [10].

I/R-induced oxidative stress is thought to be the direct cause of retinal injury. MDA, a degraded product of lipid peroxidation, produces cytotoxicity by reacting with the amino of nucleic acid [11], whereas SOD, GSH-Px and CAT are major scavengers of reactive oxygen species (ROS), which are known to trigger apoptosis and are massively generated during I/R [12]. Previous study showed that changes of MDA levels and SOD activities were indicators for lipid peroxidation degrees and therefore reflected the severity of tissue damage [13]. Therefore, in the present study, in order to elucidate whether CDDP exerts protective effects on the retina under ischemic conditions against oxidative stress, we examined the MDA level and the activities of SOD, GSH-Px, and CAT in the retina tissues.

## 2. Materials and Methods

### 2.1. Research Objects and Grouping

Fifty-four adult male SD rats, weighing from 200 to 350 g, were provided by Joinn Laboratories (Suzhou). The animal care strictly conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research. The animals were housed under controlled conditions: a 12-hour light/dark cycle; a temperature of 20°C to 26°C, and humidity in the range of 40% to 70%. The animals had free access to standard food and drinking water.

Rats were randomly divided into three groups: sham (group A) ( $n = 18$ ), I/R (group B) ( $n = 18$ ), and I/R plus CDDP (group C) ( $n = 18$ ).

### 2.2. Experiment Medicine

CDDP was provided by Tasly Pharmaceutical Co., Ltd. (Tianjin, China). The CDDP was dissolved in a certain amount of water to a certain concentration of per milliliter of drug solution. Group C was treated with drug solution at a dose of 450 mg/kg/d for seven days. Meanwhile, Group A and Group B were treated with the same volume of 0.9% saline solution.

### 2.3. Experiment Methods

#### 2.3.1. Establishment of RIRI Model in Rats

Animals were anesthetized intraperitoneally with 1% pentobarbital sodium (0.7 ml/100g). Body temperature was maintained at 36.5°C - 37°C using a heated blanket. For corneal analgesia, one drop of 0.4% oxybuprocaine hydrochloride was used. Pupillary dilation was achieved with 0.5% tropicamide and 0.5% phenylephrine.

Retinal ischemia was induced by increasing the IOP in the right eye to 110 mmHg for 60 min. After dilation of the pupil, the anterior chamber of the right eye (only the right eye was used experimentally) was cannulated with a 30-gauge needle connected to a physiological saline bag. The IOP was raised to 110 mmHg by keeping

the bag at 150 cm above the eye. Retinal ischemia was confirmed when there was whitening of the iris and loss of the red reflex of the retina. After 60 min of ischemia, the needle was removed and the IOP was returned to normal pressure. For the sham group, the procedure was performed without elevating the saline bag.

### 2.3.2. Determination of the Levels of MDA and Activities of SOD, GSH-Px, and CAT

The levels of MDA and the activities of SOD, GSH-Px, and CAT were determined in each retina 1 day and 7 days after the ischemic insult.

The retina samples were prepared as a 10% homogenate in 0.9% saline using a homogenizer on ice according to their respective weight. Then, the homogenate was centrifuged at 3500 rpm for 15 min, and the supernatant was collected and diluted.

MDA levels were determined using the double heating method. Briefly, 2.5 ml of thiobarbituric acid (TBA) means thiobarbituric acid solution (100 g/L) was added to 0.5 mL of homogenate in each centrifuge tube, and the tubes were placed in boiling water for 15 min. After cooling with flowing water, the tubes were centrifuged at 1000 rpm for 10 min, 2 mL of the supernatant was added to 1 mL of the TBA solution (6.7 g/L), and the tube was placed in boiling water for another 15 min. After cooling, the amount of thiobarbituric acid-reactive species was measured at 532 nm. The MDA concentration was calculated from the absorbance coefficient of the MDA-TBA complex.

SOD activity was determined through the inhibition of nitrotetrazolium blue (NTB) reduction by the xanthine/xanthine oxidase system as a superoxide generator. The activity was assessed in the supernatant after the addition of 1.0 mL of ethanol/chloroform (5/3, v/v) to the same volume of sample and centrifugation for 15 min at 3000 rpm. The production of formazan was determined at 560 nm. One unit of SOD activity was defined as the amount of protein that inhibited the rate of NTB reduction by 50%.

GSH-Px activity was measured by the method of Paglia and Valentine. The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide, and glutathione reductase was initiated by the addition of H<sub>2</sub>O<sub>2</sub>, and the change in absorbance at 340 nm was monitored by spectrophotometry. The data are expressed as U/g of protein.

CAT activity was measured by the method of Cohen. The principle of the assay was based on the determination of the rate constant (s<sup>-1</sup>, k) of H<sub>2</sub>O<sub>2</sub> decomposition. The rate constant of the enzyme was determined by measuring the absorbance change per minute. The data are expressed as k/g of protein.

## 2.4. Statistical Methods

Data were represented as the mean ± SEM. To determine the significance of differences, analysis of variance (ANOVA) were carried out using SPSS 18.0 software. P values less than 0.05 were considered statistically significant in this study.

## 3. Results

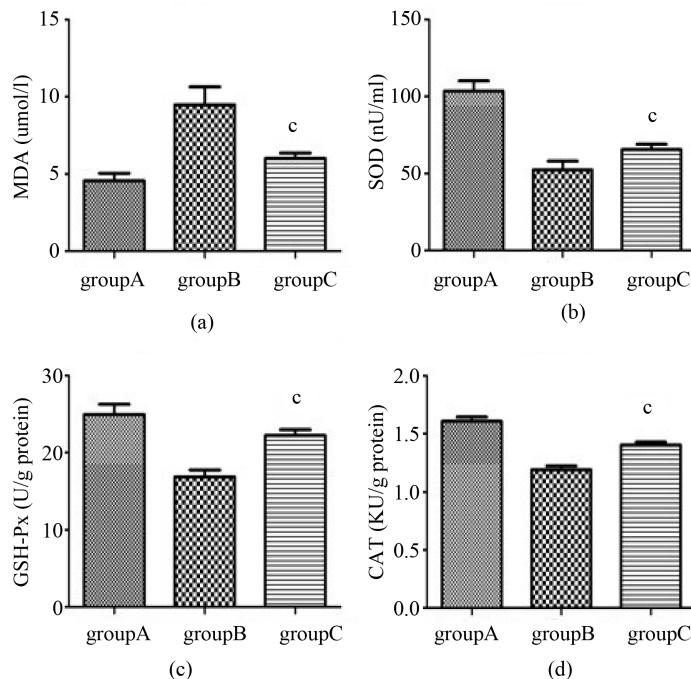
### 3.1. Effects of CDDP on the MDA Level and Activities of SOD, GSH-Px, and CAT on d1 after RIRI

To estimate the anti-oxidative effect of CDDP, the MDA level and the activities of SOD, GSH-Px, and CAT of the retina tissue were measured on d1 after I/R injury. As a marker of lipid peroxidation, the MDA level in the retina tissues of group B was remarkably higher than that in group A ( $p < 0.01$ ). CDDP significantly inhibited MDA production in the retina tissue after RIRI when compared with group B ( $p < 0.01$ ). Data are shown in **Figure 1(a)**.

SOD, GSH-Px, and CAT are pivotal enzymes scavenging ROS in vivo. The activities of SOD, GSH-Px, and CAT were greatly reduced in group B than in group A after I/R injury ( $p < 0.01$ ). Compared with group B, CDDP could significantly increase the activities SOD, GSH-Px, and CAT ( $p < 0.01$ ). Data are shown in **Figures 1(b)-(d)**, respectively.

### 3.2. Effects of CDDP on the MDA Level and Activities of SOD, GSH-Px, and CAT on d7 after RIRI

Seven days after RIRI, the MDA level in the group B was lower than those of day 1. However, compared with



**Figure 1.** Effects of CDDP on MDA levels and the activities of SOD, GSH-Px and CAT on the retina tissue from I/R-induced retinal injury on d1. Data are expressed as mean  $\pm$  SD and are representative of 3 experiments ( $n = 9$ ).  $^c p < 0.01$  vs. group B with group C.

group C, the MDA levels were still significantly higher in group B ( $p < 0.01$ ). Data are shown in **Figure 2(a)**. The activities of SOD, GSH-Px, and CAT were higher than those of d1. Compared with group C, the activities were still significantly lower in the group B ( $p < 0.01$ ). Data are shown in **Figures 2(b)-(d)**, respectively.

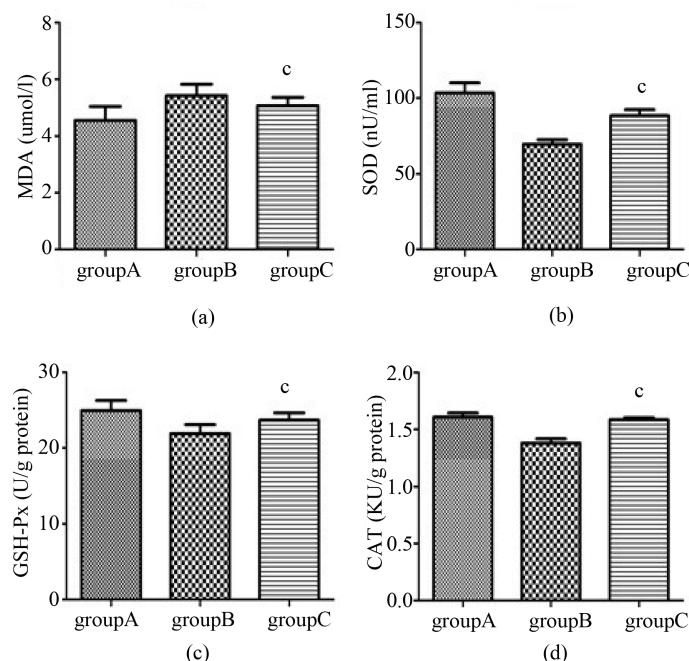
#### 4. Discussion

The present study was performed to evaluate the protective effects of CDDP against oxidative stress on the retina following RIRI in the rats. We found that CDDP improved the oxidative parameters of the retinal tissue in the I/R model, which suggested that CDDP might be a potential choice for the treatment of RIRI-related eye diseases.

A number of ocular diseases have been associated with retinal ischemia reperfusion injury, including retinal vascular occlusion, acute glaucoma, diabetic retinopathy, and retinopathy of prematurity [14]. Oxygen-free radicals [15], endogenous excitatory amino acids [16], calcium imbalances [17], and nitric oxide [18] [19], are involved in the pathogenesis of I/R injury. The exact mechanism of RIRI is complicated, mediated by a variety of factors, which makes it a hot pot problem around the world. Accordingly, finding a way to reduce or prevent RIRI, thus reduce visual impairment is of great importance.

Oxygen is crucial for life and metabolic processes, however, reactive metabolites of oxygen can be toxic to cells [20] [21]. Particularly, the sudden reintroduction of oxygen into tissues during reperfusion may exacerbate the cellular damage secondary to ischemia. ROS is considered to be a critical mediator of ischemia reperfusion injury. The generation of excess ROS can affect subcellular structures and functions, resulting in mitochondrial dysfunction, lipid peroxidation of polyunsaturated fatty acids in membranes, and alterations in protein or DNA structure [21]-[23].

Abundant studies of retinal tissue have shown that oxidative stress was a fundamental factor in cellular damage during IR injury. MDA is a naturally occurring product of lipid peroxidation, a process in which unsaturated fatty acids are oxidized to form radicals [24]. The level of MDA is currently used to assess oxidative tissue damage after I/R injury [24]. Free radicals produced in living organisms can be scavenged by intrinsic anti-oxidant enzymes, including SOD, CAT, and GSH-Px, thus, the activities of these enzymes may reflect the anti-



**Figure 2.** Effects of CDDP on MDA levels and the activities of SOD, GSH-Px and CAT on the retina tissue from I/R-induced retinal injury on d7. Data are expressed as mean  $\pm$  SD and are representative of 3 experiments ( $n = 9$ ).  $^*p < 0.01$  vs. group B with group C.

oxidative ability of the body [25]-[27].

Therefore, the aim of this study is to investigate the protective effects of CDDP against oxidative stress on the retina following RIRI in the rats through examining the MDA level and the activities of SOD, GSH-Px, and CAT in the retina tissues.

In the present study, we examined the changes in the levels of MDA, SOD, CAT, and GSH-Px, to elucidate the mechanism by which CDDP protects retinal cell injuries from oxidative stress. The results of d1 and d7 after RIRI have shown that I/R injury resulted in a significant increase in the MDA levels and decrease in the activities of SOD, CAT, and GSH-Px in the retina. However, CDDP could significantly decrease the MDA level and enhance the antioxidant enzyme activity in retinal tissue, implying that CDDP ameliorated the injury. The anti-oxidative stress effect of CDDP may be part of its protective mechanisms in RIRI.

CDDP is a new drug compound preparation in treating cardiovascular diseases. The chemical analysis of CDDP reported previously shows that the main ingredients are salvia miltorrhiza and panax notoginseng [4]. One pill of CDDP (obtained from Tasly Pharmaceutical, Tianjin, China) contains 9 mg of salvia miltorrhiza and 1.76 mg of panax notoginseng. Salvia miltorrhiza could improve cardiac function through improving antioxidant activity [5], and through preventing cardiomyocyte apoptosis [6]. Panax notoginseng could protect against myocardial damage and decrease lipid peroxidation [7]. With the combination of multiple components, CDDP hits multiple targets, and exerts the synergistic therapeutic effects. CDDP has been reported to be effective in the prevention of tissue damage in the heart and brain after IR injury, whereas there are few studies on the protective effect of CDDP against retinal damage caused by I/R, which is commonly seen in clinical practice. Thus, our studies investigated the protective effect and the underlying mechanisms.

In this study, the dose of CDDP chosen was the high-dose (450 mg/kg/d) used in the Li *et al.* study [28]. Our previous study demonstrated that the high-dose CDDP (450 mg/kg/d) could exert protective effects on the retina under ischemic conditions through examining resultant ERG and histological findings [10]. Those positive results provided proof for the use of the dosage of CDDP.

However, it should be noted that the anti-oxidative stress effect may not be the sole protective mechanism of CDDP. Various other mechanisms, such as anti-inflammatory and anti-apoptosis activities, could also play a role in the observed protective effects of CDDP during I/R injury. Further investigations should be carried out to

clarify this.

## 5. Conclusion

In conclusion, the present study demonstrates that CDDP is effective at protecting the retina during I/R injury. CDDP protected the retina from IR injury, possibly via a mechanism involving the regulation of oxidative parameters.

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