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Original Article

Edaravone ameliorates experimental autoimmune thyroiditis in rats through HO-1-dependent STAT3/PI3K/Akt pathway

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Abstract: Autoimmune thyroiditis is among the most prevalent of all the autoimmunities in population. It is characterized as both cellular immune responses with T, B cells infiltrating to the thyroid gland followed by hypothyroidism as a result of destruction of the thyroid follicles and fibrous replacement of the parenchymal tissue, as well as immune response for TPO and Tg-antibody production. Experimental autoimmune thyroiditis (EAT) has been proven to be an ideal model to study autoimmune thyroiditis. In the present study, we induced an EAT model in rats and examined the effect of edaravone, a hydroxyl radical scavenging agent, on EAT severity and explored the mechanism. The results showed that edaravone reduced the severity score of thyroiditis dose-dependently and the levels of serum TPOAb, TgAb, T3 and T4. Edaravone significantly decreased the mRNA level of IL-17, but increased the mRNA level of IL-10, IL-4, TNF-α and IFN-γ. EAT model significantly induced oxidative stress, which was inhibited by the treatment of 10 mg/kg, 20 mg/kg or 40 mg/kg of edaravone. The EAT model significantly increased the Akt and STAT3 phosphorylation, but when rats were treated with 20 mg/kg or 40 mg/kg edaravone, they were significantly inhibited. The HO-1 expression was greatly increased by 20 mg/kg or 40 mg/kg edaravone. The PI3K inhibitor LY294002, Akt inhibitor triciribine or STAT3 inhibitor WP1066 all significantly decreased the severity score of thyroiditis in the EAT model group, while the HO-1 inhibitor ZnPP-IX increased the severity score of thyroiditis. These results confirm the invlovment of ROS and HO-1-dependent STAT3/PI3K/Akt pathway in the process of Hashimoto's thyroiditis and suggest the potential usage of edaravone in the therapy of it.

Keywords: Edaravone, experimental autoimmune thyroiditis, HO-1, STAT3, PI3K/Akt

Introduction

Experimental autoimmune thyroiditis (EAT) has been used to simulate human autoimmune thyroid disease for decades [1]. It can be induced by injecting mouse thyroglobulin (MTg) and adjuvant directly into rats or by transferr ing MTg-primed donor spleen cells which has been previously activated with MTg to syngeneic recipient rats [2]. Infiltration of inflammatory cells and destruction of thyroid follicles are the main charactors shown in the thyroid lesions in EAT model, which reach maximal severity three weeks after cell transfer [3]. Chronic inflammation was also present, usually demostrated by increased level of proinflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α [4]. The human autoimmune thyroid diseases simulated by EAT include Hashimoto's thyroiditis (HT), the most common and extensively organ-specific autoimmune disease in the world. HT is characterized by infiltration of the thyroid gland by inflammatory cells and production of autoantibodies to thyroglobulin (Tg) and thyroperoxidase (TPO) [5].

Treatments for HT include nonspecific immunosuppressant or anti-inflammatory agents. These medicine are very effective to decrease helper T cell (TH) 1 cytokines or increase Th2 cytokine production, but could enhance the susceptibility of some complications as result of depressed immune function [6], making the

Table 1. Primer sequences for the PCR amplification

Primer	Sequence (5'-3')
IL-4	Forward: CGAGTTGACCGTAACAGACAT
	Reverse: CGTCTTTAGCCTTTCCAAGAAG
IL-10	Forward: CATTCATGGCCTTGTAGACACCTT
	Reverse: TCTCCCCTGTGAAAATAAGAGCAAG
IL-17	Forward: GTCGTTCAGATTGAGAGACAAGG
	Reverse: CTCATCCTTCAAAGACAGCCTCA
INF-γ	Forward: CTGGTGGACCACTCGGATGA
	Reverse: TTACTACCTTCTTCAGCAACAGCAA
TNF-α	Forward: AGGCAATAGGTTTTGAGGGCCATG
	Reverse: ACACACAAGCATCAAGGATAC
β-actin	Forward: GGAGATTACTGCCCTGGCTCCTA
	Reverse: GACTCATCGTACTCCTGCTTGCTG

development of new approaches to treat HT neccesary. Many studies have shown the connection between autoimmune thyroiditis and reactive oxygen species (ROS). Intracellular adhesion molecule-1 adhesion (ICAM-1) in thyrocytes plays an important role in the in an inflammatory response in HT by directing the localization of inflammatory mononuclear cells. The expression of ICAM-1 can be promoted by many stimuli, including oxidants [7]. In autoimmune thyroiditis, ROS can be produced in the iodine organification process in the thyroid follicle cell. Increased accomlation of ROS may be the first step in the ICAM-1 expression and induction of inflammatory response in autoimmune thyroiditis [7]. It is shown that an anti-oxidant diphenyleneiodium (DPI) decreased the generation of ROS and ICAM-1 expression in thyrocytes, indicating that anti-oxidant therapy may be effective in preventing autoimmune thyroiditis [7].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a hydroxyl radical scavenging agent which has been reported to protect against cerebral injury induced by cerebral ischemia [8], as well as intracerebral and subarachnoid hemorrhage [9] in Japan and China by elimating oxygen radicals and reducing pro-inflammatory factors [10]. However, the effect of edaravone in EAT has not been explored yet. Hence, in the current study, we studied the potential role of edaravone against EAT in rats. We induced a mouse model of EAT and tested the effects of edaravone on EAT severity, serum triiodothyronine (T3), thyroxine (T4), anti-thyroglobulin antibodies (TgAb) and thyroid peroxidase antibody (TPOAb), as well as the mRNA expression of interleukin (IL)-4, IL-10, IL-17, TNF-α and INF-γ. The oxidative products (protein carbonyl, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and malondialdehyde (MDA)) in thyroid tissues were also measured to evaluate the role of ROS in EAT. Finally, the role of heme oxygenase (HO)-1 and signal transducer and activator of transcription 3 (STAT3)/PI3K/Akt pathway was exlopred to clarify the mechanism invloved in the protection of edaravone against EAT.

Materials and methods

Animals and chemicals

Adult male Sprague-Dawley rats (220-250 g) were used in this study. Rats were procured from Shanghai University of Traditional Chinese Medicine Animal Center. They were housed in stainless steel cages (four rats/cage) and were given food and water *ad libitum* under room temperature (25°C) and a 12-h day/night cycle. All animals were acclimatized to the laboratory conditions for 1 week prior to the experiments. The experimental protocol was approved by the institutional animal care and use committee of Shanghai University of Traditional Chinese Medicine. Edavarone was brought from Double-crane Pharmaceutical Co., Ltd. (Beijing, China). Porcine thyroglobulin (pTg), HO-1 inhibitor zinc protoporphyrin IX (ZnPP-IX), PI3K inhibitor LY-294002, Akt inhibitor Triciribine and STAT3 inhibitor WP1066 were bought from Sigma Aldrich (CA, USA).

Experimental design

In the first part of study, seventy rats were randomly assigned to seven groups: Control, Sham, Model, Edaravone (10 mg/kg), Edaravone (20 mg/kg), Edaravone (40 mg/kg) and Edaravone-only (40 mg/kg), 10 rats in each group. Rats in the Control group received no treatment; rats in the Sham group received similar treatment to Model, except that they were given saline; rats in Model group received a EAT treatment; rats in Edaravone (10 mg/kg), Edaravone (20 mg/kg) and Edaravone (40 mg/kg) groups received EAT treatment and edaravone at different dosages. Edaravone was i.p. injected once a day during the EAT induction. Rats in Edaravone-only group received edara-

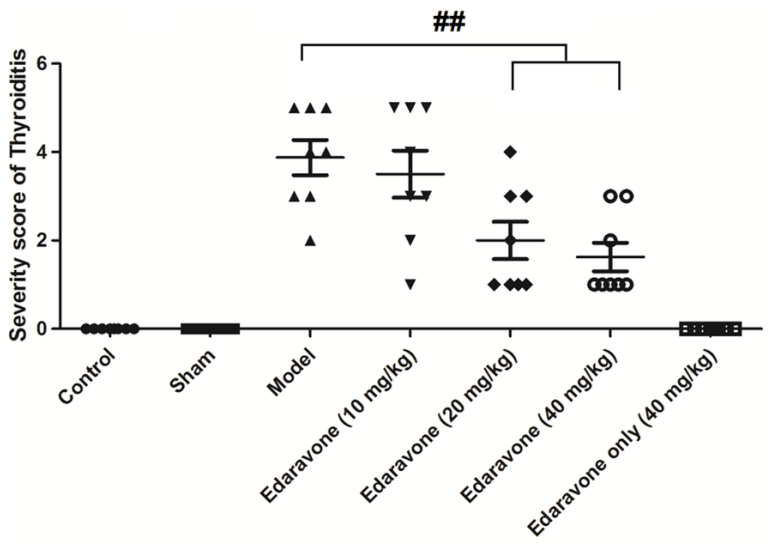

Figure 1. Severity score of thyroiditis. The score of Control group was 0. The average severity score of Model group was 3.9. When rats were treated with 10 mg/kg edaravone, the severity score of thyroiditis was slightly decreased to 3.5. When rats were treated with 20 mg/kg and 40 mg/kg edaravone, the severity score of thyroiditis was significantly decreased to 2.0 and 1.6. Control: rats received no treatment; Sham: rats received similar treatment to Model group, except that they were given saline; Model: rats received an EAT induction; Edaravone (10 mg/kg), Edaravone (20 mg/kg) and Edaravone (40 mg/kg): rats received an EAT induction and edaravone at 10 mg/kg, 20 mg/kg or 40 mg/kg. Edaravone-only group (40 mg/kg): rats received 40 mg/kg edaravone but no EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. ##: P<0.01. N = 10.

vone without EAT induction. In the second part of study, fifty rats were randomly assigned to five groups: Control, LY294002, Triciribine, WP1066 and ZnPP-IX, 10 rats in each group. LY294002 (100 mg/kg of body weight), triciribine (2 mg/kg of body weight), WP1066 (40 mg/kg) or ZnPP-IX (3 mg/kg of body weight) was administered intraperitoneally once a day during the EAT induction.

Induction of EAT

The induction of EAT was similar to the study of Song et al [6]. Briefly, pTg was first dissolved in phosphate buffer saline (PBS) to archive a final concentration of 2 mg/ml. Next, it was emulsified with complete Freund's adjuvant (Sigma, USA) or incomplete Freund's adjuvant (Sigma, USA). The final concentration of pTg in Freund's adjuvant was 1 mg/ml. On the first day of EAT induction, 100 ml of pTg emulsion in complete Freund's adjuvant was injected subcutaneously into rats on the inside of the hind leg. On day 14 and 21, 10 ml of pTg emulsion in incomplete Freund's adjuvant was injected subcutaneously into rats.

Evaluation of thyroiditis

Similarly to Chen et al [11], after rats were sacrificed, thyroids were collected and scored quantitatively for EAT severity using hematoxylin and eosin (H&E) method. A scale of 1+ to 5+ was used to evaluate the EAT severity: 1+, an infiltrate of at least 125 cells in one or several foci; 2+, 10-20 foci of cellular infiltration involving up to 25% of the gland; 3+, 25-50% of the gland is infiltrated; 4+, >50% of the gland is destroyed by infiltrating inflammatory cells; 5+, complete destruction of the thyroid with few or no remaining follicles.

Biochemical analysis

The biochemical analysis was similar to the study of Song et al [6]. After rats were sacrificed, serum samples were collected by centrifuging blood at 3500 × g for 20 min. The levels of serum T3, T4, TgAb and TPOAb were measured by radioimmunoassay method with commercially available kits (Biotecx Labs, Houston, TX) according to the manufacturers' instructions using T3 and T4 are expressed as ng/ml of serum. TgAb and TPOAb are expressed as IU/ml of serum.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RT-qPCR procedure was similar to the study of Ma et al [12]. Firstly, total RNA was extracted from thyroiditis cells using TRIzol reagent according to the manufacturer's protocol. The qPCR was performed using SYBR-Green on Applied Biosystems 7500 (Software v2.0.6, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Primer sequences for the PCR amplification of IL-4, IL-10, IL-17, TNF-α and INF-γ mRNA are listed in **Table 1**. The PCR conditions consisted of denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. The mRNA level was normalized to that of β-actin. The amplification results for PCR were calculated using the $2^{-\Delta\Delta Cq}$

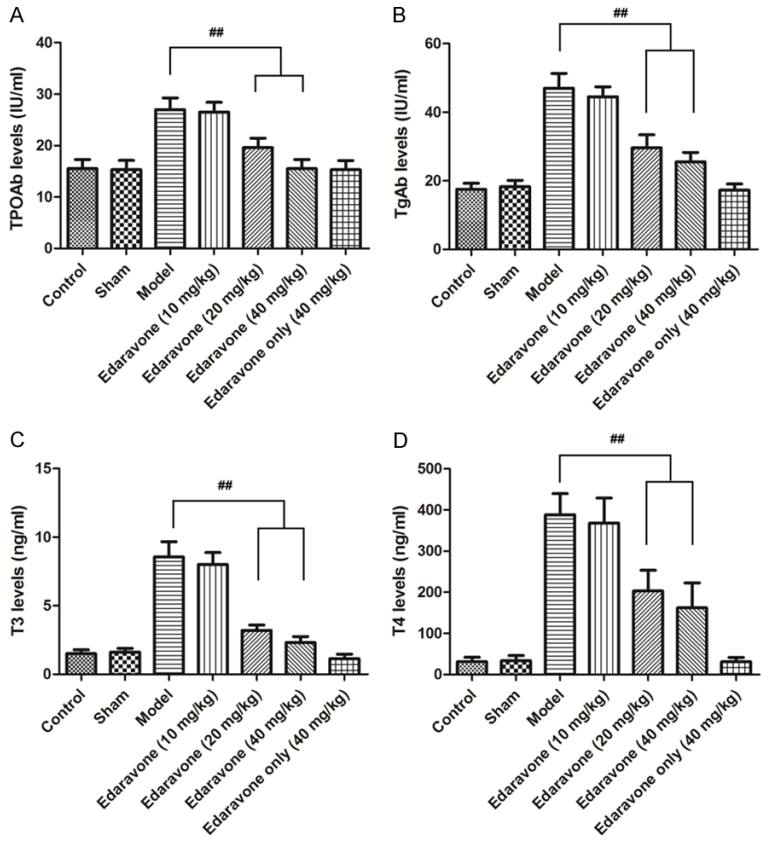

Figure 2. Levels of serum TPOAb, TgAb, T3 and T4. There was no significant change in the levels of serum TPOAb, TgAb, T3 and T4 in Sham group. The serum levels of TPOAb, TgAb, T3 and T4 in the Edaravone (20 mg/kg) and Edaravone (40 mg/kg) group were significantly decreased. Control: rats received no treatment; Sham: rats received similar treatment to Model group, except that they were given saline; Model: rats received an EAT induction; Edaravone (10 mg/kg), Edaravone (20 mg/kg) and Edaravone (40 mg/kg): rats received an EAT induction and edaravone at 10 mg/kg, 20 mg/kg or 40 mg/kg. Edaravone-only group (40 mg/kg): rats received 40 mg/kg edaravone but no EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. ##: P<0.01. N = 10.

method. All the PCR reactions were run in triplicate.

Measurement of oxidative products (protein carbonyl, 8-OHdG and MDA) in thyroiditis

First of all, 100 mg of thyroiditis tissue was homogenized in PBS and centrifuged at 12,000 g for 20 min. Next, the levels of oxidative products (protein carbonyl, 8-OHdG and MDA) in the supernatant were measured according the instruction of assay kits. Protein carbonyls were measured with a commercial quantitative assay kit (Cayman Chemical, Company). Results were expressed as nmol/mg protein. 8-OHdG assay was measured with a DNA Extraction Kit (DNA Extractor Wb Kit, Wako Chemica, Japan) and anti-8-OHdG antibody (Fukuroi, Japan) and incubated IgG, Streptavidin-Horseradish Peroxidase and 3, 3', 5, 5'-tetramethylbenzidine. Results were expressed as pg 8-OHdG /g protein. MDA was measured using a commercial kit (Nanjing Jiancheng, Nanjing, China). Results were expressed as μmol/mg protein. The protein concentration was determined using a standard BCA protein assay kit (Nanjing Jiancheng, Nanjing, China).

Western blot

For western blot assay, 50 μg protein was loaded on SDS-PAGE (10% gel), transferred to polyvinylidene fluoride (PVDF) membranes, incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA). The density of bands was determined by enhanced chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ, USA) using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Philadelphia, USA). The densitometric analysis was conducted with Image J 1.43 (National Institutes of Health).

Statistical analysis

Statistical calculations were performed using SPSS (version 20.0; SPSS, Chicago, IL, USA). Data were expressed as the Mean ± S.E.M. They were statistically analyzed with one-way ANOVA followed by Tukey's post hoc test. *P* value less than 0.05 were considered significant.

Results

Edaravone reduced the severity score of thyroiditis dose dependently

As shown in **Figure 1**, rats in Sham group had no change in the severity score of thyroiditis compared to Control (score = 0). The severity

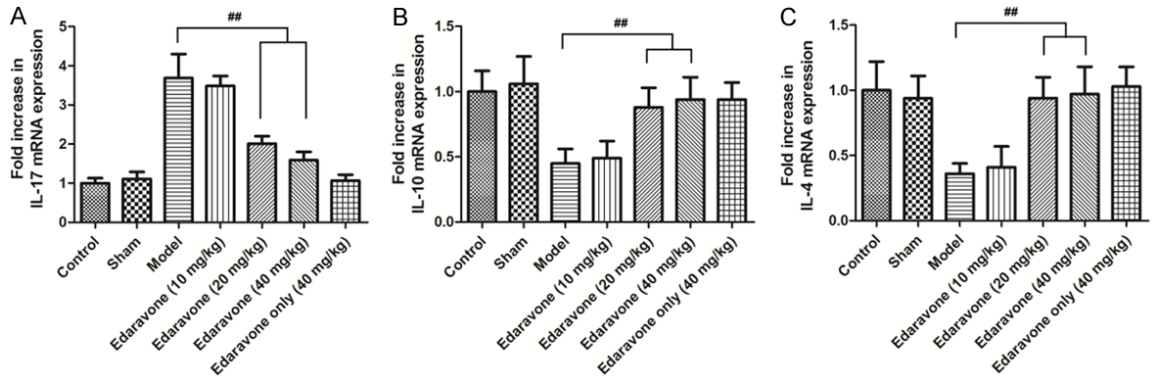

Figure 3. The mRNA levels of IL-17, IL-10 and IL-4. The mRNA level of IL-17 was significantly increased in the Model group, but decrased in the 20 mg/kg or 40 mg/kg of edaravone group. The IL-10 and IL-4 were significantly decreased in the Model group, but significantly increased in the 20 mg/kg or 40 mg/kg of edaravone group. Control: rats received no treatment; Sham: rats received similar treatment to Model group, except that they were given saline; Model: rats received an EAT induction; Edaravone (10 mg/kg), Edaravone (20 mg/kg) and Edaravone (40 mg/kg): rats received an EAT induction and edaravone at 10 mg/kg, 20 mg/kg or 40 mg/kg. Edaravone-only group (40 mg/kg): rats received 40 mg/kg edaravone but no EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. ##: P<0.01. N = 10.

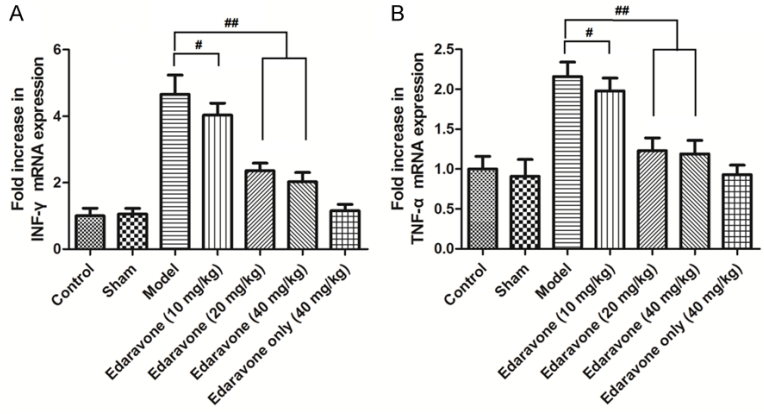

Figure 4. The mRNA levels of TNF-α and IFN-γ. The mRNA levels of TNF-α and IFN-γ were greatly increased by EAT model, but significantly decreased by the treatment with 20 mg/kg or 40 mg/kg of edaravone. Control: rats received no treatment; Sham: rats received similar treatment to Model group, except that they were given saline; Model: rats received an EAT induction; Edaravone (10 mg/kg), Edaravone (20 mg/kg) and Edaravone (40 mg/kg): rats received an EAT induction and edaravone at 10 mg/kg, 20 mg/kg or 40 mg/kg. Edaravone-only group (40 mg/kg): rats received 40 mg/kg edaravone but no EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. #: P<0.05. ##: P<0.01. N = 10.

score of thyroiditis in the Model group was significantly increased compared to the Control and the Sham group. The average severity score was 3.9. When rats were treated with 10 mg/kg edaravone, the severity score of thyroiditis was slightly decreased to 3.5 (P>0.05 compared to Model group). When rats were treated with 20 mg/kg and 40 mg/kg edaravone, the severity score of thyroiditis was significantly decreased to 2.0 and 1.6 (P<0.05 compared to Model group). Treatment of 40 mg/kg of edaravone only had no impact on the severity score of thyroiditis.

Edaravone decreased the levels of serum TPOAb, TgAb, T3 and T4

Figure 2 shows the changes of levels of serum TPOAb, TgAb, T3 and T4. Similarly to the results of severity score of thyroiditis, rats in Sham group had no change in the levels of serum TPOAb, TgAb, T3 and T4 compared to Control. There is no significant change difference between Model and Edaravone (10 mg/kg) group, but rats in the Edaravone (20 mg/kg) and Edaravone (40 mg/kg) group has significant lower levels of serum TPOAb, TgAb, T3 and T4 (P<0.05 compared to Model group). Treatment of 40 mg/kg of edaravone only had no impact on the levels of serum TPOAb, TgAb, T3 and T4.

Edaravone changed the mRNA levels of IL-17, IL-10 and IL-4 in EAT

Figure 3 shows the changes of inflammatory factors on mRNA levels. As shown in **Figure 3A**, the mRNA level of IL-17 was significantly increased in the Model group. After rats were

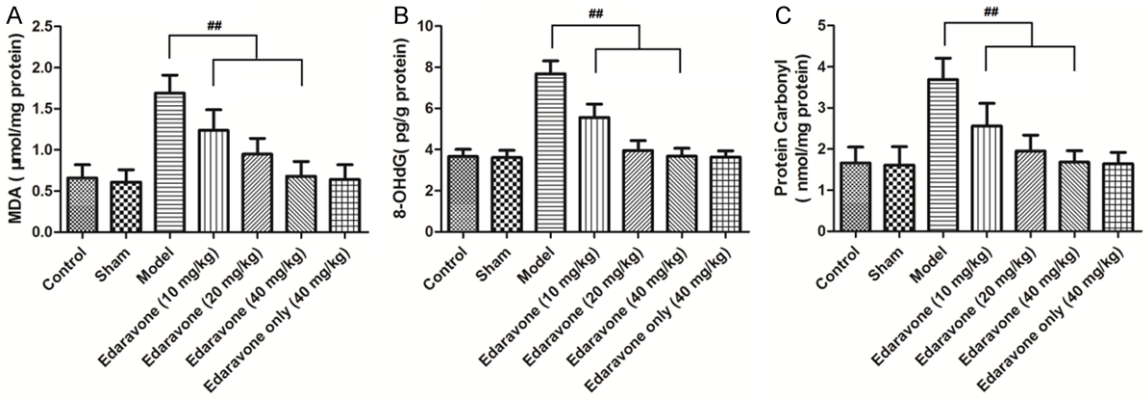

Figure 5. Levels of oxidative products (protein carbonyl, 8-OHdG and MDA) in thyroid. EAT model significantly induced oxidative stress, as shown by the dramatic increases in the MDA, protein carbonyl and 8-OHdG levels. Treatment of 10 mg/kg, 20 mg/kg or 40 mg/kg of edaravone caused significant reduction of the levels of these oxidative parameters. Control: rats received no treatment; Sham: rats received similar treatment to Model group, except that they were given saline; Model: rats received an EAT induction; Edaravone (10 mg/kg), Edaravone (20 mg/kg) and Edaravone (40 mg/kg): rats received an EAT induction and edaravone at 10 mg/kg, 20 mg/kg or 40 mg/kg. Edaravone-only group (40 mg/kg): rats received 40 mg/kg edaravone but no EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. ##: P<0.01. N = 10.

treated with 10 mg/kg of edaravone, the mRNA level of IL-17 was not significantly changed compared to the Model group. After rats were treated with 20 mg/kg or 40 mg/kg of edaravone, the mRNA level of IL-17 was significantly decreased compared to Model group (P<0.05). As shown in **Figure 3B** and **3C**, the IL-10 and IL-4 were significantly decreased in the Model group. The treatment with 10 mg/kg of edaravone did not significantly change the mRNA level of IL-10 and IL-4, but treatment with 20 mg/kg or 40 mg/kg of edaravone significantly increased the mRNA level of IL-10 and IL-4 compared to the Model group (P<0.05). Treatment of 40 mg/kg of edaravone only had no impact on the mRNA levels of IL-17, IL-10 and IL-4.

Edaravone decreases the mRNA levels of TNF-α and IFN-γ in EAT

Figure 4 shows the changes of mRNA levels of TNF-α and IFN-γ. The mRNA levels of TNF-α and IFN-γ were greatly increased by EAT model, but significantly decreased by the treatment with 20 mg/kg or 40 mg/kg of edaravone (P<0.05 compared to Model). Treatment of 40 mg/kg of edaravone only had no impact on the mRNA levels of TNF-α and IFN-γ.

Edaravone decreases the oxidative stress (protein carbonyl, 8-OHdG and MDA) in EAT

As shown in **Figure 5**, the MDA, protein carbonyl and 8-OHdG assays demonstrated that Sham operation caused no oxidative stress (Sham group versus Control group, P>0.05). EAT model significantly induced oxidative stress, as shown by the dramatic increases in the MDA, protein carbonyl and 8-OHdG levels compared to Sham group (P<0.05). Treatment of 10 mg/kg, 20 mg/kg or 40 mg/kg of edaravone caused significant reduction of these oxidative parameters levels (P<0.05 versus Model group). The treatment of 40 mg/kg edaravone only had no effect on oxidative stress.

Edaravone activated PI3K/Akt pathway, STAT3 and HO-1 expression

As shown in **Figure 6A**, the EAT model significantly increased the Akt phosphorylation (P<0.05), but when rats were treated with 20 mg/kg or 40 mg/kg edaravone, the Akt phosphorylation was significantly inhibited compared to the Model group. Similarly to the Akt phosphorylation, the STAT3 phosphorylation was significantly increased in the EAT model group, but decreased in the 20 mg/kg or 40 mg/kg edaravone groups. Treatment of 40 mg/kg edaravone only had no impact on the Akt or STAT3 phosphorylation. The HO-1 expression was not altered by EAT model, but greatly increased by 20 mg/kg or 40 mg/kg edaravone (P<0.05 compared to Model). Treatment of 40 mg/kg edaravone also increased the HO-1 expression compared to Control (P<0.05).

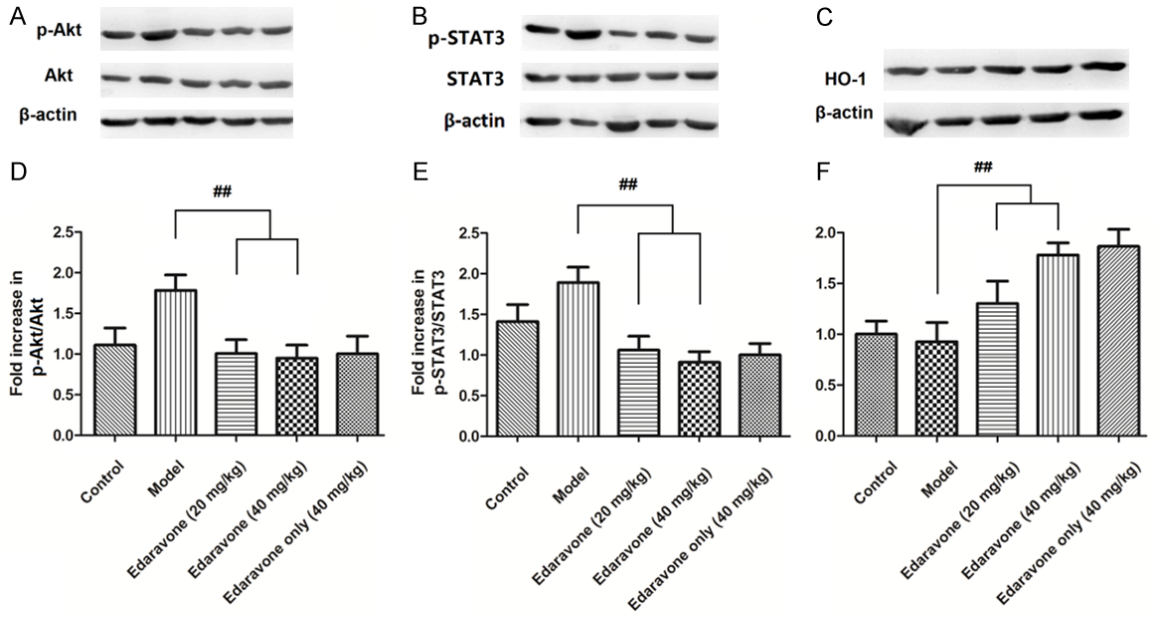

Figure 6. Phosphorylation of Akt, STAT3 and expression of HO-1. EAT model significantly increased the p-Akt and p-STAT3, which were inhibited by edaravone. The HO-1 expression was not altered by EAT model, but greatly increased by 20 mg/kg or 40 mg/kg edaravone. Control: rats received no treatment; Model: rats received an EAT induction; Edaravone (20 mg/kg) and Edaravone (40 mg/kg): rats received an EAT induction and edaravone at 20 mg/kg or 40 mg/kg. Edaravone-only group (40 mg/kg): rats received 40 mg/kg edaravone but no EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. ##: P<0.01. N = 10.

Effects of the inhibitors of STAT3/PI3K/Akt pathway or HO-1 on severity score of thyroiditis

The EAT model was induced in rats, and they were treated with PI3K inhibitor LY294002, Akt inhibitor triciribine, STAT3 inhibitor WP1066, or HO-1 inhibitor ZnPP-IX, then the severity score of thyroiditis was measured. As shown in **Figure 7**, the PI3K inhibitor LY294002, Akt inhibitor triciribine or STAT3 inhibitor WP1066 all significantly decreased the severity score of thyroiditis in the Model group (P<0.05 compared to Vehicle), while the HO-1 inhibitor ZnPP-IX increased the severity score of thyroiditis.

Discussion

In the present study, we first discovered that edaravone reduces the severity score of thyroiditis dose dependently and decreased the levels of serum TPOAb, TgAb, T3 and T4. Edaravone significantly decreased the mRNA level of IL-17, but increased the mRNA level of IL-10, IL-4, TNF-α and IFN-γ. EAT model significantly induced oxidative stress, which was inhibited by the treatment of 10 mg/kg, 20 mg/kg or 40 mg/kg of edaravone. The EAT model significantly increased the Akt and STAT3 phosphorylation, but when rats were treated with 20 mg/kg or 40 mg/kg edaravone, the Akt and STAT3 phosphorylation were significantly inhibited. The HO-1 expression was greatly increased by 20 mg/kg or 40 mg/kg edaravone. The PI3K inhibitor LY294002, Akt inhibitor triciribine or STAT3 inhibitor WP1066 all significantly decreased the severity score of thyroiditis in the EAT model group, while the HO-1 inhibitor ZnPP-IX increased the severity score of thyroiditis. These results confirm the invlovment of ROS in the process of Hashimoto's thyroiditis and suggest the potential usage of edaravone in the therapy of it.

Autoimmune thyroiditis, also known as Hashimoto's thyroiditis, is among the most prevalent of all the autoimmunities in population [13]. It was first described by Dr. Hakaru Hashimoto in 1912. He examined the thyroid specimens of four middle-age women who had undergone thyroidectomy [14]. Autoimmune thyroiditis has become the No. 1 cause of hypothyroidism in adults [15]. Autoimmune thyroiditis is characterized as both cellular immune responses with T, B cells infiltrating to the thyroid gland followed by hypothyroidism as a result of destruction of the thyroid follicles and

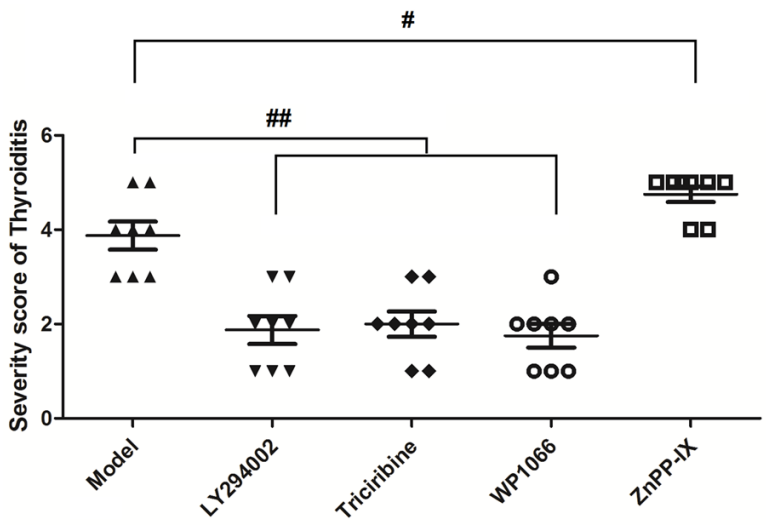

Figure 7. Effects of the inhibitors of STAT3/PI3K/Akt pathway or HO-1 on severity score of thyroiditis. The PI3K inhibitor LY294002, Akt inhibitor triciribine or STAT3 inhibitor WP1066 all significantly decreased the severity score of thyroiditis, but the HO-1 inhibitor ZnPP-IX increased it. Model: rats received an EAT induction; LY294002: PI3K inhibitor, injected daily at 100 mg/kg of body weight; Triciribine: Akt inhibitor, injected daily at 2 mg/kg of body weight. WP1066: STAT3 inhibitor, injected daily at 40 mg/kg of body weight; ZnPP-IX: HO-1 inhibitor, injected daily at 3 mg/kg of body weight. All the inhibitors were i.p. injected daily during the EAT induction. Severity score of thyroiditis were expressed as Mean ± S.E.M. #: P<0.05. ##: P<0.01. N = 10.

fibrous replacement of the parenchymal tissue, as well as immune response for TPO, Tg-antibody production [16]. EAT has been proven to be an ideal model to study autoimmune thyroiditis. In the present study, we induced an EAT model in rats and examined the severity score of thyroiditis. The severity score of thyroiditis in the Model group was significantly increased compared to the Control and the Sham group. The average severity score was 3.9. These results indicate that the EAT animal model was successful. When rats were treated with 10 mg/kg edaravone, the severity score of thyroiditis was slightly decreased to 3.5. But when rats were treated with 20 mg/kg and 40 mg/kg edaravone, the severity score of thyroiditis was significantly decreased to 2.0 and 1.6 (P<0.05 compared to Model group). These results, for the first time, show the potential effectiveness of edaravone in the treatment of autoimmune thyroiditis. Next, we found that the levels of serum TPOAb, TgAb, T3 and T4 were greatly increased in the EAT model group, which was inhibited by edaravone. There is no significant change difference between Model and Edaravone (10 mg/kg) group, but rats in the Edaravone (20 mg/kg) and Edaravone (40 mg/kg) group has significant lower levels of serum TPOAb, TgAb, T3 and T4 (P<0.05 compared to Model group).

As autoimmune thyroiditis is partly caused by the immunomodulatory cytokine secretion by thyrocytes [17], interleukin plays an important role in the inflammatory and autoimmune processes [18]. Proinflammatory cytokines are involved in the pathogenesis of HT [19]. The present study shows that the mRNA level of IL-17 was significantly increased, while the mRNA level of IL-10 and IL-4 were decreased in the EAT model group. After rats were treated with 20 mg/kg or 40 mg/kg of edaravone, the mRNA level of IL-17 was significantly decreased compared to Model group. Treatment with 20 mg/kg or 40 mg/kg of edaravone significantly increased the mRNA level of IL-10 and IL-4 compared to the Model group (P<0.05). Moreover, the mRNA levels of TNF-α and IFN-γ were significantly decreased by the treatment with 20 mg/kg or 40 mg/kg of edaravone. These results indicate that edaravone could effectively eliminate the impacts of EAT model on interleukins and proinflammatory cytokines.

Overproduction of ROS might be the cause of apoptosis and cell necrosis and thyroid dysfunction [20]. Excess ROS could overwhelm the cellular anti-oxidative defense and impair the normal structure and function of DNA, lipid and protein, leading to augmentation of cellular antioxidant capacity and thyroid dysfunction [21]. As shown by the dramatic increases in the MDA, protein carbonyl and 8-OHdG levels, EAT model significantly induced oxidative stress. The exact mechanism for oxidative stress induced in EAT model is not fully understood yet. It is possible that the iodide reacts with the iodinium cation (I+) produced during enzymatic iodide oxidation by thyroperoxidase to yield molecular iodine (I2). Interaction between endogenous peroxides and I2 may lead to the generation of ROS [22]. The result that the oxidative stress in EAT rats was inhib-

ited by the treatment of 10 mg/kg, 20 mg/kg or 40 mg/kg of edaravone suggests that the capacity of edaravone as an antioxidant may be an important mechanism to mitigate thyroiditis severity.

To explore the mechanism involved, we investigated the activation of STAT3/PI3K/Akt pathway and HO-1 expression. As shown in **Figure 6**, the EAT model significantly increased the Akt and STAT3 phosphorylation, but when rats were treated with 20 mg/kg or 40 mg/kg edaravone, the Akt and STAT3 phosphorylation were significantly inhibited. The HO-1 expression was not altered by EAT model, but greatly increased by 20 mg/kg or 40 mg/kg edaravone. Treatment of 40 mg/kg edaravone only did not change the Akt or STAT3 phosphorylation, but it increased the HO-1 expression. Finally, we induced the EAT model in rats and treated them with PI3K inhibitor LY294002, Akt inhibitor triciribine, STAT3 inhibitor WP1066, or HO-1 inhibitor ZnPP-IX, then the severity score of thyroiditis was measured. As shown in **Figure 7**, the PI3K inhibitor LY294002, Akt inhibitor triciribine or STAT3 inhibitor WP1066 all significantly decreased the severity score of thyroiditis in the Model group, while the HO-1 inhibitor ZnPP-IX increased the severity score of thyroiditis. Some studies reported the relationship between the STAT3 pathway, ROS and edaravone. Cheng et al reported that ATPγS stimulated Jak2 and STAT3 activation which were inhibited by pretreatment with edaravone in A549 cells [23]. Zhang et al found that borneol improved the efficacy of edaravone against dextran sulfate sodium-induced colitis by promoting M2 macrophages polarization via JAK2-STAT3 signaling pathway [24]. The relationship between STAT3 pathway and EAT was also indicated in some studies. Tanaka et al revealed that suppressor of cytokine signaling 1 is necessary for Th17 differentiation by suppressing antagonistic effect of IFN-γ on both STAT3 and Smads [25]. The phosphorylation of STAT3 was found in Hashimoto's thyroiditis and luteolin significantly reduced it [26]. However, this is the first study to clarify the involvement of STAT3/PI3K/Akt pathway and HO-1 in the protective effect exerted by edaravone against EAT.

Overall, the results of this study suggest that Edaravone ameliorates experimental autoimmune thyroiditis in rats. The HO-1 and STAT3/PI3K/Akt pathway was found to be invovled in the mechanism. This finding suggests the potential usage of edaravone in the therapy of Hashimoto's thyroiditis.

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Disclosure of conflict of interest

None.

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