中國1號天仙液 對氧自由基的清除作用之完整實驗報告

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SCAVENGING EFFECTS OF FRC001 (China No.1 Tian Xian Liquid) ON OXYGEN FREE RADICALS

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ABSTRACT

With ESR, BJL-chemiluminescence and other techniques, the scavenging effects of FRC001 on oxygen free radicals have been studied in aqueous, enzyme, irradiation and cell systems and compared with Vitamin C and E. It was found that FRC001 could effectively scavenge the oxygen free radicals generated from PMN stimulated with PMA, xanthine/xanthine oxidase, irradiation riboflavin/EDTA and Fenton Reaction. It was also found that it could inhibit the conjugated dienes and TBA reacted materials (TBARM) formed during lipid peroxidation of linoleic acid and liposome respectively and it could effectively scavenge $ONOO^-$.

Key words: FRC001, Oxygen free radicals,lipid peroxidation, ESR, BJL-chemiluminescence.

INTRODUCTION

Active oxygen free radicals can damage components of cell, even kill normal cells and cause aging and some very serious diseases, such as cancer and heart disease. Usually, the production and scavenging of the active oxygen free radicals are balanced in healthy human body. If there is imbalance in regular mechanism of some enzymes, such as superoxide dismutase (SOD) or catalase, there can be excessive amounts of active oxygen radicals generated in the body. It is possible that scavengers of active oxygen radicals might be beneficial for prevention of such diseases and for human health. So it is very important to search for effective scavengers of active oxygen radicals. In this paper, with ESR, BJL-chemiluminescence and other techniques, the scavenging effect of FRC001 on oxygen free radicals has been studied in aqueous. enzyme, irradiation system and cell system. It was found that FRC001 could effectively scavenge the oxygen free radicals generated from PMN stimulated with PMA, xanthine/xanthine oxidase, irradiation riboflavin/EDTA and Fenton Reaction. It was also found that it could inhibit the conjugated dienes and TBARM formed during lipid peroxidation of linoleic acid and liposome respectively.

MATERIALS AND METHODS

Agents: DMPO (5.5-dimethyl-pyrroline-1-oxide) was purchased from Sigma Chem Co. and purified by active charcoal before use PMA (phorbol myristate acetate), linoleic acid, lipoxidase, SOD (6500 U/mg), xanthine/xanthine oxidase (2.125 U/ml) and luminol were purchased from Sigma Chem Co., PMA was dissolved in a little acetone and diluted with 50mM phosphate buffer to a proper concentration before use. Other agents made in China are AR Levels. FRC001 is supplied by China-Japan Feida Union Co., LTD.

Measurement of SOD activity included in FRC001

BJL-chemiluminescence measurement: The reaction of xanthine and xanthine oxidase can generate oxygen free radicals which give a luminol-dependent chemiluminescence. SOD can scavenge the chemiluminescence generated from this system, so the SOD activity can be measured with this system. The measurement system includes 0.2 mM luminol, 0.32 mM xanthine, and 0.09 U/ml xanthine oxidase. The chemiluminescence was measured with WDD-1 chemiluminescencemeter

The scavenging effect of FRC001 on $\cdot O_2^-$ is defined as:

$$E = \frac{ho - hx}{ho} \times 100\%$$

Here ho is the Chemiluminescence of xanthine/xanthine oxidase control system and hx is the chemiluminescence of xanthine/xanthine oxidase after addition of FRC001 solution.

The measurement system was the same as above except that different concentrations of FRC001 were added to the system. First, a standard curve of SOD activity was developed with this system. Then the scavenging curve of FRC001 on oxygen free radicals was measured in this system. By using both of these curves, the SOD activity included in FRC001 solution can be determined.

Scavenging effect of FRC001 on ·OH free radicals generated from Fenton reaction

A solution of 50 mM DMPO 1% H_2O_2 and 100 μM Fe(II) as ferrous ammonium sulfate were mixed and transferred to a quartz capillary for ESR measurement. When the scavenging effect of FRC001 was measured, different concentrations of FRC001 solution were added to the system. The scavenging effect was calculated as above but here the height of the second peak was used for calculation. ESR conditions: All ESR spectra were recorded at Varian E-109 ESR spectrometer. The conditions are: microwave power 20 mW. X-band, 100 kHz modulation with amplitude 1G, central magnetic field 3250 G, scan width 200 G, time constant 0. 128 S, room temperature.

Scavenging effect of FRC001 on $\cdot O_2^-$ generated from irradiation of riboflavin/ EDTA system

A mixture containing 0.3 mM riboflavin, 5 mM EDTA and 0.1 M DMPO was transferred to a quartz capillary and put into the cavity of ESR spectrometer. After irradiation of the sample for 20 seconds with a xenon lamp (500 W, distance 70 cm), the ESR spectra were recorded immediately. When the scavenging effect of FRC001 was measured, different concentrations of FRC001 were added to the system. The scavenging effect was calculated as above but here the height of the first peak was used for calculation. The ESR measurement condition was the same as above.

Measurement of scavenging effect of FRC001 on conjugated dienes generated from lipid peroxidation of linoleic acid by lipoxidase

0.1mM linoleic acid in PBS was mixed with 480 U/ml lipoxidase and measured at 232 nm with time. When inhibition effects of FRC001 on the generation of conjugated dienes were measured, different concentrations of FRC001 were added into the system. The scavenging effect was calculated as above, but here the h and hx was used by reaction rate of control and sample respectively.

Measurement rate of scavenging effect of FRC001 on TBA reacted materials (TBARM) generated from lipid peroxidation of liposome initiated by Fe^{2+}

Liposome made from lecithine (10 mg/ml) was peroxidized by addition of Fe^{2+} (100 μM), then 95 ℃ mixed with 6.7 mg/ml TBA and 0.05 M HCL. The sample was incubated for 60 minutes at 95 ℃, then cooled to room temperature. The TBARM was extracted by butanol: methanol (85:15) and measured at 532 nm. When the inhibition effects of FRC001 were measured, different concentrations of FRC001 were added in the system. The scavenging effect was calculated as above.

Scavenging effect of FRC001 on oxygen free radicals generated from PMA stimulated PMN

Isolation of PMN: Fresh whole blood of healthy donor was purchased from Red Cross Blood Center of Beijing. PMN were separated from other cellular components by using 6% dextran sendimentation, hypersonic lysis of remained red cells and Ficoll density gradient centrifugation separation of mononuclear cells.

Production and measurement of active oxygen free radicals generated from PMN stimulated with PMA: In a typical experiment, a mixture containing 10^7 /ml PMN, 0.1 mM DETAPAC (diethylentriaminepentacetic acid) and 100 ng/ml PMA was incubated for 2 minutes at 37 ℃, then 0.1 M luminal was added and mixed homogeneously before measurement with BJL-chemiluminescence. When the scavenging effect of FRC001 was measured, different concentrations of FRC001 were added into the system. The scavenging effect was calculated as above.

Scavenging effect of FRC001 on $ONOO^-$

Peroxynitrite synthesis: Peroxynitrite was synthesized in a quenched flow reactor [12]. Solutions of (i) 0.6mol/L $NaNO_2$, and (ii) 0.6 mol/L HCL/0.7 mol/L H_2O_2 were pumped at 26 ml/min into a T-junction and mixed in a 3 mm diameter by 2.5 cm glass tube. The acid catalyzed reaction of nitrous acid with H_2O_2 to form peroxynitrous acid was quenched by pumping 15 mol/L NaOH at the same rate into a second T-junction at the end of the glass tube. Excess H_2O_2, was removed by passage over a 1 X5 cm column filled with 4g of granular MnO_2. The solution was frozen at -20 ℃ for as long as a week. Peroxynitrite tended to form a yellow top layer due to freeze fractionation, which was scraped for further studies. The concentration of peroxynitrite was determined by the absorbance at 302 nm in 1mol/L NaOH (E_{302nm}=1670 $mol^{-1}cm^{-1}$) Peroxynitrite can oxidize luminol and give a very strong chemiluminescence. The scavenging effect of FRC001 on peroxynitrite was measured by chemiluminescence method and the scavenging effect was calculated as above.

RESULTS and DISCUSSION

1. Scavenging effects of FRC001 on oxygen free radicals generated from xanthine/ xanthine oxidase system.

A standard curve of scavenging effect of SOD on free radicals generated from xanthine/ xanthine oxidase was shown in Figure 1.

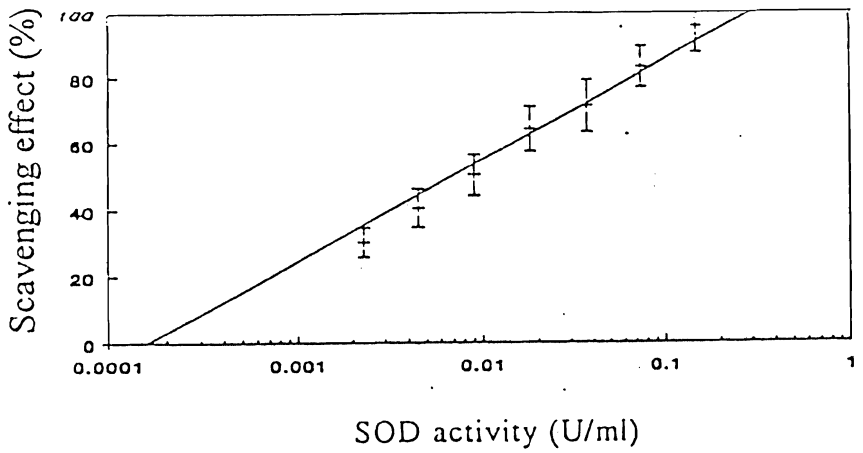


Fig.1. The standard curve of scavenging effect of SOD on oxygen free radical generated from the reaction of xanthine/xanthine oxidase system.

The scavenging effect of FRC001 on oxygen free radicals generated from the reaction of xanthine/xanthine oxidase system was shown in Figure 2.

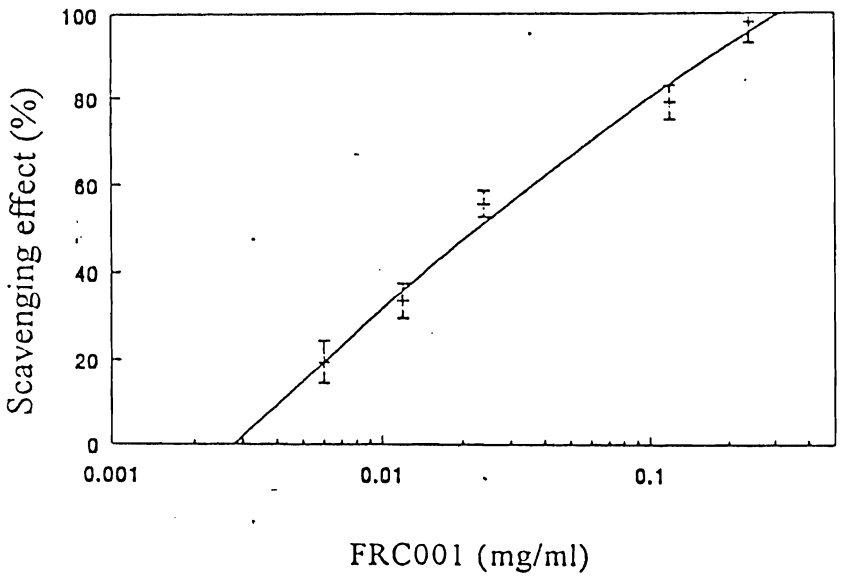


Fig 2 The scavenging effect of FRC001 on oxygen free radicals generated from xanthine/ xanthine oxidase system

From above two curves, the SOD activity included in 1g FRC001 is calculated and equal to 300.000U/ml.

2. Scavenging effects of FRC001 on oxygen free radicals generated from PMN stimulated with PMA.

When PMN are stimulated or they are in phagocytes.,there will be a respiratory burst and production of active oxygen free radicals. The active oxygen radicals produced in this process play an important role in microbicidal and tumorcidal processes and in protecting the healthy body from diseases. But if there are excess

active oxygen radicals in the body, they will damage the components of cells and even kill the normal cells and cause aging and very serious diseases, such as heart disease and cancer. Here it was used to examine effect of FRC001 on the oxygen free radicals. Figure 3 shows the scavenging effect of FRC001 on oxygen free radicals generated from PMA stimulated PMN measured by BJL-chemiluminescence. It was determined C_{50}=0.62 mg/ml which was smaller than that of Vitamin C (C_{50}=0.2mg/ml) and bigger than that of Vitamin E.

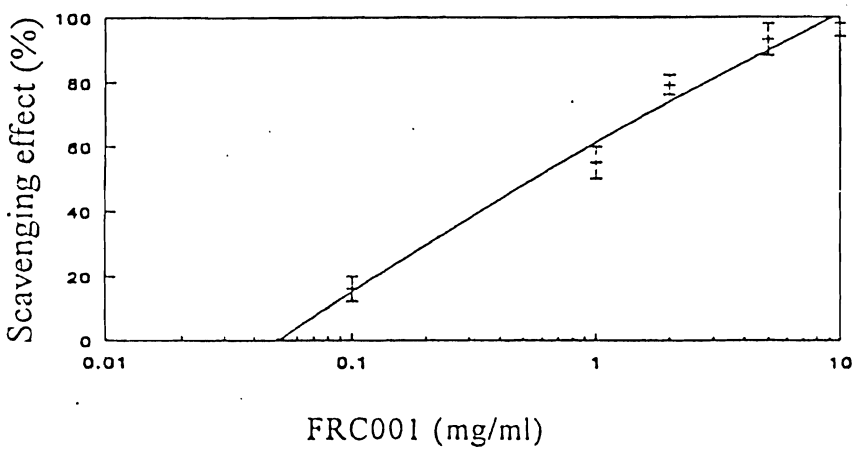


Fig. 3. Scavenging effect of FRC001 on oxygen free radicals generated from PMA stimulated PMN.

3. Scavenging effect of FRC001 on · OH free radicals generated from Fenton's Reaction

Fenton's Reaction can generate · OH and has been used for examination of scavengers of · OH free radicals

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$$

Here it was used for examination of the scavenging effect of FRC001 on · OH free radicals. The ESR spectrum of DMPO-OH was shown in Fig.4b (a_N=a_H=14.9 G). The scavenging effect of FRC001 on · OH was shows in Figure 5.

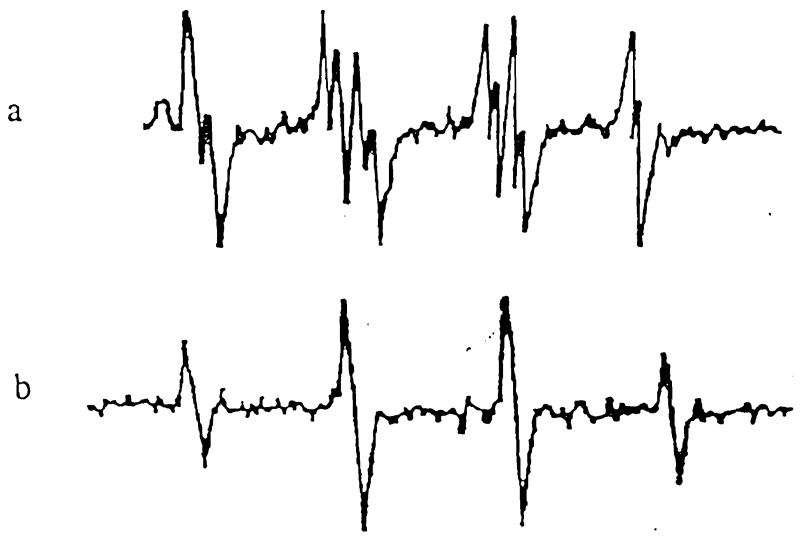


Fig.4.ESR spectra of DMPO spin trapped $\cdot O_2^-$ generated from irradiated riboflavin/EDTA system (a) and $\cdot$ OH free radicals generated froin Fenton rection (b).

The scavenging effect of FRC001 was shown in Figure 5 FRC001 can effectively scavenge the hydroxyl free radicals generated from Fenton Reaction but Vitamin C just has a little scavenging effect on the hydroxyl free radical Vitamin E only give a scavenging effect of 37.5% at the concentration of 5mg/ml.

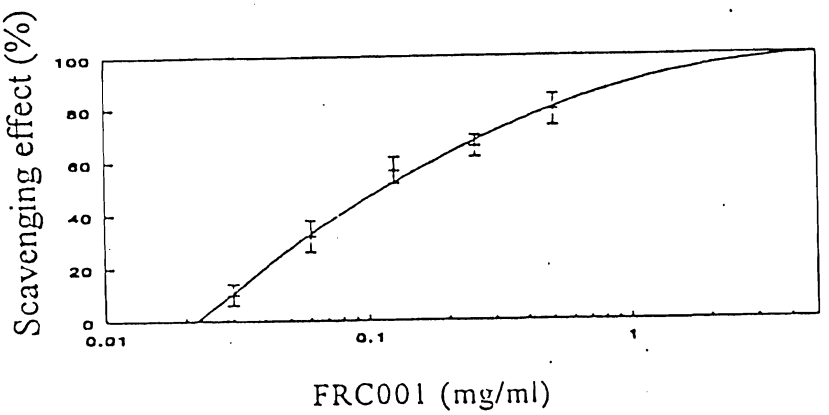


Fig.5. Scavenging effect of FRC001 on $\cdot$ OH free radicals generated from Fenton reaction

4. Scavenging effect of FRC001 on $\cdot O_2^-$ generated from irradiated riboflavin/EDTA system

Irradiated riboflavin/EDTA has been used for generation of $\cdot O_2^-$ and examination of scavenger of $\cdot O_2^-$ in photo system. Here it was used for examining the scavenging effect of FRC001 on $\cdot O_2^-$. The ESR spectrum of $\cdot O_2^-$ spin adducts DMPO-OOH generated from irradiated riboflavin was shows in Figure 4a (a_N=14.3, a_H^{β}=11.3G,a_H^{γ}=1.25G).

According to the definition of the scavenging effect, the curve of scavenging effect of FRC001 on $\cdot O_2^-$ generated from irradiated riboflavin/EDTA system was shown in Fig.6. The concentration of FRC001 for 50% scavenging is about 17mg/ml. Its scavenging effect is smaller than that of Vitamin C (C_{50}=0.0009 mg/ml) but stronger than that of Vitamin E.

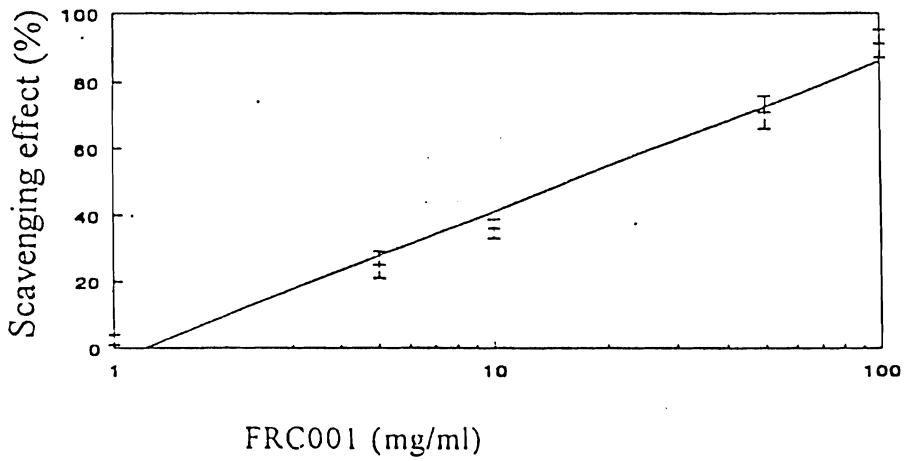


Fig.6. Scavenging effects of FRC001 on $\cdot O_2^-$ generated from irradiated riboflavin/EDTA system.

5. Inhibition effect of FRC001 on conjugated dienes generated from lipid peroxidation of Linoleic acid by lipoxidase

Conjugated dienes were generated at the first step of lipid peroxidation, which has a absorbance at 233nm. The inhibition effects of FRC001 on the generation of conjugated dienes from lipid peroxidation of linoleic acid catalyzed by lipoxidase were shown in Figure 7. From the curves, it could be found that the inhibition effects were increased with the concentrations of FRC001 added in the system. When the concentrations of FRC001 was 0.35mg/ml, about 20% of conjugated dienes was scavenged. When the concentration was increased, the absorbance at 233nm would increase, which disturbed the determination at high concentration.

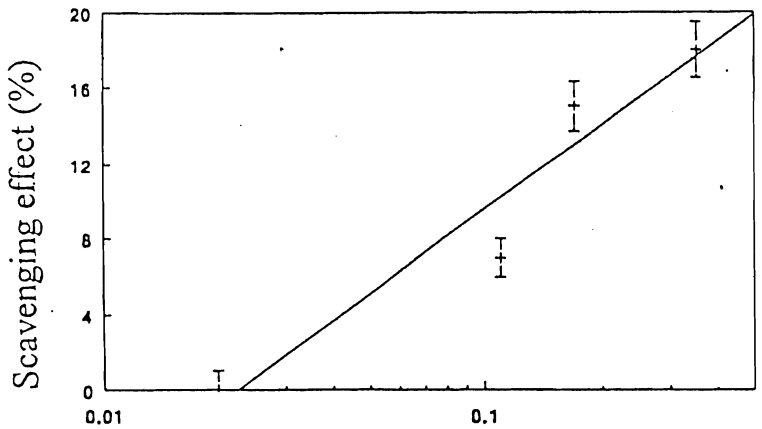


Fig.6. Inhibition effects of FRC001 on the generation of conjugated dienes from the lipid peroxidation of linoleic acid by lipoxidase.

6.Inhibition effects of FRC001 on TEA reacted materials (TBARM) generated from lipid peroxidation of liposome

The inhibition effects of FRC001 on TBA reaction materials generated from lipid peroxidation of liposome were shown in Figure 7. From the curve, it can be found that the inhibition effect on TBARM was increased with the increase of concentration of FRC001. The concentration of inhibition for 50% is about 19 mg/ml. Its inhibitory effect on TBA reaction materials is smaller than that of Vitamin C (C_{50}=1mg/ml) but larger than that of Vitamin E.

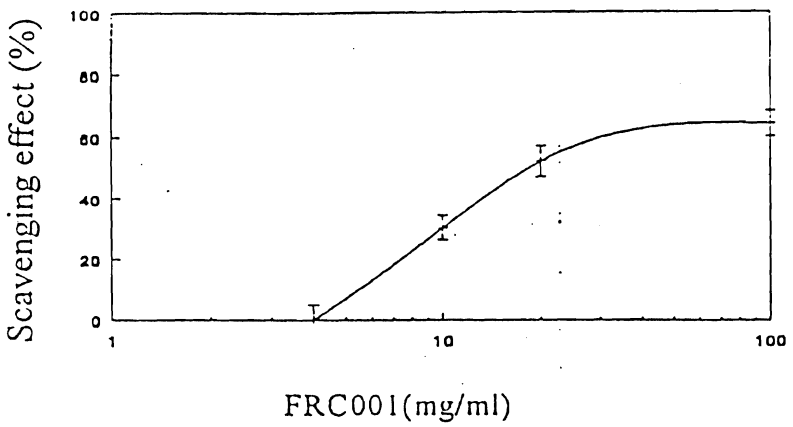


Fig.7 Inhibition effects of FRC001 on TBARM generated from lipid peroxidation of liposome initiated by Fe^{2+}

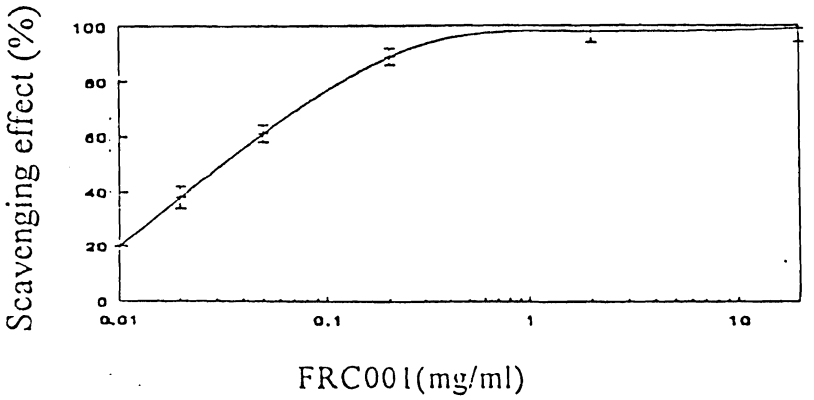
7. Scavenging effect of FRC001 on peroxynitrite

Nitric oxide has many biological functions, such as the endothelium-derived relaxing factor (EDRF), which can relax vascular smooth cell and inhibit platelet coagulation, and reverse messenger in neuron transmission. Biosynthesis of nitric oxide from L-arginine may be a pathway for the regulation of cell function and communication. Macrophages produce nitric oxide as part of their cytotoxic armamentarium. On the other hand, nitric oxide, which contains an unpaired electron, is a paramagnetic and active free radical, it can react with $\cdot O_2^-$ to form peroxynitrite anion ($ONOO^-$). In alkaline solutions, $ONOO^-$ is stable but has a pKa of 6.6 at 0 °C and decays rapidly once protonated, to give a species with hydroxyl radical-like and NO_2 free radicals respectively, according to the following reaction.

$$\cdot O_2^- + NO \rightarrow ONOO^- + H^+ \rightarrow ONOOH \rightarrow \cdot OH + NO_2$$

Recently, it is proposed that nitric oxide reacts with $\cdot O_2^-$ in many pathological

cases to yield cytotoxic species The investigation of peroxynitrite has been given much attention. Its oxidation of sulfhydryls and membrane lipid, which cause cell toxicity and some diseases. Here the scavenging effect of FRC001 on $ONOO^-$ was measured and it was showed in Figure 8 . It can be found that FRC001 could effectively scavenge $ONOO^-$ (C_{50}=0.03 mg/ ml). Its scavenging effect on peroxynitrite is smaller than that of Vitamin C (C_{50}=0.00003 mg/ml) but stronger than that of Vitamin E.



FRC001 is a drug designed on the basis of the theory of scavenging free radicals. From above experiment results, it can be found that FRC001 can effectively scavenge the oxygen free radicals generated from PMN stimulated with PMA xanthine/xanthine oxidase, irradiation riboflavin/EDTA and Fenton's Reaction. It was also found that it could inhibit the conjugated dienes and TBA reacted materials (TBARM) formed during lipid peroxidation of linoleic acid and liposome respectively and scavenge $ONOO^-$. These results suggest that the therapy effect of FRC001 on diseases maybe pass through the pathway of scavenging toxic oxygen free radicals in human body.

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