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Collagen Peptides Protects Human Hepatocytes Against Hydrogen Peroxide (H_2O_2)-induced Oxidative Stress Damage:Involvement of the Nrf2 Transcription Factor*

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ABSTRACT Objective: Oxidative-stress damage has been proved to play an important role in the liver injury. Collagen Peptides are the natural substance and they are also antioxidant that reduce oxidative stress in animals. Recent years Collagen Peptides have been proposed for treatment of liver injury, but there are few studies about the molecular mechanism of the protective effect. This study was to investigate whether Collagen Peptides against hydrogen peroxide (H_2O_2)-induced oxidative stress damage in a normal human hepatocyte cell line, and explore the mechanism of the protective effect of Collagen Peptides. **Methods:** Five groups including control, H_2O_2 model, Collagen Peptides 10 μg/ml, Collagen Peptides 100 μg/ml, Collagen Peptides 200 μg/ml groups were set up in this study. Cells were cultured with blank control medium or Collagen Peptides of the fixed dosages for 12 h, and then were stimulated with 300 μM H_2O_2 for 12 h.CCK8 assay and lactate dehydrogenase (LDH) release were analyzed cytotoxicity. Superoxide dismutase (SOD), catalase (CAT), reactive oxygen species (ROS) levels, and malondialdehyde (MDA) formation were estimated antioxidant activity and lipid peroxidation. The expression levels of Nrf2 in HL7702 cells were measured by Western blot. **Results:** The results showed that Collagen Peptides protected HL7702 cells against H_2O_2-induced oxidative stress damage. The mechanism of the protective effect of Collagen Peptides was correlated with the promotion of SOD and CAT activity, the scavenging of ROS, and the prevention of lipid peroxidation. **Conclusions:** In conclusion, the present study proved that Collagen Peptides protected HL7702 cells against H_2O_2-induced oxidative stress damage through enabling Nrf2 expression and the antioxidative effect. These results will provide new insight to the molecular mechanism of Collagen Peptides, which will be benefit for preventing oxidative stress-induced liver injury.

Key words: Collagen Peptides; Hydrogen peroxide; HL7702 cells; Antioxidant enzymes; Nrf2

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Introduction

Modern scientific research confirmed that reactive oxygen species(ROS) plays an important role in the process of hepatic diseases. Oxidative stress which are able to cause cell damages quickly as the accumulation of reactive oxygen species (ROS), breaking the balance of intracellular oxidation and anti-oxidation [1]. H_2O_2 is the major source of intracellular ROS. Since its chemical character is very active, H_2O_2 is prone to flowing into or out of the cells, which makes it possible to be the ideal inducer in oxidative-stress damage[2-5].

Previous studies have confirmed that antioxidant therapy appears as the most reasonable treatment of a variety of liver diseases [6]. There are various antioxidant that have shown to be useful to reduce oxidative stress in hepatocytes, such as curcumin, L-carnitine, S-adenosylmethionine, and vitamin E [7-9]. Collagen Peptides have been used for treatment of liver injury. Lv et al. showed that Collagen peptides protect against thioacetamide-induced hepatic fibrosis in rats [10]. And Li et al. determined that Collagen peptide alleviated alcohol-induced liver damage in NH mice [11]. In addition, Collagen Peptide has protective effect of on liver in diabetic rats induced by alloxan[12]. Recently, Lin et al. found that Collagen Peptides have a protective effect on early alcoholic liver injury in rats by their antioxidative activity [13]. Thus, antioxidant activity of Collagen Peptides have potential as a therapeutic target for hepatic disease.

Nrf2 belongs to the family of leucine zipper transcription factors that interact with Keap1 and resistance to oxidative stress[14]. Under normal circumstances, Nrf2 are suppressed by Keap1. When oxidative stress events occur, Nrf2 is activated through disentangling from Keap1, then starts the antioxidant response element and increases expression of some genes encoding antioxidant enzymes against oxidative stress [15-17]. Nrf2-Keap1 perform important role in the protective mechanism of liver injury, fatty liver, liver fibrosis, and so on. Previous study conformed that Nrf2-deficient mice delay wound-healing response to CCl_4-induved liver in-

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jury significantly and Nrf2-deficient mice aggravate the inflammatory response and liver fibrosis [18]. Rubiolo et al reported that Resveratrol increases the level of Nrf2 to protect hepatocytes against oxidative stress damage [19]. Hence, we speculate that Nrf2 may be as intracellular target the hepatoprotective effect of Collagen Peptides.

Collagen Peptides have been used for treatment of liver injury in animal experiments, but there are few studies about the molecular mechanism of the protective effect. Therefore, the study undertaken was to determine whether Collagen Peptides exerts cytoprotective effect against ROS induced cell death and Nrf2 may have protective effect on oxidative stress-induced liver injury. Normal human hepatocyte HL7702 was treated by H_2O_2, which was prone to flowing into or out of the cells and has been extensively the ideal inducer in oxidative-stress models.

1 Materials and methods

1.1 Reagents and drugs

H_2O_2 and 2',7'-dichlorfluorescein-diacetate (DCFH-DA) were purchased from sigma-Aldrich (St Louis, MO, USA). CCK8 were purchased from Dojindo Laboratories (Kumamot, Japan). Lactate dehydrogenase assay (LDH) kit, Superoxide dismutase (SOD), catalase (CAT), and malonaldehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit polyclonal antibody against Nrf2 was purchased from Abcam. Beta-actin antibody was purchased from Beijing Biosynthesis Biotechnology (Beijing, China).

1.2 Cells culture and treatment

Human normal hepatocyte cell line HL7702 was purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in RPMI 1640 medium (15% FBS), penicillin (100 units/ml) and streptomycin (100 mg/ml) and maintained at 37 ℃ in 5% CO_2 incubator. Cells were seeded in 60 mm plates until they enter the logarithmic growth phase and fusion up to 70-80%. Then cells were pretreated with different concentrations of Collagen Peptides for 12 h. After the completion of pretreatment, 300 μM H_2O_2 was added for 12 h. Control cells were cultured in RPMI 1640 medium without H_2O_2.

1.3 Cell viability analysis

CCK8 assay was analyzed cell viability. HL7702 cells were cultured in 96-well plates (5×10^3 cells/well). Collagen Peptides at different concentrations (10 ~10000 μg/ml) was added and pretreateded for 12 h. After H_2O_2 treatment for 12 h, medium was replaced with fresh medium containing 10% CCK8, then cells continued to be incubated at 37℃ for 3 h. The plate was read using a microplate reader at a wavelength of 450 nm.

1.4 Measurement of lactate dehydrogenase (LDH) release

Cells were seeded in 24-well plates until they enter the logarithmic growth phase and fusion up to 70-80%. Pretreatment with collagen Peptides (10, 100, and 200 μg/ml) were for 12 h, then H_2O_2 exposer for 12 h. Intracellular LDH activity was measured by using a Lactate dehydrogenase assay (LDH) kit according to the manufacturer's instructions. LDH activity was detected by automatic biochemical analyzer with its commercial bio-kit at 340 nm.

1.5 Measurement of superoxide dismutase (SOD), catalase (CAT) activity and malondialdehyde (MDA) level

The total SOD, CAT activities and MDA levels were measured using assay kits according to manufacturer's instructions. Then the total of SOD, CAT, and MDA levels were measured by Multiskan Spectrum. The protein content of the supernatant was determined using coomassie blue staining.

1.6 Intracellular reactive oxygen species (ROS) measurement

After H_2O_2 treatment, cells were harvested by centrifugation, washed with cold serum-free medium for three times and collected by centrifugation, resuspended in serum-free medium containing 10 μM DCFH-DA, incubated for 30 min at 37℃. Washed with cold PBS for three times, resuspended in PBS. DCF fluorescence was examined with fluorescence microscope (Olympus, made in Japan.). The fluorescence intensity (relative fluorescence units) was measured at 485 nm excitation and 530 nm emission in a Fluorescence Spectrometer.

1.7 Western blot

Cells were harvested, washed with ice-cold PBS three times, and lysed in lysis buffer (Solarbio, Beijing, China) at 4 ℃ for 60 min. Cell lysates were collected by centrifugation (12000× g, 10 min, 4 ℃). Protein were subjected to SDS-PAGE electrophoresis, and transferred to PVDF membranes (Millipore Corporation, Bedford, MA). The blots were probed with primary antibodies in fat-free milk, secondary antibodies in PBST for one hour. Western blots were then visualized with the enhanced chemiluminescence (ECL) detection system (Detail) and quantified with Bio-Rad TDS Quantity One Software.

1.8 Statistical analysis

All data were expressed as mean ± standard derivation. Statistical analysis was performed with SPSS 17.0. Statistical significance was determined by the Student t-test or by analysis of variance followed by Dunnett's multiple comparison test as appropriate. Statistical significance was determined when P-values were less than 0.05.

2 Results

2.1 Inhibitory effects of Collagen Peptides on HL7702 cells cytotoxicity induced by H_2O_2

CCK8 were used to measure cell viability. As shown in the upper curve in Figure 1, co-cultured with Collagen Peptides, concentrations ranging from 10 to 10000 μg/ml, cell growth were not be inhibited, but at a higher concentration of 100000 μg/ml, Collagen Peptides were observed significant inhibitory effect($P< 0.05$). This result suggests the toxicity of Collagen Peptides can happen

above100000 μg/ml.

The lower curve in Figure 1 showed that, cells were signal treated 300 μM H_2O_2 for 12 h, HL7702 cells displayed significantly decreased viability compared to normal group. Concentration of Collagen Peptides ranging from 10 to10000 μg/ml, improved the cell viability significantly. Concentration of Collagen Peptides ranging from10 to 200 μg/ml, Collagen Peptides showed a dose response relationship. Therefore, we select 10, 100, 200 μg/ml Collagen Peptides for our following experiments.

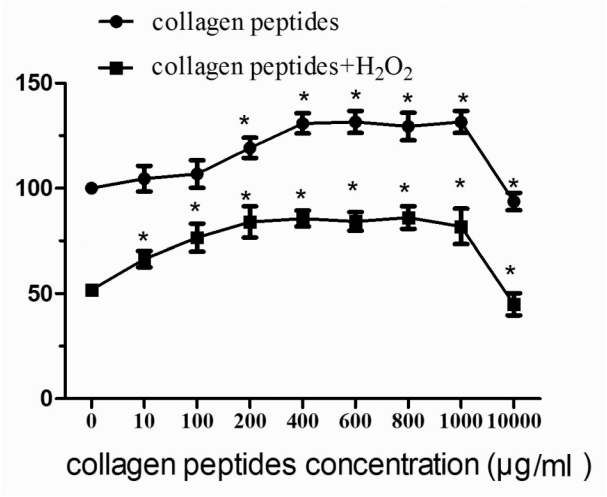

Fig. 1 The cytotoxicity of Collagen Peptides and its protective effect on H_2O_2-induced toxicity in HL7702 Cells

Note: The upper curve: HL7702 cells were incubated with Collagen Peptides for 24 h. The lower curve: Cells were treated with or without Collagen Peptides prior to H_2O_2 challenge (300 μM, 12 h). Cell viability was assayed by CCK8 assay. The percentage of cell viability in the control group was treated as 100%. Each value represents the mean of three replicates. *P< 0.05 vs. control; *P< 0.05, vs. H_2O_2 alone.

2.2 Collagen Peptides inhibited H_2O_2-induced LDH release in HL7702 cells

LDH activity can be used as an indicator of cell membrane integrity. As Figure 2A, H_2O_2 induced LDH released significantly compared with control cells (P<0.05). Pretreatment with 10~200 μ g/ml Collagen Peptides reduced H_2O_2-induced LDH release in a dose-dependent pattern.

2.3 Collagen Peptides increased SOD, CAT activities against HL7702 cells oxidative stress induced by H_2O_2

SOD, CAT activities were measured to examine the antioxidative effect of Collagen Peptides on HL7702 cells damaged by H_2O_2. As Figure 2, compared to the control group, the activities of total SOD and CAT in the H_2O_2 treated-group were remarkably decreased (P<0.05). Compared with the H_2O_2 treated-group, 10~200 μg/ml Collagen Peptides reversed the activities of SOD and CAT (P<0.05).

2.4 Collagen Peptides inhibited lipid peroxidation in HL7702 cells induced by H_2O_2

Malondialdehyde (MDA) concentration is a widely used method to analyse lipid peroxidation. As shown in Figure 2D, compared to the control group, the levels of MDA in H_2O_2 group were increased significantly. The levels of MDA among the three Collagen Peptides treated group reduced in a dose-dependent pattern.

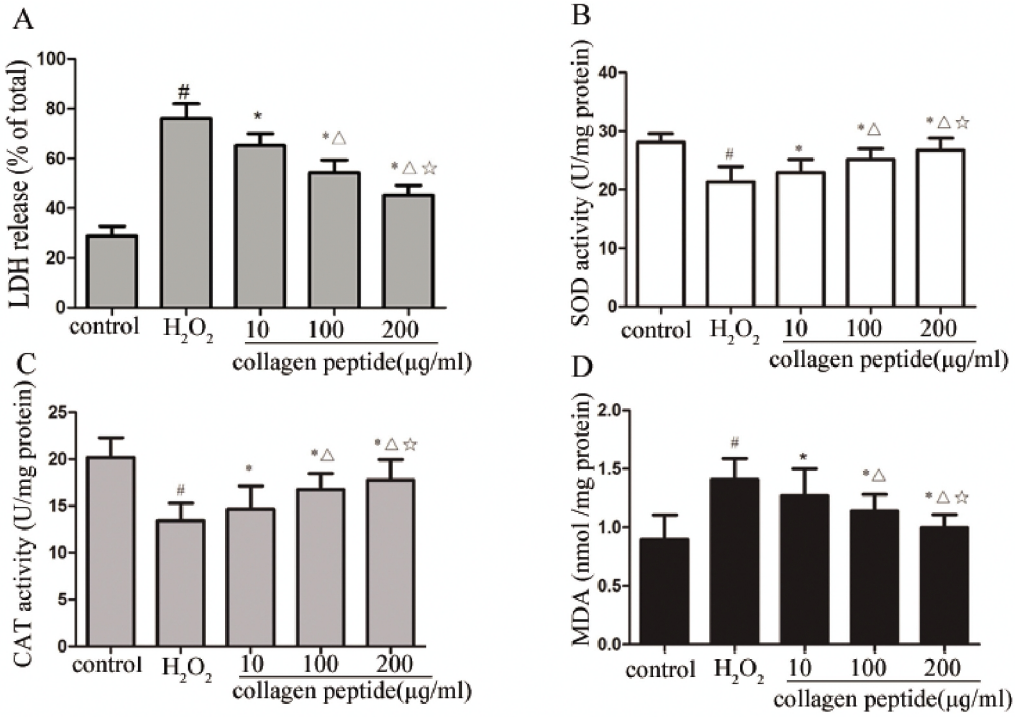

Fig. 2 Effect of Collagen Peptides on the LDH release, the activities SOD and CAT, and MDA formation in H_2O_2-damaged HL7702 cells

Note: Cells were treated with Collagen Peptides for 12 h and followed by the treatment of H_2O_2 (300 μM) for 12 h. A: LDH release represents the percentage of LDH in the culture medium relative to the total LDH. B: Total SOD activities were calculated in units of activity per mg of total protein. C: Total CAT activities were calculated in units of activity per mg of total protein. D: MDA formation in HL7702 cells. Each value represents the mean of three replicates. #P< 0.01 vs. control; *P < 0.05 vs. H_2O_2 alone; △P< 0.05 vs 10 μg/ml; ☆P < 0.05 vs 100 μg/ml

2.5 Collagen Peptides inhibited H_2O_2-induced ROS production in HL7702 cells

The levels of intracellular ROS are proportional to DCF fluorescence intensity. As shown in Figure 3 and Figure 4, the ROS were generated from HL7702 cells during H_2O_2 treated, while the three Collagen Peptides inhibited H_2O_2-induced ROS production.

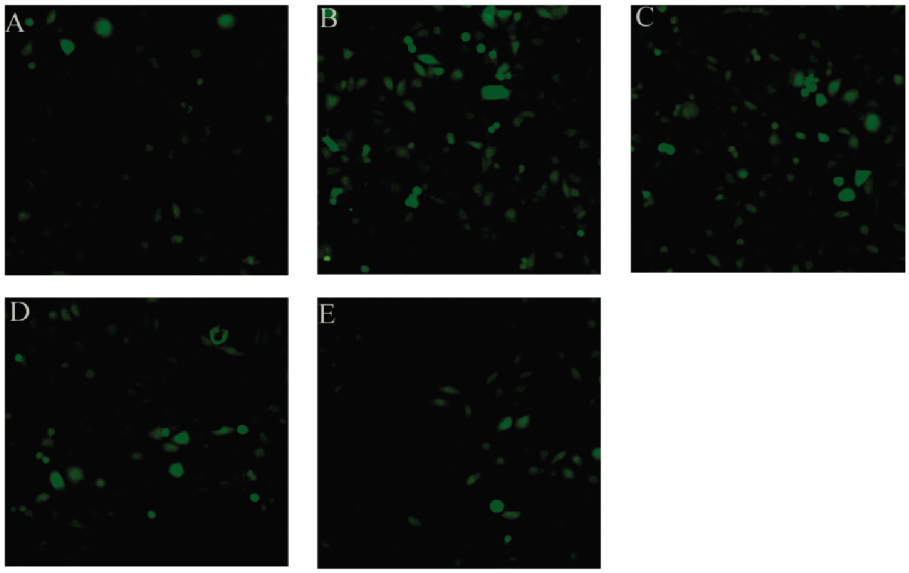

Fig. 3 Effect of Collagen Peptides e on intracellular ROS levels after H_2O_2 exposure in HL7702 cells(× 200)

Note: A: Control group; B: H_2O_2 group; C: H_2O_2+10 μg/ml Collagen Peptides; D: H_2O_2+100 μg/ml Collagen Peptides; E: H_2O_2+200 μg/ml Collagen Peptides.

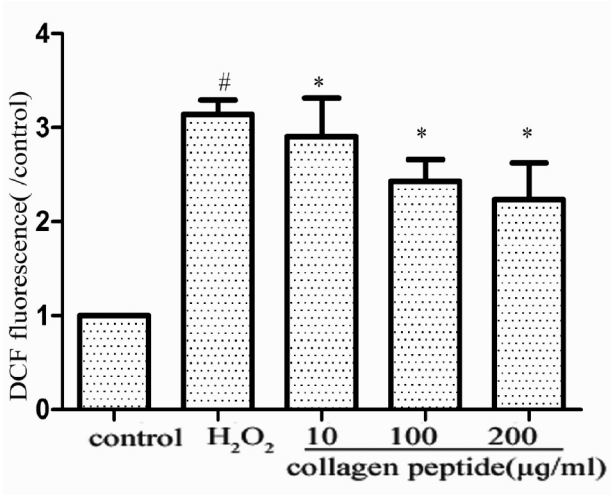

Fig. 4 Effect of Collagen Peptides e on intracellular ROS levels after H_2O_2 exposure in HL7702 cells

Note: ROS levels were measured using fluorescent probe DCFH-DA. Each value represents the mean of three replicates. #P< 0.01 vs. control; *P < 0.05 vs. H_2O_2 alone.

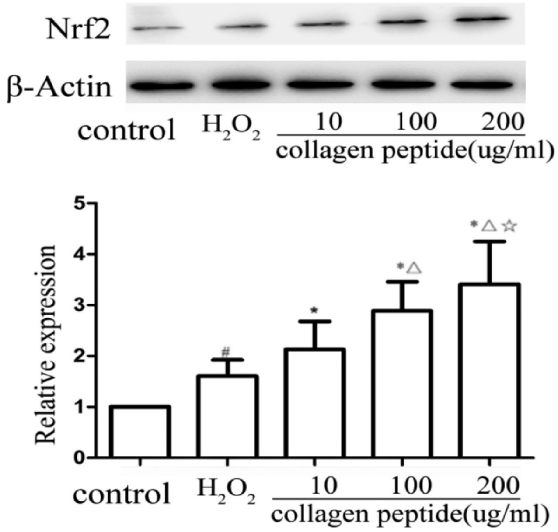

Fig. 5 Effect of Collagen Peptides on expression levels of Nrf2 in HL7702 cells

Note: The expression levels of Nrf2 in HL7702 cells were measured by Western blot. Each value represents the mean of three replicates. #P < 0.01 vs. control; *P<0.05 vs. H_2O_2 alone.

2.6 Effect of Collagen Peptides on expression levels of Nrf2 in HL7702 cells

The expression levels of Nrf2 in HL7702 cells are measured by Western blot. As shown in Figure 5, compared to the control group, the levels of protein Nrf2 in H_2O_2 group were increased significantly. Compared to H_2O_2 group, the levels of Nrf2 among the three Collagen Peptides treated group increased in a dose-dependent pattern.

3 Discussions

Since its chemical character is very active, H_2O_2 is prone to flowing into or out of the cells, which makes it possible to be the ideal inducer in oxidative-stress damage [20]. Cytotoxicity which is caused by a cell or chemical substance can lead to cell killing. Cytotoxicity test is mainly based on the cell membrane permeability change. There are several ways to detect Cytotoxicity, such as

cck8, XTT, and LDH, etc. CCK8 were used to measure cell viability. LDH activity which exists in the cell culture supernatant can determine the extent of cell damage. Cytotoxicity test be used to conduct drug screen. Our results indicated Collagen Peptides at 10~10000 μg/ml is conducive to the growth of HL7702 cells. However, cell growth was inhibited when Collagen Peptides of concentration was higher than 100000 μg/ml. Our results also demonstrated that Collagen Peptides at 10~10000 μg/ml had significant inhibition cells damage induced by H_2O_2. Collagen Peptides at 10~200 μg/ml had significant inhibition releases of LDH in a dose-dependent manner. Above all, these experimental results suggest that Collagen Peptides are able to reduce H_2O_2-induced cytotoxicity in HL7702 cells.

Previous study confirms that H_2O_2 exposure could increase intracellular reactive oxygen species (ROS)[20]. Excessive ROS production could break the balance of intracellular oxidation and anti-oxidation, which lead to oxidative damage[21]. In order to further study the mechanism of the protective effect of Collagen Peptides, the levels of intracellular ROS was examined. Compared to control group, the level of intracellular ROS is higher in H_2O_2 exposure group. However, pretreatment with Collagen Peptides led to reduced ROS levels in a dose-dependent pattern. The abnormal formation of ROS includes many endogenous defense mechanisms [22]. For example, enzymes such as SOD and CAT can scavenge ROS [23,24]. Li et al. reported that SOD and CAT involved in H_2O_2-oxidative stress damage [8]. Thus, we found that the inhibited activities of SOD and CAT by H_2O_2, but the activities of SOD and CAT were recovered by Collagen Peptides. These founding suggested that Collagen Peptides could protect HL7702 cells from H_2O_2-induced cell damage by its antioxidant functions. Collagen peptide can increase the activity of intracellular oxidative defense systems, scavenge oxygen radical, which protects against oxidative stress damage in HL7702 cells.

ROS generate the lipid peroxidation process in an organism [25]. MDA, as an end product of lipid peroxidation in the cells, is commonly known as a marker of oxidative stress[25]. Previous studies showed that elevation of MDA caused by pathological conditions were prevented by Collagen Peptides [9,26]. We observed that H_2O_2 exposure increased MDA level in HL7702 cells. However, pretreatment with Collagen Peptides reduced the levels of MDA.

H_2O_2, due to its small molecular weight, allows easy entry into cells to starting off lipid peroxidation. Then the combined effect of both H_2O_2 and cytokines cause the damage of the cell through the oxidative stress. Previous study confirmed that Nrf2 played an important role in the regulation of lipid peroxidation, such as acetaminophen-induced hepatic injury, or dimethylnitrosamine- induced hepatic injury[27]. In our experiments, we discovered that 300 umol/L H_2O_2 treated HL7702 cells for 12 h, with the result that increased the expression level of Nrf2. H_2O_2 -induced oxi dative stress unwinds Nrf2 from Keap1, thereby enabling gene activity. Compared with H_2O_2 group, Collagen peptide pretreatment for 12 h unwind Nrf2 from Keap1 significantly, thereby enabling Nrf2 expression. It has been reported that the transcription factor Nrf2 regulates the expression of antioxidase system, such as SOD, CAT [28]. From the results, we hypothesized that Collagen peptide might enabling Nrf2 activity, and then activate SOD and CAT, thereby inhibit hepatic cells damage induced by H_2O_2.

In conclusion, the present study proved that Collagen Peptides prevented HL7702 oxidative stress induced by H_2O_2. Collagen Peptides increased in membrane integrity through reduction in the degree of lipid peroxidation. Our study confirmed that Collagen Peptides extract have protective effects of were found with the up regulation ability of ROS scavenging enzymes and Nrf2 activity. The mechanism of how Collagen Peptides up-regulate the expression of Nrf2 is unclear. An increasing number of studies have found that Nrf2 has emerged as a potential therapeutic target in liver disease. Pharmaceutical research based on Nrf2 has been paying more and more attention. In view of the liver protection of Collagen Peptides, researchers believe that Nrf2-targeted medicine is promising for medical applications.

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胶原蛋白肽经由 Nrf2 信号通路对 H_2O_2 诱导的肝细胞氧化损伤的保护作用*

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摘要 目的:氧化应激在肝脏疾病中扮演着重要的角色。胶原蛋白肽是天然的抗氧化剂,其在动物实验中已经被证实有抑制氧化应激的作用。最新研究证实胶原蛋白肽将有可能被应用在肝脏疾病的预防中,但是很少有研究报道其分子作用机制。因此本研究在胶原蛋白肽是对 H_2O_2 诱导的正常人的肝细胞系 HL7702 氧化损伤有保护作用的基础上,并探索其分子作用机制。**方法:**实验设空白对照组,H_2O_2 模型组,胶原蛋白肽低、中、高剂量组(10, 100, 200 μg/ml)。胶原蛋白肽各组加入相应浓度的药物预处理 12 h 后,与模型组一起加入 300 μM H_2O_2 的 H_2O_2 共同培养 12 h ,空白对照组正常培养。细胞毒性是由 CCK8 和乳酸脱氢酶(LDH)的释放检测。抗氧化试剂盒检测细胞内活性氧的水平,超氧化物歧化酶(SOD)、过氧化氢酶(CAT)活性和丙二醛(MDA)含量的变化。Western blot 检测细胞内 Nrf2 蛋白的表达水平。**结果:** 胶原蛋白肽对 H_2O_2 诱导的正常人的肝细胞系 HL7702 氧化损伤有保护作用。胶原蛋白肽能够及时清除细胞内的活性氧,增加 Nrf2 的蛋白表达水平,提高超氧化物歧化酶(SOD)、过氧化氢酶(CAT)的活性,减轻脂质过氧化反应,从而保护正常人的肝细胞系 HL7702。**结论:**总之,胶原蛋白肽通过增加 Nrf2 的蛋白表达水平,提高抗氧化活性,对 H_2O_2 诱导损伤的肝细胞发挥保护作用。本研究为胶原蛋白肽的分子作用机制提供了新的证据,将有助于预防氧化应激所致的肝损伤。

关键词:胶原蛋白肽;过氧化氢;HL7702;抗氧化酶;Nrf2

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