

# EFFECTS OF CURCUMINOIDS EXTRACTS CAPSULE ON ANTIOXIDANT ENZYMES IN LEBER'S HEREDITARY OPTIC NEUROPATHY

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**ABSTRACT:** Leber's hereditary optic neuropathy (LHON) is a mitochondria genetic disease which involves genes encoding complex I subunit of respiratory chain. LHON mutation shows consistent reduction in complex I electron transfer activity. Finally, reactive oxygen species (ROS) increase resulting in optic nerve degeneration and blindness. The aim of this study was to investigate the oxidative stress status and antioxidant activities in blood circulation of LHON patients before and after administration of curcuminoids extracts capsules. Thirty two LHON patients participated in this randomized, double blind, placebo-control study. Patients received placebo or 250 mg of curcuminoids extracts capsules twice a day for 12 months. Malondialdehyde (MDA) levels in plasma and antioxidant enzymes activities in red blood cells were measured at 0, 3, 6 and 12 month. MDA levels were in normal range. In curcuminoids group, the activities of enzyme glutathione peroxidase at 3 and 6 month decreased 24.1 % and 12.6 % ( $p < 0.05$ ) respectively when compare to baseline. In addition, the activities of superoxide dismutase at 3 month were decreased 12.6 % ( $p < 0.05$ ) from baseline. We can conclude that oxidative stress was not found in blood circulation of LHON patients. The use of curcuminoids extracts capsules in LHON patients may scavenge the reactive oxygen species resulting in the reduction of enzyme glutathione peroxidase and superoxide dismutase activities.

**Keywords:** Leber's hereditary optic neuropathy, Curcuminoids, Malondialdehyde, Antioxidant enzymes

## INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is a mitochondria genetic disease and affected remain asymptomatic until they begin with clouding or blurring of central vision in one eye. The other eye can be affected either simultaneously or sequentially. The characteristic field defect in LHON is a centrocaecal scotoma but papillary reflexes are preserved and report no pain on eye movement. All patients with LHON showed mitochondrial dysfunction in complex I. Over 95% of LHON cases are the result of one of three mitochondrial DNA (mtDNA) point mutations, G11778A, G3460A and T14484C, which all affect genes encoding complex I subunits of mitochondrial respiratory chain [1], cause overproduction of reactive oxygen species (ROS), and resulting in retinal ganglion cell degeneration in LHON [2]. There are substantial evidences supporting ROS as a key role in the pathogenesis of LHON. The decrease in antioxidant defense including glutathione peroxidase (GPx) and Mn-superoxide dismutase (MnSOD) in cells carrying

LHON mutations was especially evident in cells harboring 3460 and 11778 mutations, which are associated with the most severe clinical phenotype. Some *in vitro* study showed fibroblasts from LHON have over production of ROS [3]. Patients with LHON and asymptomatic carriers were found to have a decrease of  $\alpha$ -tocopherol/lipid ratio in their plasma [4]. Despite knowledge in clinical, genetic and pathogenesis are revealed, no treatment of LHON has proven effective. Since oxidative stress is one of important mechanism leading to optic nerve degeneration in LHON patients, the antioxidant may be a possible potential treatment to improve visual outcome in LHON [5].

Curcuminoids, a group of principle yellow pigments isolated from turmeric (*Curcuma longa* Linn), is known as a potent antioxidant comparable to  $\alpha$ -tocopherol. Its antioxidative activities have been studied in several *in vitro* models [6]. There also have been many studies about the use of curcuminoids for treating various diseases associated to oxidative stress such as thalassemia [7], Parkinson's disease [8] and various proinflammatory diseases [9]. Regarding to the safety of curcuminoids in animals, it was confirmed a lack of significant toxicity [10].

The aim of this study was to investigate the effect

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of curcuminoids capsules in LHON patients with 11778 mutation on oxidative stress status and endogenous antioxidant enzyme activities in red blood cell, such as catalase (CAT), superoxide dismutase (SOD), total glutathione (GSH), oxidized glutathione (GSSG) and glutathione peroxidase (GPx).

## MATERIALS AND METHODS

Thirty-two Leber's hereditary optic neuropathy patients with G11778A DNA mutation were recruited from the neuro-ophthalmology clinic. The patients had to discontinue any antioxidant substances such as coenzyme Q10, vitamin B, vitamin C, vitamin E and steroid for at least one month before starting the intervention. This is a randomized, double blind, placebo-control study. The protocol was reviewed and approved by the Ethical Committee on research involving human subject, Faculty of Medicine, Siriraj Hospital, Mahidol University. Patients were asked not to take any medication for their vision at least 1 month before the beginning of the study. They received placebo or 250 mg of curcuminoids extracts capsules twice a day for 12 months. The antioxidant enzymes were monitored at 0, 3, 6 and 12 months.

### Blood samples and erythrolysates

Ten ml of venous peripheral blood was collected into EDTA vacutainer tubes. The plasma was separated by centrifugation at 1200 g for 15 min. Plasma was collected for detection of malondialdehyde level (MDA). Then, the buffy coat was discharged and the remaining erythrocytes were washed and centrifuged repeatedly three times with 0.9% normal saline (1,200 g at 4 °C for 15 min). Finally, a known volume of erythrocytes was lysated with equal volume of cold pure water.

### Determination of plasma MDA levels

One ml of diluted plasma or standard solution was mixed with 50 µl of 100 µM butylated hydroxytoluene (BHT) to inhibit lipid peroxidation occurring during the process, then 1.0 ml of 10% trichloroacetic acid was added and mixed for 1 min. A 0.5 ml of 5 mM EDTA, 0.5 ml of 8% sodium dodecyl sulfate and 1.5 ml of 0.6% thiobarbituric acid, were added and vortexed, respectively. The mixture reaction was boiled in water bath at 95 °C for 1 hr. After cooling, 4 ml of butanol was added. The mixture was vigorously vortexed for 1 min then centrifuged at 1,500 g for 10 min. Fluorescence of the butanol extracts was measured at an excitation wavelength of 515 nm and an emission wavelength of 553 nm [11].

### Determination of Catalase activity

The determination of catalase activity was performed by a spectrophotometric assay based on the catalyzed decomposition of H<sub>2</sub>O<sub>2</sub> [12]. To determine the catalase activity, 3 ml of 10 mM H<sub>2</sub>O<sub>2</sub> in 50 mM

potassium phosphate buffer was pre-warmed in cuvet at 25°C for 5 min. Then add 20 µl of hemolysate and record the change in absorbance at 240 nm between 30 and 210 sec. Catalase activities are expressed as KU/g Hb. One unit of activity is defined arbitrarily as the amount of enzyme which induces a change in A<sub>240</sub> of 0.43 during the 3 min incubation.

### Determination of Glutathione Peroxidase activity

The formation of GSSG catalyzed by GPx is coupled to the recycling of GSSG back to GSH using glutathione reductase. NADPH is oxidized to NADP<sup>+</sup>. The change in A<sub>340</sub> due to NADPH oxidation was monitored and was indicative of GPx activity. The reaction mixture consisted of 5 mM ethylene diamine tetraacetic acid-Na salt (EDTA), 0.1 M glutathione (GSH), 10 unit/ml glutathione reductase (GSH-Rd), Tris-HCl buffer pH 8.0. The hemolysate was added to the mixture and allowed to incubate for 5 min at 37°C. The enzyme reaction was initiated by the addition of 7 mM cumene hydroperoxide that was used as the peroxide substrate (ROOH). The rate of decrease in the absorbance at 340 nm was measured. Erythrocyte GPx activities were expressed as U/g Hb [13].

### Determination of total Glutathione

Determination of total glutathione was based on the GSH recycling method [14]. In this reaction GSH reacts with 5, 5'-dithiobis, 2-nitrobenzoic acid (DTNB) to form disulfide GSTNB and the yellow colored compound, 5-thio-2-nitrobenzoic acid (TNB). The disulfide product (GSTNB) is then reduced by glutathione reductase in the presence of NADPH. The rate of TNB formation was followed at 405 nm and was proportional to the concentration of GSH in the sample. Preparation of sample by adding 0.5 ml of 4% sulfosalicylic into 0.5 ml of the hemolysate, then centrifuging at 12,000 rpm for 15 min at 4°C. Diluted sample (20 µl), was transferred to 96-well microplate. Then add 80 µl of 0.01 sodium phosphate buffer with 1 mM EDTA pH 7.5. Subsequently, 100 µl of reaction mixture containing 1 mM of DTNB, 0.5 mM of NADPH, and 1 iu of GSH reductase dissolved in 0.01 M of Sodium Phosphate buffer with 1 mM of EDTA pH 7.5, was added immediately, color development was recorded at 405 nm for 4 min.

### Determination of oxidized Glutathione

GSSG was determined with the same method of total glutathione, but free GSH must be masked first by 1-methyl-2-vinylpyridinium trifluoromethane sulfonate (M2VP) [14].

### Determination of Superoxide Dismutases

SOD activity was measured in hemolysate using a modified method based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT)

by superoxide anions generated by xanthine and xanthine oxidase reaction [15]. Briefly, 5 µl of diluted hemolysate was added to 170 µl of mixed substrate (0.05 M xanthine, 0.025 M NBT, in 50 mM sodium carbonate buffer with EDTA pH 10.2), then reaction was started with 25 µl xanthine oxidase (60 unit/L). Immediately record a change of absorbance at 560 nm every 30 sec for 4 min.

### Determination of Hemoglobin

This procedure is based on the oxidation of hemoglobin and its derivatives to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin which has a maximum absorption at 540 nm. Determine the total hemoglobin concentration (mg/ml) from the calibration curve of cyanmethemoglobin standard solution [16].

### Statistical Analysis

The data were analyzed by using SPSS version 13.0 and expressed as mean  $\pm$  SE. Mann-Whitney-U Test was used to test for comparison between groups. The correlation analyses were assessed by Spearman correlation. *P* value < 0.05 was considered as significant difference.

## RESULTS

### Subject characteristics

The physical characteristics of LHON patients were presented in Table 1. The ages of LHON patients were in a range of 12-67 years with mean age of onset between 18 and 35 years (*p* = 0.405). The other diseases have been found in some patients. In placebo group, four patients have one of the following diseases: hypertension, peptic ulcer and hemorrhoid. Whereas in curcuminoids group, two cases of allergy, one case of anemia and one case of hypertension with hyperlipidemia, were found. Smoking and alcohol intake were found in placebo more than curcuminoids group.

### The Malondialdehyde (MDA) level

The MDA levels in two groups of LHON patients at 0, 3, 6 and 12 month were not significantly difference (Figure 1). The positive correlation between age and

MDA level in LHON patients was found with the *r*-value of 0.41 (*p* < 0.05)

### Antioxidant enzyme activity

Catalase activities, total glutathione (GSH), oxidized glutathione (GSSG) and the percentage of GSSG with respect to GSH were not significant difference between curcuminoids and placebo group (data not show). SOD activities were not significant difference between curcuminoids and placebo group but in curcuminoids group, only SOD activities at 3 month were decreased 12.6% significantly from baseline (Figure 2).

The activities of glutathione peroxidase at 3 month in curcuminoids group were significantly decreased when compare to placebo group (*p* < 0.05). In addition, the GPx activities at 3 and 6 month in curcuminoids group were significantly decreased 24.1% and 12.6% from baseline, respectively (Figure 3).

## DISCUSSION

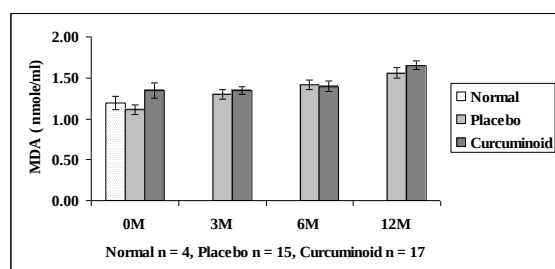
LHON discovered with mean age of onset between 18 and 35 years. In our study, the age of LHON patients were in a range of 12-67 years. The positive correlation between MDA level and age (*r* = 0.407, *p* < 0.05) was found and indicated that the older patients had higher oxidative stress status than the younger ones. The correlation between the generation of lipid peroxidation products and aging has also been reported [17]. We also found that MDA level in female was higher than male. So we suggested that the higher level of MDA at baseline in curcuminoids group might be due to the higher age and higher number of female compared to placebo group. In addition, patients in curcuminoids group had other diseases such as allergy, hypertension and liver function impairment that have been reported higher MDA levels [18, 19]. Although, the percentages of smoke and alcohol consumption in placebo group were higher than those in curcuminoids group, the oxidative stress was not different between both groups. However, the association between tobacco or alcohol consumption and the increased risk of developing symptoms in LHON has been found equivocal [2]. Since the pathogenesis of LHON was selectively on optic nerve degeneration. In our study, MDA levels from plasma of 11778 LHON mutation were not significantly different from normal individuals, indicating that oxidative stress was not present in plasma of LHON patients and cannot refer to oxidative stress in the optic nerve and pathogenesis of LHON. However, we cannot directly measure the oxidative stress in optic nerve. The investigation of endogenous activities of antioxidant enzymes in red blood cells of LHON patients found that the activities of CAT, SOD and GPx were not significantly different from normal individuals, indicating that endogenous enzyme activities were

**Table 1** Physical characteristics of LHON patients

| Parameters                            | Placebo<br>(n=15)                | Curcuminoids<br>(n=17)           |
|---------------------------------------|----------------------------------|----------------------------------|
| Age (years) <sup>1</sup>              | 29.3 $\pm$ 3.33<br>(15-52 years) | 33.8 $\pm$ 3.95<br>(12-67 years) |
| Sex (M/F)                             | 14 M / 1 F                       | 13 M / 4 F                       |
| Weight (kg) <sup>1</sup>              | 60.7 $\pm$ 1.50                  | 53.7 $\pm$ 2.25                  |
| Height (cm) <sup>1</sup>              | 168.0 $\pm$ 2.36                 | 161.4 $\pm$ 2.07                 |
| Smoking                               | 73.3 %                           | 23.5 %                           |
| Alcohol                               | 73.3 %                           | 29.4 %                           |
| Head injury                           | 60.0 %                           | 35.3 %                           |
| History of affected<br>LHON in family | 46.7 %                           | 82.4 %                           |

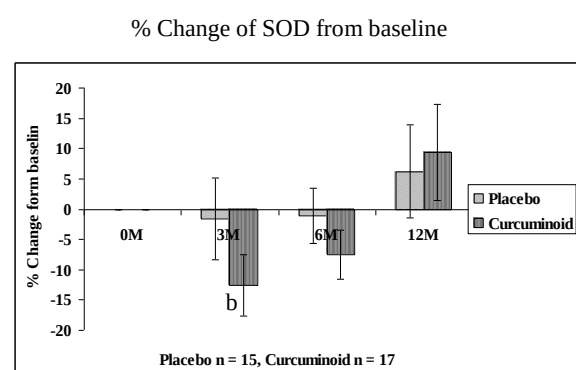
**Figure 1** The malondialdehyde levels in LHON patients. Results are expressed as nmole/ml. Each bar represents the mean value and the vertical lines show the SE.

<sup>b</sup> Significant difference from baseline at  $p$  value  $< 0.05$ .



**Figure 2** Percent change from baseline of superoxide dismutase activities. Each bar represents the mean value and the vertical lines show the SE.

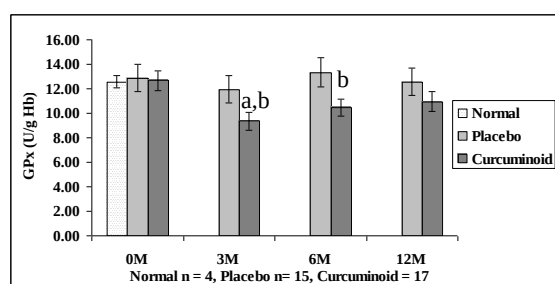
<sup>b</sup> Significant difference from baseline at  $p$  value  $< 0.05$ .



**Figure 3** Glutathione peroxidase enzyme activities in LHON patients. Results are expressed as U/g Hb. Each bar represents the mean value and the vertical lines show the SE.

<sup>a</sup> Significant difference from the placebo at  $p$  value  $< 0.05$ .

<sup>b</sup> Significant difference from baseline at  $p$  value  $< 0.05$ .



in normal. However, we found some effects of curcuminoids capsules on antioxidant enzyme activities.

The superoxide dismutase is an enzyme that catalyzes removal of superoxide anion from cell. The activities of SOD in red blood cell were declined 12.6% from baseline when treated with curcuminoids capsules for 3 month. There was evidence that curcuminoids have a radical scavenging activity due to phenolic group and  $\beta$ -diketone moiety on the structure of curcuminoids [20]. So we suggested that curcuminoids might be

used to scavenge superoxide anion generated in red blood cells instead of SOD, the normal antioxidant enzyme defense. This effect was seem to disappears at 6 and 12 months-administration of curcuminoids, might be due to its property to enhance activities of SOD or a pro-oxidant activity of curcumin. However, the relevance of the pro-oxidant nature of curcumin in an in vivo cellular scenario is not clearly determined [21]. Hydrogen peroxide is another ROS found in biological systems. It is catalyzed by catalase and glutathione peroxidase enzyme. The activities of catalase and glutathione peroxidase vary from tissue to tissue. For example both enzymes are extremely active in the liver (where considerable  $H_2O_2$  is formed) but only glutathione peroxidase is active in the brain (where  $H_2O_2$  product is much less). In human erythrocytes, the principal antioxidant enzyme for the detoxification of  $H_2O_2$  is GPx, as CAT has much lower affinity for  $H_2O_2$  than GPx [22]. In this study, the catalase activities in erythrocyte of both groups were not significantly different but GPx activities in curcuminoids group were declined 24% from baseline ( $p < 0.05$ ). GPx enzyme activities were also found to be decreased 18.9% in twenty-four thalassemia patients received 500 mg curcuminoids capsules daily for 6 months [7].

GSH is a major antioxidant in human tissues and serve as a co-substrate for the glutathione peroxidase to reduce hydrogen peroxide and organic peroxide to water and alcohol, respectively. During this process GSH become GSSG. Determination of percentage of GSSG/GSH was a useful indicator of oxidative stress. GSSG/GSH balance was important to homeostasis, facilitating cellular performance and survival [23, 24]. In this study, the percentages of GSSG/GSH in both groups of LHON patients were not significantly different and were in normal range [25]. Our result indicated that percentages of GSSG/GSH in both groups were in balance and oxidative stress was not occurred in red blood cells of LHON patients.

In this study we found the positive correlation between antioxidant enzymes and MDA level only in placebo group (Table 2). The positive correlation between SOD and CAT was found since SOD and CAT enzymes work in sequence to scavenge the superoxide anion and hydrogen peroxide, respectively. We also found the positive correlation between GSH and CAT since GSH work as a hydrogen donor to hydrogen peroxide or lipid peroxide by glutathione peroxidase then GSH were convert to GSSG. While CAT enzyme work to decompose hydrogen peroxide to oxygen and water. In addition, we found the positive correlation between MDA level and GSSG level, indicating that high level of lipid peroxidation resulting high level of GSSG. These correlations were found only in placebo group because of the normal antioxidant defense system. However these correlations were

**Table 2** Correlation between oxidative parameter in LHON patients

| Parameter    |              | Placebo group<br><i>r</i> ( <i>p</i> -value) | Curcuminoids group<br><i>r</i> ( <i>p</i> -value) |
|--------------|--------------|----------------------------------------------|---------------------------------------------------|
| MDA vs. Age  |              | 0.41 ( <i>p</i> <0.05)                       |                                                   |
| SOD vs. CAT  | at 3 month   | 0.74 ( <i>p</i> <0.05)                       | 0.256 ( <i>p</i> =0.274)                          |
|              | at 6 months  | 0.73 ( <i>p</i> <0.05)                       | 0.361 ( <i>p</i> =0.170)                          |
|              | at 12 months | 0.79 ( <i>p</i> <0.05)                       | 0.405 ( <i>p</i> =0.054)                          |
| CAT vs. GSH  | at 6 months  | 0.54 ( <i>p</i> <0.05)                       | 0.396 ( <i>p</i> =0.129)                          |
|              | at 12 months | 0.75 ( <i>p</i> <0.05)                       | 0.403 ( <i>p</i> =0.053)                          |
| GPx vs. GSH  | at 3 months  | 0.77 ( <i>p</i> <0.05)                       | 0.036 ( <i>p</i> =0.130)                          |
|              | at 12 months | 0.54 ( <i>p</i> <0.05)                       | 0.403 ( <i>p</i> =0.053)                          |
| MDA vs. GSSG | at 3 months  | 0.68 ( <i>p</i> <0.05)                       | 0.028 ( <i>p</i> =0.911)                          |
|              |              | 0.56 ( <i>p</i> <0.05)                       | 0.011 ( <i>p</i> =0.968)                          |

disappeared in the curcuminoids group. We suggested that the scavenging oxidants property of curcuminoids might be help normal antioxidant defense.

In conclusion, the results shown that the MDA levels and antioxidant enzyme activities in circulation were in normal range, indicating that oxidative stress was not present in circulation of LHON patients. However, the findings of this study suggested that curcuminoids were able to function as antioxidants in red blood cells of LHON patients. The limitation of the present study was some different characteristics of LHON patients in placebo and curcuminoids group including smoking and alcohol consumption and lack of the investigation in oxidative stress markers in tissue involve in pathogenesis. Further study the effects of curcuminoids on antioxidant enzyme activities or ROS generation in the specific tissue in LHON patients should be done.

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