*Free Rad. Res. Comms.*, Vol. 3, No. 6, pp. 373-377
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Printed in Great Britain

# EFFECT OF SILIBININ ON THE ACTIVITY AND EXPRESSION OF SUPEROXIDE DISMUTASE IN LYMPHOCYTES FROM PATIENTS WITH CHRONIC ALCOHOLIC LIVER DISEASE

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*(Received May 20th 1986)*

The *in vitro* and *in vivo* effects of the naturally occuring flavolignan hepatoprotective agent silibinin† on the expression and activity of superoxide dismutase (SOD) enzyme were studied in lymphocytes from patients with chronic alcoholic liver disease. *In vitro* incubation with silibinin in a concentration corresponding to the usual therapeutic dosage markedly increased the SOD — expression of lymphocytes as measured by flow-cytofluorimetry following staining with monoclonal anti-Cu, Zn-SOD — antibody and FITC-conjugated anti-mouse Ig. *In vivo* treatment with the drug restored the originally low SOD activity of the patients' lymphocytes. These data indirectly suggest that antioxidant activity might be one of the important factors in the hepatoprotective action of silibinin.

KEY WORDS: Silibinin, lymphocyte, alcoholic liver disease, superoxide dismutase

## INTRODUCTION

Since the free radical mechanism is supposed to play a major role in the pathogenesis of liver diseases[1-6], antioxidant activity is one of the important mechanisms of action of potent hepatoprotective drugs. As the naturally occuring substance silymarin (Legalon®, Madaus, FRG) belongs to the group of free radical scavenger agents[7,8] we studied its effects on one of the most important antioxidant enzymes, superoxide dismutase (SOD, E.C.1.15.1.1.) in lymphocytes of patients with chronic alcoholic liver disease.

## MATERIALS AND METHODS

Twelve patients with chronic alcoholic liver disease (histological diagnosis: fatty degeneration or micronodular cirrhosis) and as control twelve age and sex matched healthy subjects were studied. All patients were receiving a one month treatment with daily 280 mg of silymarin (2 xl capsules Legalon 140). The control group was not treated.

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†Silibinin is the major component of silymarin (Legalon®)

The SOD activity of lymphocytes was determined by the original method of Misra and Fridovich[9], modified by us for lymphocytes. The method is based on the spontaneous autooxidation of epinephrine to adrenochrome in the presence of air at pH 10.2. The inhibition of this process depends on the amount of SOD. Results are given in units. One unit refers to the amount of enzyme causing a fifty percent inhibition of autooxidation during one minute. Spectrophotometric measurements were performed with a Spectromom 204 equipment at 37°C and 480 nm.

Lymphocytes were separated from heparinized venous blood on Ficoll-Uromiro gradient[10]. Phagocytic cells were removed by carbonyl-iron treatment. Viability as judged by trypan blue test was greater than 95%. Monocyte contamination was less than 2%. Cells were suspended in phosphate buffered saline (PBS). Lymphocyte membranes were disrupted by sonication in icecool PBS. After centrifugation enzyme analysis was performed from the supernatants. All data were expressed as units of total SOD/ml sample.

Statistical analysis was performed by student's *t*-test.

For the flow-cytofluorimetric evaluation of SOD-expression in lymphocytes separated peripheral blood mononuclear cells were depleted of phagocytic cells by carbonyl-iron treatment. $10^7$ lymphocytes were incubated at 4°C with 100 $\mu$l monoclonal mouse anti-calf-Cu, Zn-SOD-antibody[11], diluted to 1:200 in PBS. Following two washings in PBS, cells were stained with 5 $\mu$l rabbit anti-mouse immmunoglobulin, conjugated with FITC (DAKO) for 30 min at 4°C. After three washings in cold PBS lymphocyte counts were adjusted to 2–3 M cells/ml and fluorescence patterns of cell suspensions were evaluated using an TC 4800 A cytofluorograph (Bio Physics System, Inc.).

## RESULTS

### *Effect of in vitro treatment with Silibinin on the SOD expression of lymphocytes*

SOD is one of the most important enzymatic components of the natural defence mechanism protecting the cell from oxygen stress. Therefore we studied the effect of silibinin on the SOD expression of lymphocytes of patients with chronic alcoholic liver disease, measured by flow cytofluorimetry. Six hr incubation with 10 $\mu$g/ml silibinin markedly increased the originally low SOD expression of patients' lymphocytes (Figure 1: dotted line as compared to unbroken line). The originally normal SOD-expression of healthy lymphocytes had only been moderately increased by the same concentration of the drug (data not shown).

### *Effect of in vivo treatment with Silymarin on the SOD activity of lymphocytes*

Total SOD activities of lymphocytes of patients and of healthy control subjects are demonstrated in Figure 2. Patients with chronic alcoholic liver disease had significantly lower lymphocyte SOD activities (column II) than the mean values of the healthy control group (column I). After one month treatment with Silymarin the originally decreased SOD values of the patients had significantly increased (Column III).

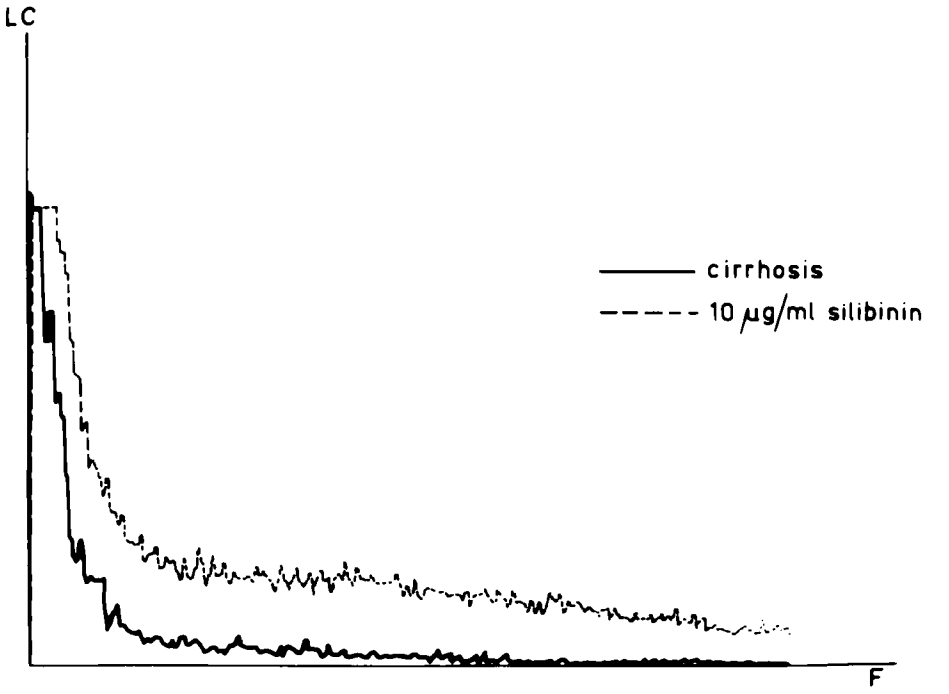


FIGURE 1 Flow-cytofluorimetric evaluation of the effect of *in vitro* treatment with silibinin on the SOD-expression of lymphocytes from a patient with alcoholic liver cirrhosis. Fluorescence distribution histogram. LC: lymphocyte count F: fluorescence

## DISCUSSION

The effective therapy of chronic liver diseases is still unsolved. Recently, a naturally occuring flavolignan silymarin (Legalon®) gained much interest in the treatment of these disorders. Legalon could normalize decreased hepatic functions in experimental cases of liver damage[12–17] and exerted benefitial effects in clinical studies,[18–21]. Since the antioxidant effect is supposed to be one of the important factors in the mechanism of action of potent hepatoprotective agents[22], and previous data suggested the free radical scavenger activity of silibinin[2,7], we have investigated its *in vitro* and *in vivo* effects on the expression and activity of superoxide dismutase. Cu, Zn-SOD has been considered to be the central component of the intracellular defence mechanism of human cells against oxygen stress[23].

We postulated that the change in SOD-expression of lymphocytes following *in vitro* incubation with the potential antioxidant silibinin may indirectly indicate its capacity to exert a free radical scavenger effect.

In our hands *in vitro* incubation with silibinin in a concentration corresponding to the usual therapeutic dosage markedly increased the SOD expression of lymphocytes. Similarly, *in vivo* treatment with the drug restored the originally low SOD activity of the patients' lymphocytes. Decreased SOD expression and SOD activity of the

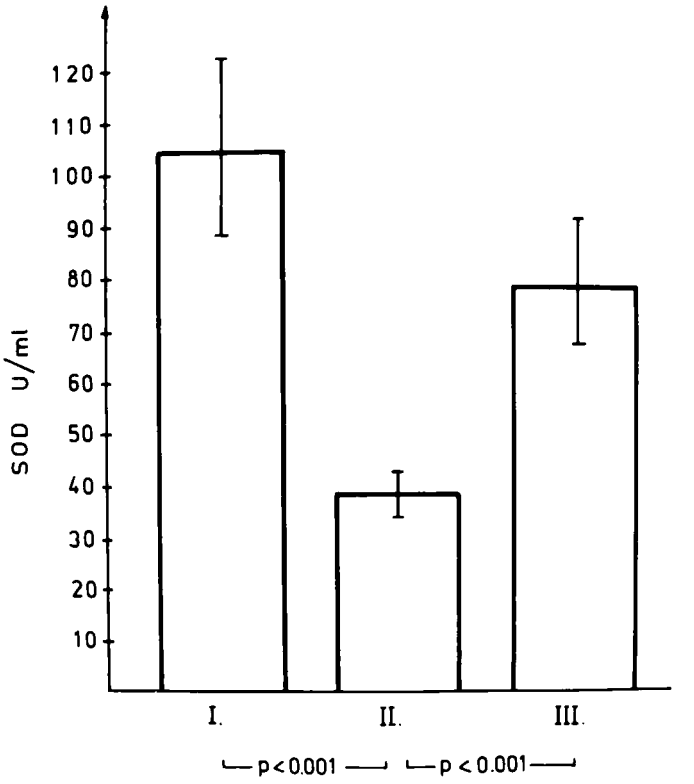


FIGURE 2 Effect of *in vivo* treatment with silibinin on the SOD-activity of lymphocytes from patients with chronic alcoholic liver disease.

lymphocytes of the patients may reflect an increased enzyme consumption as a consequence of enchanced production of oxygen free radicals. The fact that both *in vitro* and *in vivo* treatment with silibinin increased SOD expression and activity, respectively, may indirectly suggest the *in vitro* and *in vivo* antioxidant effect of the drug.

Further studies are under way in our laboratory to elucidate the possible significance of the decreased SOD-expression and activity of lymphocytes in patients with chronic alcoholid liver disease and its partial restoration following *in vitro* and *in vivo* treatment with the hepatoprotective agent silibinin.

ACKNOWLEDGEMENT

This work was supported by grants from the Hungarian Academy of Sciences and Ministry of Health.

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**Accepted by Dr. J.V. Bannister**