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Effects of *Rhus verniciflua* Stokes (RVS) on Cell-associated Detoxificant Enzymes and Glucose Oxidase-mediated Toxicity in Cultured Mouse Hepatocytes

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ABSTRACT : *The ethanol extract of Rhus verniciflua Stokes (RVS), the Korean Lacquer tree, was subsequentely isolated and fractioned into two portions using distilled water (SED) and 99% ethanol (SEE) as elution buffers through silica gel column (4×28 cm, 22 Å, 28~200 mesh). To know the antioxidative effect of the RVS extracts, primary hepatocytes were exposed to hydroxyl radical generated by 20 mU/ml glucose oxidase with SED or SEE for 4 hr. The addition of 100 μg/ml SED in culture medium showed good protection from glucose oxidase (GO)-mediated cytotoxicity of hepatocytes, showing approximately equivalent to control. When the hepatocytes were incubated with 100 μg/ml SED or SEE only for 4 hr, the activities of cell-associated superoxide dismutase (SOD) and catalase were elevated up to 1.22 fold and 1.4 fold, respectively, compared to control. Further increase, 1.88 fold in SOD activity or 1.64 fold in catalase activity, was also observed when the hepatocytes were incubated with 100 units/ml of commercial SOD or catalase for 4 hr. Moreover, the glucose oxidase-mediated cytotoxicity in cultured hepatocytes was generally reduced upon addition of lysate obtained from SED or SEE-stimulated hepatocytes in a dose-dependent manner. From these results, we suggest that, in cultured hepatocytes, RVS ethanol extract can efficiently reduce cytotoxicity induced by glucose oxidase and may increase the activity of cell-associated SOD and/or catalase, thereby preventing and/or scavenging superoxides and hydroxyl radicals in this experiment.*

Key Words : *Rhus verniciflua Stokes (RVS) ethanol extract, Hepatocytes, Glucose oxidase, Catalase, Superoxide dismutase*

I. INTRODUCTION

Reactive oxidants such as superoxides ($O_2^{\cdot}$) and hydroxyl radicals (· OH) can react directly with membrane lipids to produce peroxide stimulating the free radical chain reaction of lipid peroxidation and damage proteins and DNA in organism (Bracco *et al.*, 1991; Halliwell, 1991; Hall and Braughler, 1996). The protection from their oxidative damage in organism depends on the ability of antioxidant defenses to prevent and scavenge such a radical production (Frei *et al.*, 1992).

The major antioxidants of primary intracellular defenses are the enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. These enzymes represent an essential defense against the potential toxicity of reactive oxidants generated by severe oxidative conditions such as chronic inflammation and abnormal metabolism (Shigenaga, 1994). Catalase is a widely occurring enzyme which converts hydrogen peroxide to oxygen and water very rapidly (Woodbury *et al.*, 1971). Superoxide dismutase has been also detected in a wide range of living things and implicated as being an important defense against the cytotoxicity of oxygen (Imlay and Fridovich, 1991). Therefore, the balance between antioxidant defenses and generation of free radicals is a core factor to maintain normal metabolism of living cells.

It was well-known that detoxifying enzymes including SOD and catalase in animals as well as plants represented extraordinary defensive system against the toxic materials derived from endogenous and exogenous sources (Kyle *et al.*, 1988, Mittal and Dubey, 1991). Togashi *et al.* (1990) reported that levels of

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superoxide dismutase and catalase, two of the reactive oxidants-scavenging enzymes in liver, have been shown to be low in the diseased human liver, thereby potentially increasing the susceptibility of the liver to damage caused by reactive oxidants.

Bland and Bralley (1992) suggested that many antioxidant substances in foods may contribute to the activating of proper hepatic detoxification. It was also found that cultured hepatic cells could take up very rapidly exogenous detoxificant enzymes dissolved in the extracellular fluid. This implys that the intracellular damage caused by reactive oxidants in hepatocytes can be protected by supplementation of natural antioxidants. Moreover, it has been reported that experimental diet with antioxidant-depleted nutrients resulted in amplifying of superoxide and other radical production (Videla *et al.*, 1990). In contrast, it was shown that the diet containing augmented levels of the antioxidative nutrients maintains tissue levels of protective nutrients and prevents organisms from oxidative damage (Thompson *et al.*, 1983). Taken together, it is believed that improved activity of such detoxifying enzymes in the liver can help protect tissue injury from reactive radicals produced in a wide range of physiological processes and/or pathological conditions.

We have studied *in vivo* and *in vitro*, biological functions of *Rhus verniciflua* Stokes (RVS), Korean Lacquer tree, on hydroxyl radical-mediated toxicity of neurocytes, CT-26-induced tumorgenesis, cholesterol transformation, etc (Lee *et al.*, 1999; Lim and Shim, 1997). Considering a beneficial role of RVS in those studies, it is supposed that, in cultured hepatocytes, exogenous RVS extracts added to culture medium can directly scavenge · OH generated by glucose oxidase and may activate a cell-associated detoxifying enzyme, thus improving a function as preventer against cytotoxicity of free radicals.

The goal of this report, therefore, pointed effects of RVS ethanol extract on cytotoxicity induced by hydroxyl radical and on activation of superoxide dismutase and catalase in cultured hepatocytes.

II. MATERIALS AND METHODS

1. Materials

Glucose oxidase (GO; G8135), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; M2128), silica gel (28~200 mesh, 22 Å, S4883), penicillin G (PEN-NA), streptomycin (S6501), superoxide dismutase (SOD; S2515), catalase (C40), hypoxanthine (H9377), xanthine oxidase (X1875) and trypsin (T4549) from Sigma (St. Louis, USA) and nitrotetrazolium blue chloride (NBT; N1540-5) from Aldrich (Milwaukee, USA) were obtained. Proteinase K from Promega (Madison, USA) and Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) from Gibco BRL (Grand Island, N.Y., USA) were also purchased. Other chemicals and reagents were of the highest quality available from Sigma or Aldrich. Mice (ICR) were purchased from Daehan Animal Research Center (Daejeon, Korea).

2. Extraction and Isolation of *Rhus verniciflua* Stokes (RVS)

RVS was obtained from natural growth at Hwasoon, Chonnam province in October, 1998. The small-pieced branches were soaked in 99% ethanol for ten months and then filtered with Whatman filter paper (No. 2). The ethanol extract was lyophilized and eluted firstly in distilled water and then in 99% ethanol through silica gel column (4×28 cm, 22 Å, 28~200 mesh). The eluted samples in distilled water (SED) and 99% ethanol (SEE) were lyophilized under vacuum (Freeze dry system, Samwon Ltd, Co., SFDSM06). The dried powders were stored at -20°C for further experiment.

3. Liver Cell Culture

Hepatocyte cultures were prepared from ICR mouse liver of embryonic day 18~19. The liver tissues were cut out and washed thoroughly and dissected in phosphate buffered saline (PBS; pH 7.4) containing 5% FBS, 100 unit/*ml* penicillin, 100 μg/*ml* streptomycin, 5 mM calcium chloride and 0.05% collagenase. Red blood cells were removed by incubation at 37°C for 3 min in 160 mM ammonia chloride Tris buffer (pH 7.4). The cells were centrifuged at 1,000 × g for 10 min, and then at 800 × g for 10 min. The pellets were gently resuspended by pipetting in culture medium (CM; DMEM supplemented with 10 μg/*ml* insulin, 0.1 μM CuSO4, 50 pM $ZnSO_4$, 50 μg/*ml* linoleic acid, 50 unit/*ml* penicillin, 50 μg/*ml* streptomycin and

5% FBS). The cells at a concentration of 1×10^6 cells/ml were divided into 35 mm culture dishes for detoxifying enzyme assay or 96 multiwell flat-bottom plates for glucose oxidase-mediated cytotoxicity assay. The average viability of hepatocytes was estimated over 80% by trypan blue staining. Cells were grown in a CO_2 incubator under 5% CO_2/95% air atmosphere at 37°C during experimental period.

4. Glucose Oxidase-mediated Cytotoxicity Assay

On days 3, the cultured hepatocytes in 96 multiwell were washed with serum-free CM and exposed to hydroxyl radical generated by GO system (DMEM containing 20 mU/*ml* glucose oxidase and 0.5% D-glucose) with SED or SEE for 4 hr. After the exposure, MTT assay was performed according to the previous method (Mosmann, 1983). Briefly, MTT stock solution was dissolved in PBS at 5 mg/*ml* and filtered. After 4 hr incubation with GO system, 10 μ*l* of MTT stock solution was added to all wells and the plates were incubated under 5% CO_2 at 37°C for 4 hr. Acidic isopropanol (70 μ*l*) was then added into all wells. After 10 min, plates were read on a microplate reader (Dynatech Microelisa Reader, SINUVT06235, USA) using a wavelength of 570 nm. Results were expressed as relative percentage (%) of optical density of control which was incubated without exposure against the radical generating system.

5. Detoxifying Enzyme Assay

On days 2, the hepatocytes in 35 mm culture dish were gently washed with CM and coincubated with 100 μg/*ml* SED or SEE under 5% CO_2 at 37°C for various times. Superoxide dismutase activity in the lysate was determined spectrophotometrically following the standard curve on inhibition rate of NBT reduction employing commercial SOD at 560 nm. In brief, the SED or SEE-stimulated hepatocytes were lysed with extraction buffer [0.2% triton ×-100, 50 mM potassium phosphate buffer, and 0.1 mM EDTA (pH 7.8)] by resuspension in liquid nitrogen (Beauchamp and Fridovich, 1971). The lysate (1~10 μ*l*) was mixed with reaction mixtures containing 25 μM NBT, 0.1 mM xanthine, 0.1 mM EDTA and 0.05 M Na_2CO_3 (pH 10.2) in total volume of 3.0 *ml*. Xanthine oxidase (2 mU/*ml*) was then added into the mixtures and maintained at 25°C for 15 min. NBT reduction was determined spectrophotometrically at 560 nm. Result in SOD activity was expressed as unit/mg protein of each lysate. On the other hand, to assess the catalase activity, hepatocytes coincubated with SED or SEE were resuspended in lysis buffer [0.2% Triton ×-100, 0.1% NP-40 and 10 mM HEPES (pH 7.4)] and centrifuged at 15,000 × g for 30 min. Supernatants were assayed spectrophotometrically according to Thomson's method (1978). Briefly, the adequate amounts (1~100 μ*l*) of the lysate were mixed with 2.8 *ml* of 50 mM phosphate buffer (pH 7.4). After stand at 30°C for 5 min, 200 μ*l* of 0.1 N sodium perborate-H_3PO_4 (pH 7.4) was added into the reaction mixtures. The catalase activity was measured by calculating the k values of the optical density vs time following standard curve of the disappearance of sodium perborate using commercial catalase at 220 nm with intervals of 20 sec for a period of 2 min. Results were expressed as unit/mg protein of each lysate.

6. Detoxifying Assay of Lysates from RVS-stimulated Hepatocytes against GO System

The lysates obtained from SED or SEE-stimulated hepatocytes were added into 96 multiwell plates up to 10 μ*l*/well as a maximal volume. The amount of protein was determined as 100 mg/*ml*. The plates were then exposed to 20 mU/*ml* glucose oxidase for 4 hr and processed for MTT assay. Results were determined as relative percentage of living cells of control.

7. Statistical Analysis

Results were expressed as means±SED and analyzed by ANOVA analysis and Duncan test (One way) using the SPSS program.

III. RESULTS AND DISCUSSION

1. Protective Effect of RVS Ethanol Extract against Glucose Oxidase-mediated Cytotoxicity in Hepatocytes Culture

To investigate the cytotoxic level of hydroxyl radical in the liver, the cultured hepatocytes were incubated

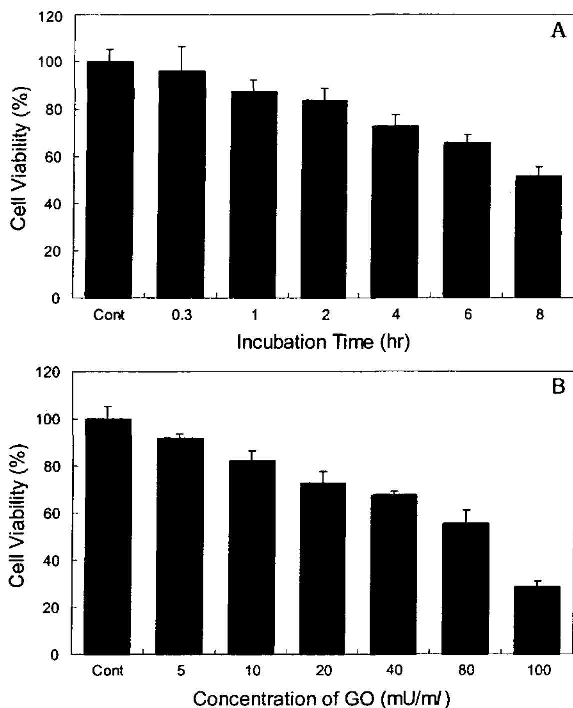

Fig. 1. Time- (A) and dose- (B) dependent cytotoxicities by glucose oxidase (GO) in cultured mouse hepatocytes. Results in cell viability are expressed as the mean±SED (n = 5).

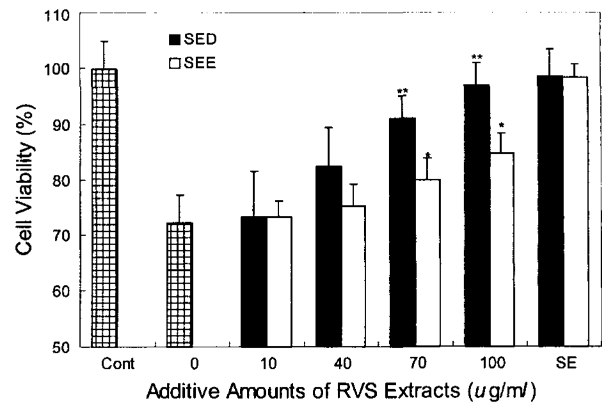

Fig. 2. Protective effects of *Rhus verniciflua* Stokes against glucose oxidase-mediated toxicity in cultured mouse hepatocytes. SED; RVS sample eluted with distilled water. SEE; RVS sample eluted with 99% ethanol. Control is the hepatocytes without SED, SEE and GO. '0' is only in presence of GO system with 0 μg/*ml* of SED or SEE. 'SE' is the hepatocytes in presence of 100 μg/*ml* SED or SEE alone. Results in viability are the mean±S.E. of the determination on five separate assays. ANOVA and Duncan test (oneway) were performed. *p < 0.05, differed significantly from GO alone. **p < 0.01, differed significantly from GO alone.

in serum-free culture media with glucose oxidase (GO) as the source of free radical by Haber-Weiss reaction. When the hepatocytes were exposed to 20 mU/*ml* GO for various times, cell viability was reduced in a time-dependent manner (Fig. 1A). Approximately 70% of hepatocytes were survived after 4 hr incubation in the presence of 20 mU/*ml* GO, while exposure to 100 mU/*ml* GO for 4 hr reduced the cell viability up to 28.8% (Fig. 1B). The addition of 100 μg/*ml* SED or SEE without GO had no effect on the viability of hepatocytes. Herein, the incubation of 20 mU/*ml* of GO for 4 hr was used as GO system in subsequent studies.

As shown in Fig. 2, the presence of SED or SEE blocked the cytotoxicity induced by hydroxyl radical generating system (GO system). The addition of 70 μg/*ml* SED in GO system increased viability up to 91% (P < 0.01) of hepatocytes, compared with control. When 100 μg/*ml* of SED was added into GO system, the viability of hepatocytes was significantly increased up to 96.9% (P < 0.01). In contrast, the viability of hepatocytes was determined to be 79.9% (P < 0.05) on the addition of 70 μg/*ml* SEE to GO system. The number of living cells was 84.7% when hepatocytes were cultured with 100 μg/*ml* SEE as maximum additional amount of RVS extract in GO system (P < 0.05).

It was found that exposure to GO system in cultured neuron cells resulted in approximately 50% mortality, indicating neuron cell containing high level of lipid membrane could sensitively damage by hydroxyl radical (Lim and Shim, 1997). Comparing the sensitivity to GO-mediated cytotoxicity between neuron cells and hepatocytes, hepatocytes showed more tolerance against hydroxyl radical than neuron cells. The reason of this tendency might be interpreted that the detoxifying enzymes such as catalase and SOD in hepatocytes were activated, and/or hydroxyl radicals were directly scavenged by RVS extracts.

2. Determination of Hepatic Catalase and Superoxide Dismutase Activities

In order to study the stimulating effects of RVS extracts on detoxifying enzyme activities in cultured hepatocytes, the cells were incubated under 5% CO_2 at 37°C in the presence of SED or SEE for various times.

SOD activity in control incubated with CM only was 11.90 units/mg protein. When the hepatocytes were coincubated for 1 hr with SED, SOD activity in lysate increased up to 14.99 units/mg protein, while the ac-

Table 1. Stimulating effects of *Rhus verniciflua* Stokes (RVS) on the cell-associated superoxide dismutase (SOD) and catalase in cultured mouse hepatocytes

Treatment	SOD activity	Catalase activity
	(unit/mg protein)	
Control	11.90±3.36	38.06±3.76
Glucose oxidase (20 mU/*ml*) + 4 hr	11.50±1.25	23.64±2.05
SED/SEE + 30 min	12.69±0.91/12.75±0.91	39.98±4.51/39.98±6.90
SED/SEE + 1 hr	14.99±2.29/13.63±1.07	50.75±6.55/48.59±3.00
SED/SEE + 2 hr	14.90±1.76/13.63±1.07	52.29±1.62/49.20±4.52
SED/SEE + 4 hr	14.82±2.63/13.76±2.27	53.50±5.72/50.21±2.80
SED/SEE + 8 hr	13.61±1.43/11.85±1.76	50.10±6.15/47.81±1.53
SED/SEE + 12 hr	12.05±1.26/11.71±2.19	49.62±1.42/40.20±8.60
SED/SEE + 16 hr	11.69±1.01/10.46±1.07	41.50±1.88/40.05±5.92
SED/SEE + 24 hr	10.68±0.80/10.08±1.43	42.30±5.92/37.50±7.36
SOD (100 unit/*ml*) + 4 hr	22.40±0.77	42.72±3.43
Catalase (100 unit/*ml*) + 4 hr	12.24±1.32	62.37±4.25

The hepatocytes were incubated in culture medium with 100 μg/*ml* SED or SEE for various times and then lysed as described in Materials and Methods. The activities of SOD and catalase in lysates were measured spectrometrically. Results are the mean±S.E. of the determination on three separate cultures.

tivity of lysate obtained after 16 hr incubation in the same condition was not increased any more. Moreover, when hepatocytes were incubated with 100 units/*ml* SOD for 4 hr, the cell-associated SOD activity was clearly amplified to 22.40 units/mg protein. SOD activity was also increased in lysate obtained from SEE-stimulated hepatocytes. Comparing the stimulating effects on SOD activity, SED was shown to be better than SEE. Additionally, coincubation with 100 units/*ml* of catalase did not give any effect to the increase of SOD activity (Table 1).

On the other hand, catalase activity was clearly elevated when the hepatocytes were incubated in CM supplemented with SED or commercial catalase. When the hepatic cells were incubated with 100 μg/*ml* SED for 4 hr, the catalase activity was elevated up to 53.50 units/mg protein, while its activity by SEE in the same condition was determined as 50.21 units/mg protein. Furthermore, catalase activity in lysate obtained after 4 hr incubation with 100 units/*ml* of catalase was 62.37 units/mg protein. In contrast, catalase activity in cultured hepatocytes was not affected through addition of 100 units/*ml* SOD alone, and both SOD and catalase activities were distinctly reduced, when the hepatocytes were exposed to GO system only.

As shown in Table 1, intracellular SOD activity was increased up to 1.22 fold, while the catalase activity was increased up to 1.4 fold when the hepatocytes were incubated with RVS extracts for 4 hr, compared to control. When the cultured hepatic cells were incubated with 100 units of SOD or catalase alone for 4 hr, SOD activity was increased to 1.88 times and catalase was 1.64 times, compared to control. These results indicate that, on the addition of RVS extracts, activities of cell-associated SOD and catalase, are elevated in hepatocytes.

It was reported that an increase of detoxifying enzymes activity by exogenous SOD or catalse comes from endocytotic ability of hepatocytes (Starke and Farber, 1985). It is suggested, therefore, that extracellular antioxidants will be also internalized to activate the intracellular defenses, depending on rate of cellular membrane recycling (Kyle *et al.*, 1988). This means that the resistance and prevention to toxic substances in eukaryotic cell may be controlled by its internalization ability. However, such an effect depends on what kinds of bioactive substances and cells. For instance, it has been reported that endothelial cells could not take up SOD in its native form at least in a short incubation in vitro, indicating endocytotic protection may depend on whether the cells could take up detoxifying enzymes or antioxidative substances (Freeman *et al.*, 1983; Hiraishi *et al.*, 1992). With such consideration, it is interesting to study whether the lysate of hepatocytes incubated with SED or SEE can protect cells from glucose oxidase-mediated cytotoxicity or not.

3. Detoxifying Effect of Lysates from RVS-stimulated Hepatocytes against GO System

To investigate the detoxifying effect of lysates obtained from RVS-stimulated hepatocytes, the cultured

Table 2. Dose-dependent protection of lysates obtained from RVS extracts-stimulated hepatocytes against glucose oxidase (GO)-mediated cytotoxicity in cultured mouse hepatocytes

Treatment	Cell viability (%)
1. Control	100±3.39
2. GO (20 mU/ml for 4 hr)	72.2±4.29
3. GO + 1 μl lysate of SED/SEE	72.15±3.38/72.15±4.91
4. GO + 2 μl lysate of SED/SEE	73.86±6.49/73.29±7.40
5. GO + 5 μl lysate of SED/SEE	75.57±3.95/73.86±4.57
6. GO + 7 μl lysate of SED/SEE	80.11±7.04/76.70±3.22
7. GO + 10 μl lysate of SED/SEE	82.38±5.45/78.40±7.95
8. GO + 10 μl lysate of SED/SEE + 100 μg SED	97.16±3.72/94.32±5.67
9. GO + 10 μl lysate of SED/SEE + 100 μg SEE	92.38±6.16/89.75±5.56
10. GO + catalase (100 unit/ml)	98.86±4.91
11. GO + SOD (100 unit/ml)	88.63±3.16

As described in "Materials and Methods", the lysates of SED or SEE-stimulated hepatocytes were added into 96 multiwell plates. After exposure to GO system for 4 hr, cell viability was measured by MTT assay. Lysate of 1 μl contains 0.1 mg protein and culture medium per each well was adjusted into 100 μl. Results represent the mean±S.E. of the determination on three separate assays.

hepatocytes were exposed to GO system with or without the lysates. Cell viability was then determined by MTT assay. Results were expressed as relative percentage of living cells to control which was incubated in CM without lysates and RVS extracts.

Table 2 shows that GO-mediated cytotoxicity in the cultured hepatocytes is generally reduced depending on the additive concentration of the lysates. When hepatocytes were exposed to GO system, cell viability was reduced up to 70%, while it was increased up to 82.38% or 78.40% in coincubation with 10 μl lysates of SED- or SEE-stimulated hepatic cells, compared to control. On the other hand, when hepatocytes were cultured in GO system with 100 units/ml catalase, cell viability was clearly increased up to 98.86%, while it was estimated as 88.63% at addition of 100 units/ml SOD, compared to control.

These results indicate that the hepatic lysate can reduce GO-mediated cytotoxicity of hepatocytes in a dose-dependent manner. Moreover, it is supposed that exogenous antioxidants and detoxifying enzymes such as SOD and catalase in hepatocytes culture are essential for protection, at least, from the glucose oxidase-mediated cytotoxicity of hepatocytes in this experiment.

Conclusively, it can be summerized that, in cultured hepatocytes, 1) hydroxyl radical generated by glucose oxidase promotes death of hepatocytes; 2) SED and/or SEE have a protective effect against glucose oxidase-mediated cytotoxicity; 3) detoxifying enzymes such as cell-associated SOD and catalase may be activated by supplementation of SED and/or SEE; 4) cell-associated SOD and catalase may be play a critical role in protecting cytotoxicity caused by superoxides and/or hydroxyl radicals.

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