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Mechanism of the toxicity induced by natural humic acid on human vascular endothelial cells

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Running title: Mechanism of the toxicity of humic acid

ABSTRACT Humic acid, a group of high-molecular weight organic compounds characterized by an ability to bind heavy metals, is normally found in natural water. Although the impairment of vascular endothelial cells in the presence of humic substances has been reported to be involved in some diseases, the mechanisms responsible for this involvement remain unclear. In this study, we examined the cytotoxicity of humic acid obtained from peatland in Central Kalimantan, Indonesia, to human vascular endothelial cells, as well as the mechanisms behind these effects. It was found that 50 mg/L humic acid showed cytotoxicity, which we considered to be mediated by apoptosis through the mitochondrial pathway due to an increase in the expression of caspases 6 and 9 in response to humic acid administration. In addition, this cytotoxicity was enhanced when cells in this experimental system were exposed to oxidative stress, while it was decreased by the addition of vitamin C. Thus, we conclude that the apoptosis induced by humic acid depends upon oxidative stress. Furthermore, an iron chelator, DFO, showed a tendency to decrease humic acid-induced cytotoxicity, suggesting that iron may potentially mediate humic acid-induced oxidative stress. In conclusion, long-term consumption of humic acid-rich water obtained from our study area may cause damage to endothelial cells and subsequent chronic health problems.

Key Words: humic acid; tropical peatland; apoptosis; endothelial cell; oxidative stress

INTRODUCTION

In Southeast Asia, peatlands ocuppy more than 26 million hectares, accounting for 69% of all tropical peatlands (Page et al., 2004). This includes the approximately 6 million hectares of peatland in Kalimantan, Indonesia (Radjagukguk, 1992). Natural lowland tropical peatlands are dominated by peat swamp forests and are important reservoirs of carbon. As a major component of dissolve organic carbon (DOC) is humic acid (HA), which has been shown to damage human endothelial cells (Hur et al., 2011; Hseu et al., 2002), the effects of the drinking water obtained from the Sebangau River on human health was investigated in this study.

HA forms a dark-colored mixture, comprising products of microbial degradation of organic matter. It is widely found in soil, coal, and well water, and is especially abundant in peat soil (Cavani et al., 2009; Avena and Wilkinson, 2002). HA is a major component of DOC, comprising up to 70%–90% of brown-colored river water (Mills et al., 1996). It contains aromatic rings and phenolic hydroxyl and carboxyl groups, which act as binding sites for metal ions (Riggle andWandruszka, 2004). Therefore, HA easily formed complexes with iron, zinc, lead, and cadmium (Kalis et al., 2006).

It had been reported that exposure to HA-containing water damages tissues and cells (Cheng et al., 2003; Hseu et al., 2008; Lu et al., 1994; Yang et al., 1996). For instance, HA has been implicated as one of the contributing factors for the development of Blackfoot disease, which is an endemic peripheral vascular disease found on the southwest coast of Taiwan (Hseu et al., 2008; Yang et al., 2002). The clinical symptoms of this disease include numbness or coldness of one or more of the extremities, resulting in black discoloration, ulceration, or gangrenous changes (Tseng et al., 2007). A causal association between consumption of well water and occurrence of blackfoot disease has

been revealed through an epidemiological study (Tseng, 2008; Yu et al., 1984). Furthermore, it was reported in the 1980s that the artesian well water in the area of Taiwan contained a high level of fluorescent compounds that induced blackening of the tail and feet of experimental mice (Cheng et al., 1999; Huang et al., 1995). The fluorescent compounds purified from this well water were identified as HA by infrared spectrophotometry and atomic absorption spectrometry; however, further studies have found that arsenic ions may also be related to Blackfoot disease (Liao et al., 2011; Selim Reza et al., 2011).

Pathological studies have revealed two types of vascular disease among patients with Blackfoot disease: arteriosclerosis and thromboangitis (Tseng, 2005). The impairment of vascular endothelial cells at an early stage has been shown to be an important aspect of both these vascular diseases (Yu et al., 1984). While HA has previously been shown to mediate damage to human vascular endothelial cells (Lu et al., 1994; Yang et al., 1996), the mechanisms of these effects are still unknown. However, some studies have revealed that HA-mediated damage on chondrocytes and human erythrocytes occurs in response to the generation of reactive oxygen species (ROS) (Hseu et al., 2002). The oxidative damage on endothelial cells plays an important role in the pathogenesis of arteriosclerosis and thrombosis (Cheng et al., 2003). Therefore, we assessed the risk of oxidative stress caused by HA in the tropical peatlands in Indonesia. The mechanism by which HA elicits these effects in human endothelial cells was investigated in this study.

MATERIALS AND METHODS

Materials

HUVECs, a cell line of human umbilical vein endothelial cells, were purchased from Cambrex Bio Science Walkersvile (Walkersvile, MD, USA). Endothelial Cell Growth Medium-2 (EGM-2) was bought from Lonza (Basel, Switzerland). HA, DAX-8, desferrioxamine (DFO), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), ribonuclease A (RNase), streptavidin-conjugated peroxidase, peroxidase-conjugated avidin, and ethidium bromide were obtained from Sigma-Aldrich (St. Louis, MO, USA). An SV total RNA isolation system, non-radioactive cytotoxicity assay kit, caspase assay kit, RT-PCR kit, and DNA marker were purchased from Promega (Madison, WI, USA). Ascorbic acid, Triton X-100, and trypan blue solution (0.5%) were purchased from Nacalai Tesque (Kyoto, Japan). The DNA 7500 assay kit and RNA 6000 nano assay kit were from Agilent Technologies (Waldbronn, Germany). Other chemicals were of analytical reagent grade.

Sampling sites of water and peat soil

Sampling was conducted at Sebangau River catchment in the suburbs of Palangka Raya City in Central Kalimantan, Indonesia. Water samples were collected 3 times from two sites, one in the artificial canal and one in the Sebangau River, in June 2009, December 2010, and August 2010. Peat soil samples were collected 2 times from 3 sites in December 2010 and August 2010. Sampling sites are shown in Figure 1.

DOC measurements

DOC was measured with a TOC-analyzer TOC-5000A (Shimadzu, Kyoto, Japan), according to the instruction manual. Five milliliters of 0.1 M HCl was added to each 25-

mL sample of water before determination of DOC content.

Preparation of purified HA

Natural HA from Indonesia was purified from the peat soil obtained in the natural forest near the Sebangau River by using DAX-8 absorption chromatography, as described by Swift (1996). Commercial HA was further purified following the methods described by Lu et al. (1988). In brief, commercial HA was acidified to pH 1 with 0.1 M HCl. Following centrifugation, the supernatant was removed, and the precipitated HA was dissolved in 0.1 M NaOH. This alkaline–acid treatment was repeated twice.

Elemental analysis of HA

The elemental contents of HA (carbon, hydrogen and nitrogen) were measured with a Micro Corder JM10 CHN analyzer (J-Science Lab, Kyoto, Japan), while oxygen content was calculated by normalizing the sum of the elements present in the samples (C, H, N, and O) to 100 wt%. The ash content was determined by weighing residual materials after elemental analysis.

Functional group analyses in HA

Organic functional group analyses (total acidity, carboxyl, and carbonyl groups) were performed using the methods described by Tsutsuki and Kawatsuka (1978). Phenolic hydroxyl groups were calculated on the basis of the difference between total acidity and carboxyl group.

Metals

The Al, Fe, Cu, Mn, Zn, and Pb contents of the HA samples were measured by inductively coupled plasma mass spectrometry using an ICP-MS Seiko SPQ-6500 mass spectrometer (Tokyo, Japan), according to the instruction manual. To digest the weighed HA samples with acid, they were heated in an oven (Muffle oven; Isuzu Seisakusho, Tokyo, Japan) at 95°C for 3 hr by using Teflon reactors with concentrated HNO3. The final volumes of the digested samples were made up to 10 mL with 1 M HNO3. The content of each metal is represented as nanograms of metal per milligram of HA.

Cell culture and exposure to HA

HUVECs were grown in EGM-2 medium with 2% FBS in 5% CO2 atmosphere. Upon reaching 90% confluence, cells were subcultured using 0.05% trypsin-EDTA, and exposed to various concentrations of HA (0–100 mg/L). Two types of HAs, HA from Indonesia and commercial HA, were assessed to compare the toxicities of different chemical compositions. Cells cultured in medium containing various concentrations of HA were also exposed to oxidant (H_2O_2; 10 μM), antioxidant (vitamin C; 10 μM), and iron chelator DFO (20 μM) to determine whether cells were experiencing oxidative stress. H_2O_2 and DFO were simultaneously added with HA, while the cells were treated with vitamin C 30 min prior to adding HA to the medium.

Determination of cell viability

Cell viability was measured by trypan blue exclusion assay. Briefly, HUVECs were incubated in medium containing 2% FBS and 0–100 mg/L of HA for 72 hr before being stained in 0.25% trypan blue in phosphate-buffered saline. The numbers of total cells and trypan blue staining cells were counted manually using a hemocytometer. Cell viabilities are expressed as the percentage of cells positive for staining against the total number of cells in each experiment. Each experiment was repeated at least 3 times.

Lactate dehydrogenase (LDH) activity assay

Cytotoxicity was assessed by measuring the amounts of LDH in the treatment medium by using a non-radioactive cytotoxicity assay kit (Promega, WI, USA). For this assay, HUVECs were cultured in medium containing 0–100 mg/L of HA and 10 μM of H_2O_2, vitamin C, or DFO. After culture, 50 μL of the medium was transferred to multi-titer plates, and the substrate mixture containing tetrazolium salts was added to the wells. After 0.5-hr incubation at 37°C, 50 μL of stop solution was added, and the amount of formazan dye formed was determined by measuring absorbance at 495 nm by using a Micro Plate Reader model 450 (BIO-RAD). Relative LDH activity was expressed as the relative change in the ratio of control groups, with the resulting values representing the cytotoxicity of HA.

RT-PCR analyses for caspases

The expression levels of caspase 6, 8, and 9 were determined by RT-PCR. Total RNA was isolated from treated HUVECs by using an SV total RNA isolation system kit

(Promega, WI, USA), according to the instruction manual. The resultant total RNA was reverse-transcribed to cDNA by using an Access RT-PCR introductory system (Promega, WI, USA). The transcriptase reaction was carried out at 48°C for 45 min followed by 94°C for 2 min, and 40 cycles of 94°C for 30 s, 60°C for 1 min, 68°C for 2 min. This was followed by a final extension time of 7 min at 72°C. The primers used in this study are presented in Table I. PCR products were electrophoresed on an Agilent Technologie DNA 7500 chip (Biosizing, USA). The expression levels of the caspases and β-actin were determined using a Digital Imaging and Analysis System Agilent Technologies 2100 Bioanalyzer (Biosizing, USA), and are expressed as amounts relative to those in the control cells.

Measurement of intracellular free-SH levels

The HUVECs were harvested after being exposed to HA for 24 and 48 hr, washed with PBS, and added to 80 μL of 10 mM HCl. Two freeze-thaw cycles were then performed to rupture the cell membranes, and the resultant solution was centrifuged at 8000 g for 10 min. Intracellular free-SH levels were determined using 200 μM DTNB. After 10-min incubation, the absorbance at 412 nm was measured with a DU-65 spectrophotometer (Beckman, CA, USA). The concentrations of free-SH in the HUVECs were determined using a molecular co-efficiency factor of 13,500 per cell number (1×10^5).

Statistical analysis

All experiments were performed a minimum of three times, and data are presented as

mean ± S.E.M. Data were analyzed using t-tests, and differences were considered statistically significant at values of $p < 0.05$ and $p < 0.1$.

RESULTS

DOC levels in the Sebangau River and artificial canal

The DOC levels in the Sebangau River were found to be 41.3–61.0 mg/L throughout the year. However, DOC levels measured in an artificial canal of the river were lower, ranging between 20.5–53.5 mg/L. This was particularly evident in December 2009 and August 2010.

HA is a major component of DOC in natural water from peatland areas, and the ratio of HA in DOC can reach up to 70%–90% (Mills et al., 1996). From the results of DOC determination in this area, HA concentrations were expected to be 40–50 mg/L and 20–40 mg/L in the Sebangau River and artificial canal, respectively. Since most residents along the Sebangau River use river water as drinking water, a concentration of 50 mg/L HA was used for cell experiments in this study.

Chemical analysis of HA

To compare the chemical compositions of the HA collected in this study and commercial HA, elemental analyses and functional group analyses were carried out as shown in Table II. The carbon content of the HAs ranged from 52% to 61%, which is in good agreement with values reported by Yonebayashi and Hattori (1988). As a whole, HA samples obtained from Indonesia had lower carbon content compared with commercial HA. Total acidity, as determined by carboxyl groups plus phenolic hydroxyl groups, ranged between 7.2 and 7.8 mmol/g in the HA obtained from Indonesia, while

commercial HA was found to have a total acidity of 6.1 mmol/g. Carboxyl and carbonyl groups, which have been reported to have positive correlation with humification (Yonebayashi and Hattori, 1988), were found to be present in higher numbers in commercial HA than in the HA collected from Indonesia (Table II).

Determination of HA metal content

The concentrations of Al, Fe, Cu, Mn, Zn, and Pb in the HA from Indonesia are shown in Table III. The concentrations of Al and Fe in HA were 67.6 and 81.0 ng/mg, respectively, while the concentration of Cu was 13.6 ng/mg. In addition, the concentration of As was 0.75 ng/mg. Since the HA samples collected in this study went through an extraction process, which could decrease ash content, HA in natural water is considered to contain higher concentrations of metal than purified HA. After DFO treatment, the concentration of Fe bound to the HA from Indonesia was decreased by around half, with a final level of 36.7 ng/mg.

Effects of HA on cell viability

To determine whether HAs affect cytotoxicity, the cell viability of HUVECs treated with HAs was studied using the trypan blue exclusion method. HUVECs were cultured with HA from Indonesia or commercial HA for 72 hr at concentrations of 0–100 mg/L. The viability of HA-treated cells was shown to decrease with an increase in HA concentration in a concentration-dependant manner [Fig. 2(a)]. The viability of HUVECs exposed to >10 mg/L HA was significantly decreased compared with that of control cells ($p < 0.05$), with cell viability decreased by approximately 60% with 100 mg/L HA treatment. However, no significant differences in cell viability were observed

between the cells treated with HA from Indonesia or commercial HA.

Effects of HA on LDH activity

To confirm the cytotoxicity of HAs, the LDH activity in the medium of cultured cells was measured 48 hr after treatment with 0–100 mg/L HA [Fig. 2(b)]. The LDH activity detected in the medium of HUVECs treated with 100 mg/L HA from Indonesia or commercial HA increased by 69% and 54%, respectively, compared with the activity in the medium of control cells. Significant increases in LDH activity were observed when cells were treated with 10–100 mg/L HA from Indonesia ($p < 0.05$), and 50–100 mg/L commercial HA ($p < 0.05$). The time-courses of LDH activity in the medium of cells treated with 50 and 100 mg/L HA from Indonesia are shown in Figure 2(c). The LDH activity in the medium was found to increase significantly 48 hr after HA treatment. As described above, the chemical characteristics of HA from Indonesia and commercial HA with regard to humification are different, and these differences are thought to be responsible for different levels of cytotoxicity. However, we observed no remarkable differences in the cytotoxicity between the HA samples from either source in this study, as determined by cell viability and LDH activity levels (Fig. 2). The cytotoxicity caused by HAs may, therefore, depend on the binding of heavy metals rather than on the functional groups present in the HAs or their levels of humification.

Effects of HA from Indonesia on caspase expression

To determine whether HA from Indonesia induces apoptosis, the expression levels of caspases 6, 8, and 9 were investigated by RT-PCR in cells exposed to HA from this source. The levels of caspase mRNA expression in the HA-treated cells are expressed as

the relative contents when the expression in untreated cells was set to 1.0. As illustrated in Figure 3, significant increases in the expression of caspases 6 and 9 were observed in cells exposed to 50 mg/L HA from Indonesia, wherein there was an approximately 2-fold increase in the expression of these caspases at 24 hr compared with that in control cells. On the other hand, the caspase 8 content decreased by approximately 50% following HA administration. These results suggest that HA from Indonesia induces apoptosis mediated by the mitochondrial pathway.

Free-SH levels in HUVECs

Glutathione (GSH), a major cellular thiol component of the cellular antioxidant system, has been shown to play an important role in preserving cells against ROS such as free radicals and peroxides (Alfonso et al., 2003). It is oxidized to GSSG when oxidative stress occurs. To estimate the intracellular oxidation status induced by HA exposure, the levels of free-SH were analyzed in the lysates from HUVECs treated with HA from Indonesia. It was found that the free-SH levels in these cells decreased in a time- and concentration-dependent manner in response to treatment with HA (Fig. 4), whereby treatment with 50 mg/L reduced the amount of free-SH by 26% and 63% at 24 hr and 48 hr after administration, respectively. This suggests that oxidative stress is increased in HUVECs in response to treatment with HA.

Effects of vitamin C on cytotoxicity caused by HA

To investigate the effects of an antioxidant (vitamin C) on HA-induced cytotoxicity in HUVECs, cells were pre-treated with 10 μM vitamin C for 30 min before being incubated with 50 or 100 mg/L HA from Indonesia (50 and 100 mg/L) for 48 hr. Our

results demonstrated that pre-treatment with vitamin C significantly decreased LDH activity in the medium of cells exposed to 50 and 100 mg/L HA ($p < 0.05$) [Fig. 5(a)], suggesting that vitamin C suppressed the cytotoxicity of HA-induced oxidative stress.

Effects of H_2O_2 on cytotoxicity caused by HA

The effects of oxidant H_2O_2 on HA-induced LDH release were also examined in HUVECs treated simultaneously with HA from Indonesia (50 and 100 mg/L) and 10 μM H_2O_2 for 48 hr. LDH activity in the medium of the cells exposed to 50 and 100 mg/L HA from Indonesia was significantly increased by the H_2O_2 treatment ($p < 0.05$) [Fig. 5(b)].

Effects of DFO on cytotoxicity caused by HA

The effects of an iron chelator, DFO, on the cytotoxicity of HA were also examined in HUVECs simultaneously treated for 48 hr with HA from Indonesia (50 and 100 mg/L) and 10 μM DFO (Fig. 6). It was found that the presence of DFO significantly inhibited the release of LDH into the medium of cells exposed to 100 mg/L HA from Indonesia ($p < 0.1$).

DISCUSSION

The structure of HA molecules can change according to the environmental background, which may explain the differences observed in the characteristics of the HA obtained from soil and water samples collected from peatland. Based on the results of the elemental analysis carried out in our study, the characteristics of HA obtained from a non-forest (NF) area were similar to those of HA from the Sebangau River (S1) (Table

II). Therefore, although residents near the Sebangau River are exposed to HA through drinking water, we used HA obtained from a NF area of Indonesia in our studies because of to the difficulty of obtaining large enough amounts of HA from water samples.

It was demonstrated in this study that 50 mg/L HA obtained from Indonesia has cytotoxicity in a cell-culture system. This HA concentration is nearly equivalent to that in the drinking water used by some residents of the Sebangau River catchment in Central Kalimantan. It must be emphasized that although HA-related heath disorders have not yet been reported in our study area, an area in Taiwan epidemic for blackfoot disease has been found to contain about 20–200 mg/L HA (Liu et al., 2003; Lu and Liu, 1986). Therefore, the long-term exposure to HA-rich water is a concern as a potential cause of chronic health injury due to the endothelial cell damage elicited by HA.

The HA-induced cytotoxicity observed in this study, as shown in Figure 2, was considered to depend on apoptosis because the expression of caspases 6 and 9 in the cells was increased by treatment with HA (Fig. 3). On the other hand, the expression of caspase 8 was decreased in response to HA administration. As the expression of caspases 8 and 9 are thought to be relevant to death receptor-mediated and mitochondrion-mediated apoptosis, respectively, it is suggested that the apoptosis induced by HA is not related to the death receptor pathway, but rather, the mitochondrial pathway. This explanation corresponds well to the findings of a previous report that showed that the exposure of endothelial cells to HA-induced DNA ladder formation detectable by ligase-mediated PCR (Hseu et al., 2002).

A variety of stimuli can induce apoptotic cell death. For instance, oxidants, including ROS, lipid hydroperoxides, and NO, are believed to be widely involved in oxidative

stress-induced apoptosis (Higuchi, 2004). The signs and symptoms of blackfoot disease are similar to those of arteriosclerosis and arterial thrombosis (Tseng, 2005), which are known to correlate well with oxidative stress damage to endothelial cells (Laude et al., 2004; Tsai et al., 2011). In addition, previous studies have shown that HA can induce oxidative stress in a variety of cells, including lung epithelial cells and human primary fibroblasts (Cheng et al., 2003; Shufan et al., 2008). This led us to speculate that apoptosis in HUVECs may also be mediated by the effects of HA-induced oxidant generation. While a recent study has indicated that high levels of As and humic substances may play a critical role in causing blackfoot disease in the Chianan plain region of Taiwan (Liao et al., 2011; Selim Reza et al., 2011), the HA examined in this study was found to have very little As content.

We also showed in this study that intracellular free-SH levels in HUVECs are decreased in an HA concentration-dependent and exposure time-dependent manner. We explored the possibility that HA treatment exposed the HUVECs to oxidative stress by examining the effects of vitamin C, an antioxidant, on cell death. As expected, vitamin C was found to inhibit cell death in response to HA [Fig. 5(a)].

There are many potential sources of ROS generation in cells, including cytosolic xanthine oxidase, membrane NADPH oxidase, and the mitochondrial electron transport system. The photosensitization of HA also yields active oxygen species such as $^1O_2$, HO·, HO_2·, and O_2· (Gau et al., 2001). Such species give rise to the generation of H_2O_2 in the environment. In addition, naturally occurring Fe(III) ions can be photochemically reduced to Fe(II) by HA (Fukushima and Tatsumi, 1999). A combination of H_2O_2 generation and Fe(III) reduction can then leads to a photo-Fenton reaction, in which the forcible oxidant HO· radical is produced according to the

following equation (Aguer and Richard, 1996).

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

The fact that the HA investigated in this study contained several metals, and iron, in particular, suggests that iron ions may have generated the reactive hydroxyl radicals. Indeed, it was shown that LDH release from the cells was increased by the addition of both H_2O_2 and HA to the medium for HUVECs [Fig. 5(b)], while an iron chelator (DFO) decreased the HA-induced lethal cell injury (Fig. 6). Although it was expected that DFO completely decreased the oxygen stress induced by HA, the effects of DFO on cell toxicity were not great. However, it should be noted that HA retained half of its Fe content after DFO treatment (Table III), but that cellular toxicity caused by HA could be prohibited if all Fe could be removed from HA. We, therefore, concluded that HA-bound iron may be responsible for mediating oxidative stress induced by HA. In normal tissue, iron rarely exists as a free ion, but rather it is bound to a variety of proteins including hemoglobin, transferrin, or ferritin. It has been suggested that HA could enhance the levels of intracellular chelatable iron in a cell, leading to an increase in ROS generation because of an increase in available iron ions. Although the HA obtained from Indonesia in this study was not found to bind significant amounts of As, oxidative stress was increased in the HUVECs in response to HA treatment. It was postulated that blackfoot disease may be related to the amount of As bound to HA, as it is rare for patients to develop blackfoot disease in Central Kalimantan in Indonesia.

CONCLUSION

In this study, it was demonstrated that HA obtained in Indonesia had cytotoxicity at concentrations greater than 50 mg/L. This concentration is similar to that found in the

water used as drinking water in the study area. While the presence of disorders involving HA has not yet been reported in the area of this study, an area of Taiwan that is epidemic for blackfoot disease has been reported to have about 20–200 mg/L HA. Therefore, the long-term exposure to HA rich water could have the potential to lead to chronic health injury due to the effects of HA on endothelial cells.

Since HA treatment resulted in increase in the expression of caspases 6 and 9, this cytotoxicity was considered dependent upon apoptosis mediated by the mitochondrial pathway (oxidative stress pathway). Supporting this, the cell toxicity caused by HA was increased by H_2O_2 treatment, and decreased by vitamin C treatment. Furthermore, an iron chelator, DFO, inhibited HA-induced lethal cell injury. These results indicate that HA-bound iron could mediate HA-induced oxidative stress, but not its functional groups or humification. To clarify the mechanism of ROS-triggered cell death in response to HA, further investigation will be needed.

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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FIGURE CAPTIONS

Fig. 1. Sampling sites in Central Kalimantan, Indonesia

Fig. 2. Cell viability (a), and LDH activity in the media (b) of HUVECs exposed to HA from Indonesia (●) and commercial (○) HAs. HUVECs were treated with or without 0–100 mg/L HAs for 72 hr. (c) Time-course of LDH activity in the medium of HUVECs exposed to 50 mg/L (●) and 100 mg/L (○) HA from Indonesia. Values are expressed as the mean ± S.E.M. of experiments performed in triplicate. * denotes $p < 0.05$ compared with the cell viability of untreated cells.

Fig. 3. Effects of HA from Indonesia on the expression of caspases 6, 8, and 9

HUVECs were treated with 0 or 50 mg/L HA from Indonesia for 24 hr before the expression of caspases in the cells was assessed, as described in the Material and Methods. Caspase contents are expressed as the relative change in the ratio of caspase expression in treated cells compared to control cells, after caspase levels were calculated as nmol/nmol β actin (fold). Values are expressed as the mean ± S.E.M. of triplicate experiments. * denotes $p < 0.05$ compared to the ratio of caspase expression in untreated cells.

Fig. 4. Concentrations of intracellular free-SH in HUVECs after incubation with or without 0–100 mg/L HA from Indonesia for 24 hr (●) or 48 hr (○). Values are expressed as the mean ± S.E.M. of triplicate experiments. * denotes $p < 0.05$ compared to the levels of free-SH in untreated cells.

Fig. 5. Effects of vitamin C antioxidant (a) and H_2O_2 (b) on cytotoxicity induced by treatment with 50 or 100 mg/L HA from Indonesia. Vitamin C was added to the medium 30 min prior to the addition of HA, while H_2O_2 (10 μM) was added concurrently. LDH activity is expressed as the relative change in the ratio of the control group (fold). Values are expressed as the mean ± S.E.M. of triplicate experiments. * denotes $p < 0.05$ compared with the LDH activity in cells exposed to HA in the absence of vitamin C or H_2O_2

Fig. 6. Effects of DFO on cytotoxicity induced by treatment with 50 or 100 mg/L HA from Indonesia. DFO (20 μM) was added to the medium in concurrence with HA. LDH activity is expressed as the relative change in the ratio of the control group (fold). Values are expressed as the mean ± S.E.M. of triplicate experiments. ♯ denotes $p < 0.1$ compared with the LDH activity in the cells exposed to HA in the absence of DFO.