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The diabetogenic effects of the combination of humic acid and arsenic: In vitro and in vivo studies

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Abstract

Black foot disease (BFD) is a peripheral arterial occlusive disease found among the inhabitants of the southwest coast of Taiwan. Moreover, within the BFD-endemic areas, diabetes mellitus occur at significantly higher rates than in other areas of Taiwan. A high concentration of humic acid (HA), and arsenic (As) are present in the artesian well water from BFD-endemic area. The aim of this paper is to study the diabetogenic effect of the combination of HA and AS. Treatment of HIT-T15 cells with HA, As, or both of them resulted loss of cell viability, apoptosis, depletion of ATP, increment of oxidative stress, activation of caspase 3, and dysfunction of insulin secretion. In addition, the plasma insulin of ICR mice, which were exposed to HA and As in drinking water for 12 weeks, was decreased in the 5, 7, and 12 weeks, and increased at early stage of exposure (3 weeks). The results reported herein reveal that HA and As exert HIT-T15 cell dysfunction and inhibited insulin secretive effects. In addition, the sub-acute peri-pancreatitis and islet damage caused by the infiltration of inflammatory cells after exposure of HA and As in drinking water for 5 weeks. Our study has important implications in the diabetogenic effect of the HA and AS which may be mediated by ROS and further information of the toxicity mechanisms will provide under our progressive studies.
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Keywords: Humic acid; Arsenic; Diabetogenic effect; Insulin; HIT-T15 cells; Oxidative stress

1. Introduction

Diabetes mellitus is a complex syndrome characterized by elevated blood glucose level. Most of the causes of diabetes mellitus are still unknown. Type 1 diabetes mellitus (T1DM) is an autoimmune disease in which antibodies to the beta cells of the islets progressively destroy the beta cells leading to an absolute deficiency of insulin (Le Roith et al., 2003). In the absence of insulin, hepatic glucose production will continue unabated, leading to hyperglycemia. Type 2 diabetes mellitus (T2DM) is a genetic disorder with strong environmental influences such as obesity, aging, stress of life, some viral infection, medications and chemicals (Tseng et al., 2002). The disorder is defined as a dual defect; with insulin resistance being detected early followed by dysfunction of the pancreatic beta cells. Clinical examples of this process are seen in patients with impaired

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glucose tolerance who progress to T2DM (Le Roith et al., 2003).

Insulin is the most potent anabolic hormone known and is essential for appropriate tissue development, growth, and maintenance of whole body glucose homeostasis. It is secreted by the β cells of the pancreatic islets of Langerhans and is a response to increase circulating levels of glucose and amino acids after a meal (Pressin and Saltiel, 2000). Insulin regulates glucose homeostasis at many sites, reducing hepatic glucose output and increasing the rate of glucose uptake of striated muscle and adipose tissue.

In addition, environmental toxicants have been suggested to play an etiologic role in diabetes development (Longnecker and Daniels, 2001). The possibility of an association between chronic environmental exposure and diabetes has important research and publishing implications. It has been proposed that oxidant free radicals play a relevant role in the etiology and pathogenesis of human diabetes mellitus and its sequels like nephropathy, neuropathy, and angioplasty (Soto et al., 2003). Abnormalities in the regulation of peroxide and transition metals metabolisms are postulated to result in establishment of the disease as well as its longer term complications (Wolff, 1993). There was considerable evidence that oxidative stress, the resulting role in the development of diabetes mellitus, as well as in mediating insulin resistance and impaired insulin secretion (Davi et al., 2005).

Black foot disease (BFD) is a peripheral arterial occlusive disease found among the inhabitants of the southwest coast of Taiwan (Tseng et al., 1961). Moreover, within the BFD-endemic areas, other diseases such as cancer (bladder, kidney, lung, skin, and liver), diabetes mellitus, cardiovascular anomalies, hypertension, cerebral apoplexy, and goiter occur at significantly higher rates than in other areas of Taiwan (Lu, 1994).

A high concentration of humic acid (HA, approximately 200 ppm), and arsenic (As, approximately 0.05–1.98 ppm) are present in the artesian well water from BFD-endemic area (Huang et al., 1994; Tseng et al., 1961). The study by Lai et al. (1994) carried out in BFD area in Taiwan is the first cross-sectional epidemiologic study indicating an associational between As exposure from drinking water to diabetes mellitus. Tseng et al. (2000, 2002) demonstrated that As exposure from the artesian well water has a dose–response relation with the incidence of diabetes mellitus.

Based on these in vitro and in vivo studies (Lai et al., 1994; Tseng et al., 2000, 2002), chronic arsenic exposure has been suggested to contribute to diabetes development. On the other hand, Navas-Acien et al. (2006) performed a systemic review of the evidence on the association of arsenic and T2DM from in vitro and in vivo; however, the current available evidence is inadequate to establish a causal role of arsenic in diabetes and needs further research.

HA is a group of high-molecular-weight polymers that comes from decomposition of organic matter, in particular dead plants. It exists abundantly in peat, soil, well water, and other sources (Hartenstein, 1981). A hypothetical "type" structure of humic acid has been exposed: a polymer is composed of aromatic rings of the di- and trihydroxybenzene type that are bridged by ether, methylene, amine, imine, and other linkage (Cheng et al., 2003). Because of its ubiquitous presence in soil and ground water and its characteristic ability to dissolve or to suspend metals (Schulze et al., 1994), HA could be used to help solubilize the metals for environmental toxicology studies (Tully et al., 2000). It has been shown that HA-metal complexes are more potent than metal ions alone in their effects on the hepatic enzymes (Lu et al., 1988; Lu and Lee, 1992).

HA was implicated as a causal factor of goiter (Lookesy et al., 1985), cancer (Lu et al., 1986), BFD (Lu et al., 1988; Lu, 1990a,b). Radioisotope tracing with iodinated HA in animals indicated that up to 60% of HA was retained in the body 24 h after administration (Huang et al., 1995). Furthermore, HA has been shown to generate reactive oxygen species (ROS) such as superoxide anion (Cheng et al., 2003), and causes a depletion of glutathione and several antioxidant enzymes (Cheng et al., 1999). The presence of free radicals in HA has been revealed using ESR spectroscopy (Lu et al., 1988). Recent investigations indicated that radicals were immobilized in the solid (macromolecular) matrix of HA (Jezierski et al., 2000). Thus, HA–As complexes may be important causative factors of diabetes mellitus in BFD area in Taiwan. The aim of this paper is to study the diabetogenic effect of the combination of HA and AS on a hamster pancreatic β cell line, HIT-T15, and ICR mice. The related biochemistical mechanisms that included apoptosis, caspase 3 activities, intracellular ROS, ATP, and GSH content, pathological examination, and insulin secretion, etc., were discussed in this study.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum, phosphate buffered saline (PBS), RPMI-1640 medium, penicillin–streptomycin (PS), and glutamine were obtained from Hyclone Co. (Hyclone, Logan, VT). Humic acid, glucose, trypan blue, propidium iodide

(PI), BCA kit, apoptosis detection kit (Apo-AC), Caspase 3 assay kit, adenosine 5′-triphosphate bioluminescent assay kit (FL-AA), 2′,7′-dichlorofluorescein diacetate (DCFDA), *N*-acetyl-cysteine (NAC) were purchased from Sigma–Aldrich Co. (St. Louis, MO). Monochlrobimane (mBCL) was obtained from Molecular Probes, Inc. (Fingene, OR). Insulin ELISA kit was obtained from Mercodia Co. (Uppsala, Sweden). All other chemicals were supplied either by Merck (Darmstadt, Germany) or Sigma–Aldrich Co. (St. Louis, MO).

2.2. Purification of HA

The chromatographic and fluorescence data indicate that Sigma–Aldrich HA is similar to HA obtained from coral skeletons, sea water, river water, soil, and plant leaves (Susic and Boto, 1989). Therefore, all experiments were performed with the same batch of HA, which was obtained from Sigma–Aldrich Co. (St. Louis, MO).

HA was first dissolved in 1N NaOH solution (pH > 10), and any undissolved material was removed by filtration. The solution was then acidified with 1N HCl to pH < 2.0 to precipitate the HA. Any precipitate formed was collected by centrifugation at 3000 × *g* for 30 min, and redissolved in 1N NaOH. Such procedure of alkaline-acid treatment was repeated thrice to get the purest HA as described by Schnitzers (1982). After the third round of acid precipitation, the precipitate was dissolved in 0.1N NaOH, and pH of the resultant solution was adjusted to 7.2–7.4. The purified HA was then freeze-dried to a powder. The HA was stored as dried powder, and was dissolved in PBS (pH 7.4) or double deionized water before experiments.

2.3. Cell culture

Hamster pancreatic β cell, HIT-T15 (ATCC number: CRL-1777), which was established from a primary culture of Syrian hamster islet cells which were transformed with SV40. Insulin secretion is stimulated by glucose and glucagon, and is suppressed by somatostatin and glucocorticoids. Cells were cultured in a RPMI-1640 medium containing 10% FBS, 100 unit/mL penicillin, and 100 unit/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The medium was exchanged every 2–3 days.

2.4. Cell viability

HIT-T15 cells (2×10^5/mL) were seeded onto 24 well culture plates, after the cells were treated for 24 h with various concentrations of HA, As, or both of them, cells were washed two times with PBS. The cells were trypsinized, stained with trypan blue, and the viable cells were counted by microscopic examination.

2.5. Programmed cell death assay

Apoptosis, or programmed cell death, was an important mechanism of most cells used to negatively select cells that are deleterious to the host. In living cells the phosphatidylserine (PS) was transported to the inner plasma membrane (Kuypers et al., 1996), and during the onset of apoptosis or necrotic, the PS was transported to the external leaflet of the plasma membrane. Apoptosis cells could be differentiated from necrotic cells by apoptosis detection kit, Annexin-Cy3 (Apo-AC, Sigma). Cells were incubated with Annexin V-Cy3 (Ann Cy3) and 6-carboxy fluorescein diacetate (6-CFDA) simultaneously. After labeled at room temperature, the cells were immediately observed by fluorescence microscopy (Axiovert 200, Zeiss). The PS was available for binding to Ann Cy3, which was observed as red fluorescence. In addition, cell viability could be measured by 6-CFDA, which was hydrolyzed to 6-CF and appears as green fluorescence. Live cells would be labeled only with 6-CF (green), while necrotic cells could be labeled only with Ann Cy3 (red). Cells in the early stage of apoptosis would be labeled with both Ann Cy3 (red) and 6-CF (green).

2.6. Caspase 3 activity assay

Caspase 3 activity in the cell lysate was determined using Caspase 3 assay kit (CASP-3-C, Sigma). Briefly, following pretreatment with HA, As, or both of them for 4 h, cells were washed with cold PBS. Then cells were lysed with lysis buffer on ice for 15 min. After centrifugation the lysed cells at 16,000 × *g* at 4 °C for 15 min, supernatants were transferred to 96 well plate and added with caspase 3 substrate AC-DEVD-pNA (*p*-nitroaniline) and assay buffer. The plate was incubated at 37 °C for 70–90 min and measured the optical density at 405 nm. Standard curve was made by measuring of various amounts of pNA (Porter and Janicke, 1999).

2.7. Intracellular ATP levels

The intracellular ATP content in the HIF-T15 cells is measured by Adenosine 5′-triphosphate bioluminescent assay kit (FL-AA, Sigma). Bioluminescent luciferase-luciferin reactions provide the basis of simple, rapid, and highly sensitive assays for ATP (Taylor et al., 1998). 2×10^5 cells/mL were grown in 96 well culture plates, after exposure to different concentrations of HA, As, or both of them for 2 h, 4 h, 8 h, or 24 h, cells were washed two times with iced PBS, and then lysed in 40 μL iced RIPA buffer and 50 μL ATP assay mix dilution buffer FL-AAB (pH 7.8) and immediately measure the amount of light produced with a luminometer (Tropix TR717).

2.8. Intracellular ROS

The amount of intracellular reactive oxygen species, especially H_2O_2 produced by HA and As was determined by using DCFDA (Gou et al., 1998). HIT-T15 cells were treated for 1 h, 2 h, or 4 h with various concentrations of HA and As.

After removing of the supernatant, cells were washed two times with PBS, and them a new medium which con-

tained 10 μM DCFDA was added and incubated for a further 20 min. Cells were lysed in 10 mM Tris and 0.25 M Sucrose in 0.05% Triton X-100 solution, pH 7.5. The cytoplasmic fluorescence intensity was measured by a fluorescence spectrometer (Perkin-Elmer LS-30) with excitation and emission wavelengths of 405 and 520 nm, respectively.

2.9. Intracellular GSH levels

After HIT-T15 cells were treated for 4 h with various concentrations of HA and As, cells were washed twice with PBS, and then a new medium which contained 60 μM mBCL, which was specified to GSH, was added and incubated for further 30 min at 37 °C, 5% CO_2. After loading the cultures with mBCL, the supernantants were discarded and cells were washed twice with PBS. Cells were lysed in lysing buffer (10 mM Tris, 0.25 M sucrose in 0.05% Triton X-100, pH 7.5). The mBCL-GSH related fluorescence in the cells was monitored by a fluorescence spectrometer (Perkin-Elmer LS-30) with excitation and emission wavelengths of 385 and 485 nm, respectively (Chatterzee et al., 1999).

2.10. Insulin levels

To measure the amount of insulin secreted, aliquots of samples were collected from the supernatant of cells, which was exposed to different concentrations of HA and As for 4 h. The amount of insulin in the supernatant was assayed according to the manufacturer's instruction of rat insulin ELISA kit (Mecodia).

2.11. The role of GSH in the insulin releasing functions of HIT-T15 cells

GSH is one of the most abundant cellular antioxidants and plays an important role in protection against ROS and toxic xenobiotics (Chatterzee et al., 1999). NAC has the same benefits as dose GSH, both through its own detoxifying and antioxidant function, as well as a precursor of GSH (Belletti et al., 2002). In this study, HIT-T15 cells were preincubated for 30 min in a medium with 5 mM NAC, and the supernatant of cells after incubation for 4 h with HA and As were collected to examine the released insulin. The amount of insulin in the supernatant was assayed according to the manufacturer's instruction of rat insulin ELISA kit (Mecodia) as described above.

2.12. Animal treatments

Male CD-1® (ICR) mice (20 ± 2 g body weight) were obtained from BLT (BioLASCO Taiwan Co. Ltd.) fed with MF-18 standard Chow (Oriental Yeast Co., Japan), and maintained at 22 ± 2 °C with 12 h light–dark cycles in laboratory animals center of Chung Shan Medical University. The animal study was conducted in accordance with the guidelines for the care and use of laboratory animals of the Animal Research Committee of the Chung Shan Medical University. The animals were divided into the following groups: (1) control group without any drug treatment for 12 weeks ($n = 14$). (2) HA group, mice received 500 mg/L humic acid in drinking water for 12 weeks ($n = 14$). (3) As group mice received 10 mg/L As_2O_3 in drinking water for 12 weeks ($n = 14$). (4) HA + As group

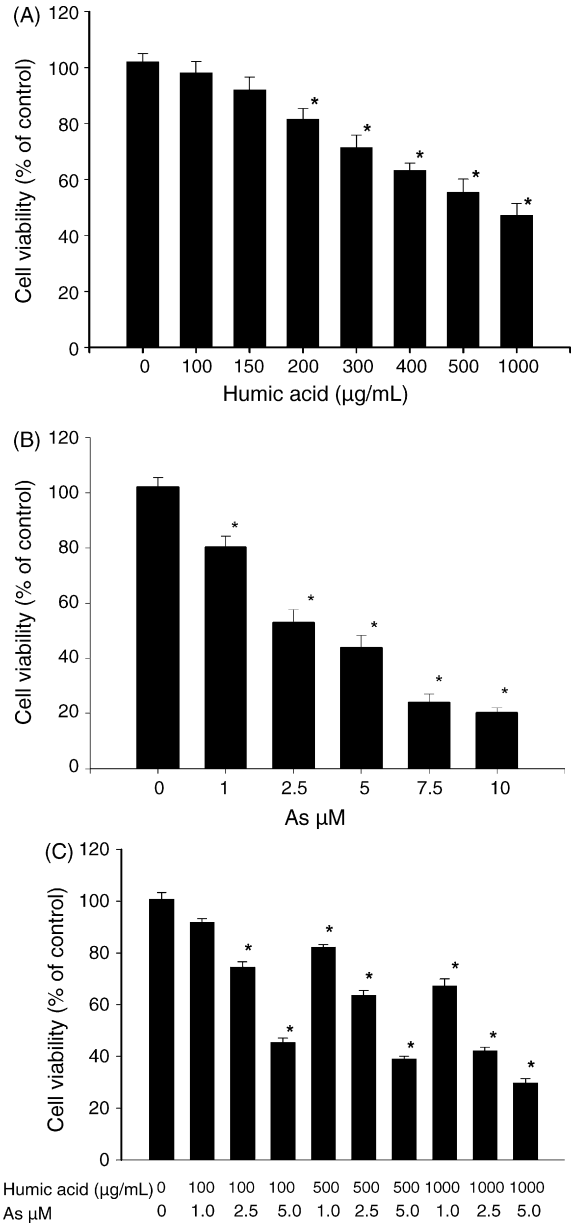

Fig. 1. Cell viability of HIT-T15 β cells after exposure of HA or As for 24 h. (A) The cells were exposed to different concentrations of HA, (B) the cells were exposed to different concentrations of As, and (C) the cells were exposed to combination of HA and As. Cells viability was determined by trypan blue exclusion method as described in Section 2. The values (%) are expressed in relation to control cells. Data are presented as mean ± S.E.M. ($n = 8$–10). $^*p < 0.05$ as compared with control cells.

mice received both 10 mg/L As_2O_3 and 500 mg/L humic acid in drinking water for 12 weeks ($n = 14$). Blood was collected from orbital vascular plexus at 3, 5, 7, and 12 weeks, and was then centrifuged at 4 °C, 1500 × g, for 10 min. The amount of insulin in the plasma was assayed according to the manufacturer's instruction of rat insulin ELISA kit (Mercodia) as described above. In addition, six mice of each group were sacrificed after 5 weeks cessation of administration and pancreas were rapid removed and used for histopathological examination. A portion of the pancreas was fixed overnight in a 10% neural formaldehyde solution and embedded in paraffin. Tissue slices were subjected to hematoxylin and eosin staining and histological study by light microscopy. Slides were coded and examined blind by the pathologist for the histological alterations (Matsumura et al., 2001).

2.13. Statistical analysis

Student's t-test was used to determine the statistical significance between treatment groups. Differences were considered statistically significant at a value of $p < 0.05$.

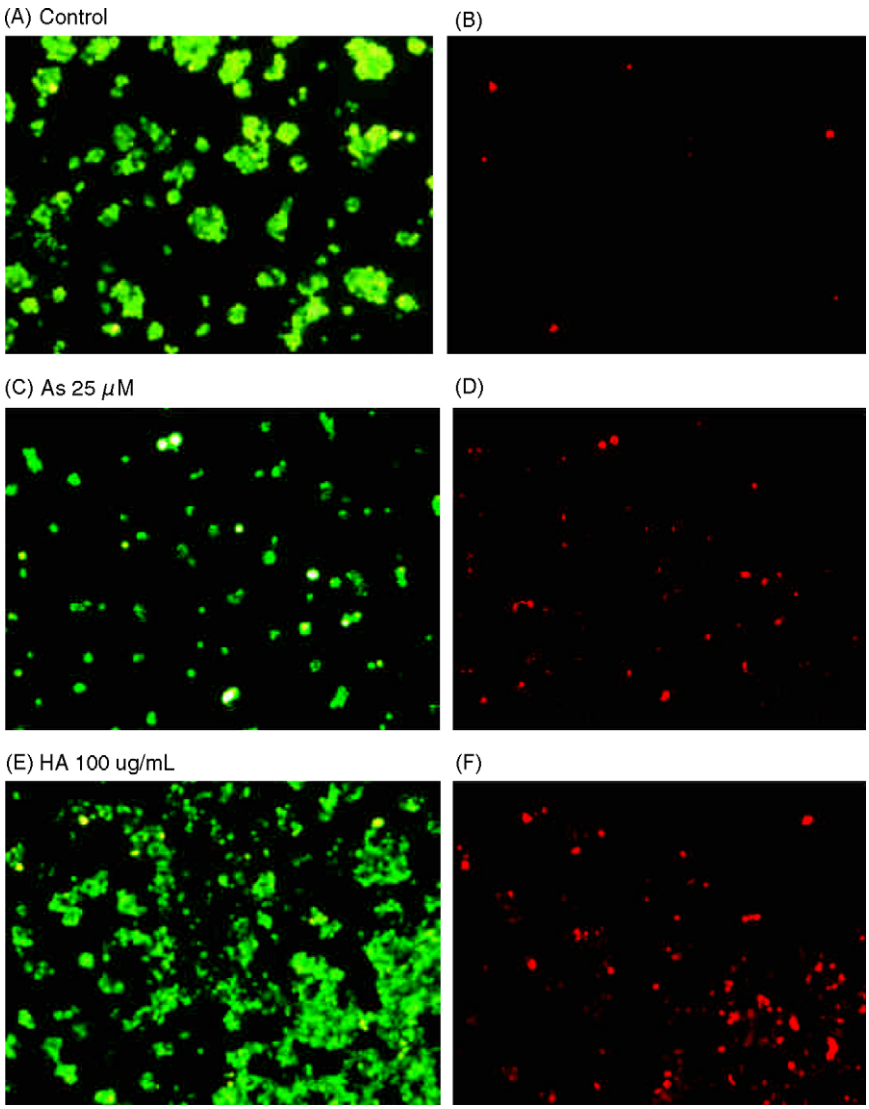

Fig. 2. Effect of HA and As on the morphology of HIT-T15 β cells. The cells were exposed to different concentrations of HA or As for 24 h, (A and B) Control, (C and D) As 2.5 μM, (E and F) HA 100 μg/mL, (G and H) HA 300 μg/mL, (I and J) HA 100 μg/mL + As 2.5 μM, and (K and L) HA 300 μg/mL + As 2.5 μM. Apoptosis cells could be differentiated from necrotic cells by incubated with Ann Cy3 and 6-CFDA simultaneously. After labeled at room temperature, the cells were immediately observed by fluorescence microscopy (200×). Live cells would be labeled only with 6-CF (green fluorescence, A, C, E, G, I, K), while necrotics cells could be labeled only with Ann Cy3 (red fluorescence, B, D, F, H, J, L). Cells in the early stage of apoptosis would be labeled with both Ann Cy3 (red fluorescence) and 6-CF (green fluorescence). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

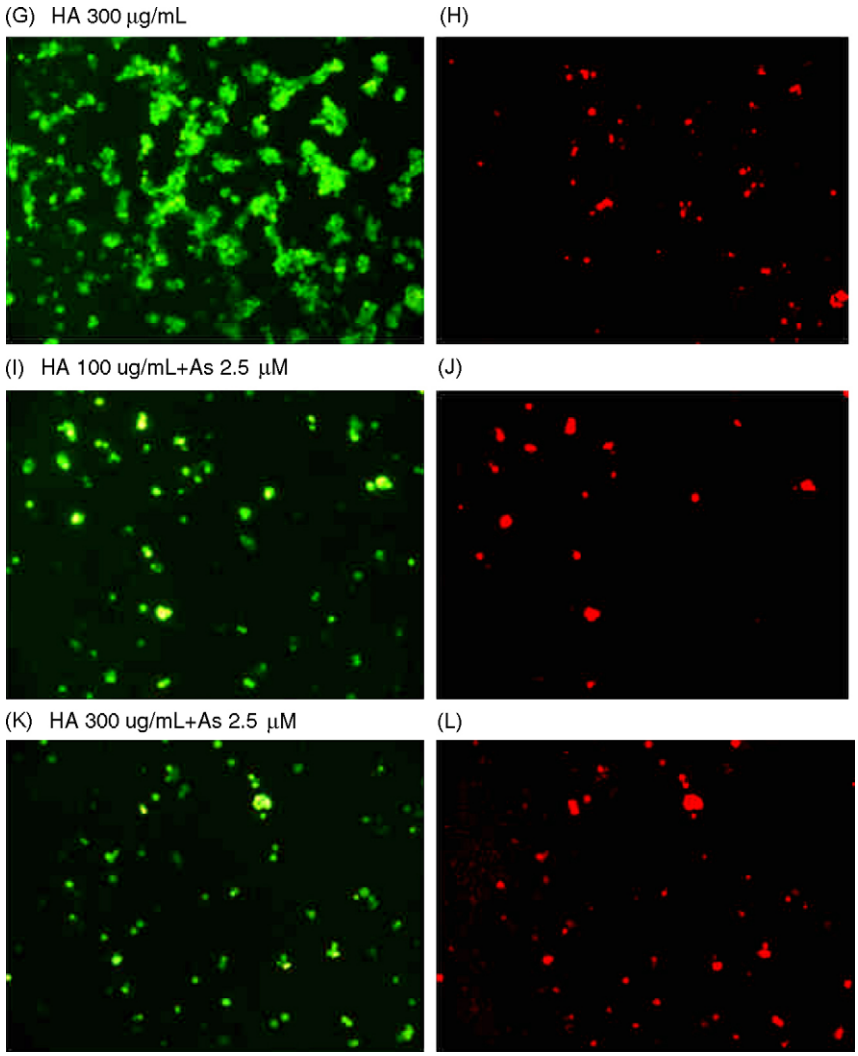

Fig. 2. (*Continued*).

3. Results

3.1. The cytotoxicities of HA and As on HIT-T15 cells

To test the toxicity of HA, HA in concentrations ranging from 100 to 1000 μg/mL with step increase was used to treat HIT-T15 cells for 24 h. It was found that the cell survivals were significantly inhibited by exposed of 300 μg/mL of HA and LD_{50} was near 1000 μg/mL (Fig. 1A). As shown in Fig. 1B, As in concentrations ranging from 1 to 10 μM significantly inhibited cell viability and LD_{50} was about 2.5 μM. In addition, the cell survivals were significantly inhibited by co-exposed of higher concentration of HA and As. On the other hand, the cytoxicity was prevented by the co-exposure of lower concentrations HA and As (100–500 μg/mL and 1–2.5 μM), respectively, Fig. 1C).

3.2. Induction of programmed cell death by HA and As

Fig. 2 shows the photographs of characteristic population of HA or As-treated HIT-T15 cells which were stained with 6-CFDA and AnnCy3 (Apoptosis detection kit, Sigma). Microscopy examination found that, after 24 h of exposure, the majority of HIT-T15 cells that were treated with HA and As were labeled both with green (6-CF) and red (AnnCy3) fluorescence, which means that the cells were in the early stage of apoptosis.

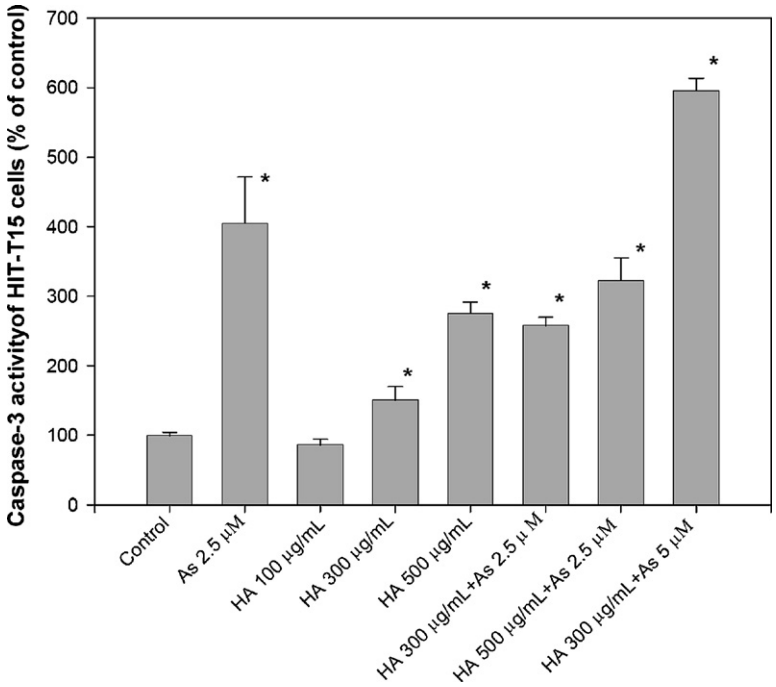

Fig. 3. Effect of HA and As on the caspase 3 activity of HIT-T15 β cells. Caspase 3 activity in the cell lysate was determined using Caspase 3 assay kit. Cells were lysed with lysis buffer on ice, and centrifuge at 16000 × *g* at 4 °C for 15 min. Supernatants were transferred to 96 well plates and added with caspase 3 substrate AC-DEVD-pNA (*p*-nitroniline) and assay buffer. The plate was incubated at 37 °C and measured the optical density at 405 nm. The average ratios (%) of caspase 3 activity were compared with control. Data are presented as mean ± S.E.M. ($n = 5$–7). $^{*}p < 0.05$ as compared with control.

3.3. Caspase 3 activity assay

As shown in Fig. 3, 2.5 μM As or 300–500 μg/mL HA-treated cells induces caspase 3 activity increased about four-fold or 1.5–3-fold of control cells after 4 h exposure, respectively ($p < 0.05$). On the other hand, co-exposure of difference concentrations HA and 2.5 μM As significantly increased caspase 3 activity than control, but which was less potency than 2.5 μM As along. Based on these observation, the apoptosis pathway could be trigged in HIT-T15 β cells after exposure of HA, As, or both of them.

3.4. Intracellular ATP levels

The intracellular ATP content in the HIT-T15 β cells was shown in Fig. 4. ATP was significantly decreased after exposure to higher concentrations of HA, As, or both of them for 2–24 h ($p < 0.05$). Furthermore, ATP contents in the cells remained over 30% of control except the exposure of higher concentrations of As and HA for 24 h, which can support the minimum energy requirement of apoptosis pathway after exposure of lower concentrations HA and As.

3.5. Intracellular ROS

We used DCFDA as a free radicals probe to study the intracellular oxidation in HIT-T15 cells which were treated for 1 h, 2 h, or 4 h with HA or As. Exposures of along or combination of these compounds could significantly induce oxidative stress in the cells ($p < 0.05$, Fig. 5). The oxidative property of HIT-T15 cells which exposed to As or co-exposed to HA and higher concentration of As for 2 h was higher than that of the exposure of 1 h or 4 h. On the other hand, ROS was significantly increased in HIT-T15 cells which exposed to HA and co-exposure of HA and lower concentration of As for 1 h and then decreased.

3.6. Intracellular GSH levels

GSH concentrations were significantly increased about 1.2–1.5-fold in HIT-T15 cells after exposing As or low concentration of HA for 4 h ($p < 0.05$, Fig. 6), excepting that higher concentration of HA could decrease intracellular GSH. On the other hand, co-exposure of HA and As could slightly increase GSH in the cell.

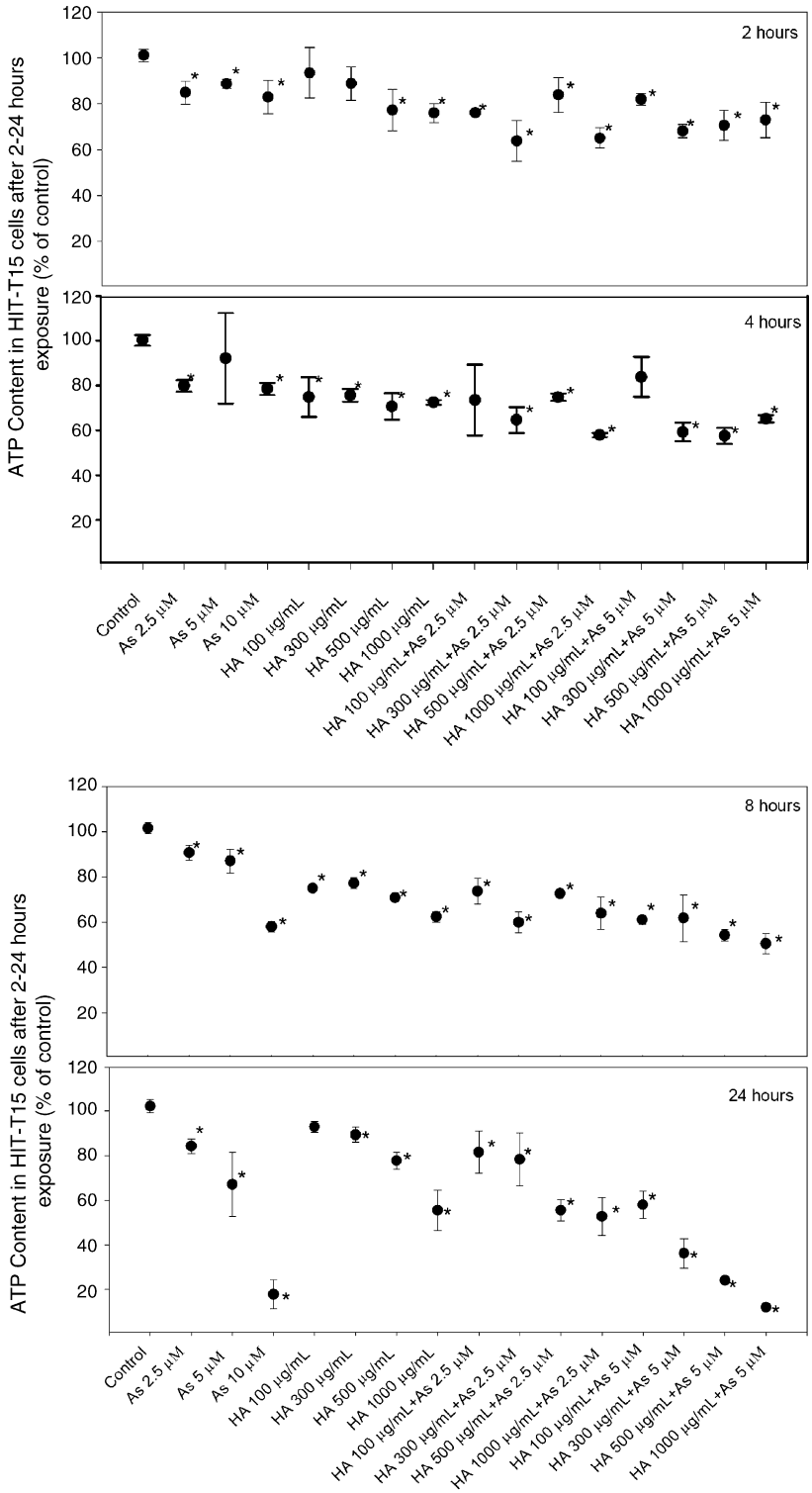

Fig. 4. Effect of HA and As on the intracellular ATP levels of HIT-T15 β cells. The intracellular ATP content in the cells was measured by Adenosine 5′-triphosphate bioluminescentassay kit. Cells (2×10^5 cells/mL) were grown in 96 wells plates, after exposed to different concentrations of HA, As, or both of them for 2 h, 4 h, 8 h, or 24 h. Cells were washed two times with iced PBS, and then lysed in 40 μL iced RIPA buffer and 50 μL ATP assay mix dilution buffer FL-AAB (pH 7.8) and immediately measured the amount of light produced with a luminometer. The values (%) are expressed in relation to control cells. Data are presented as mean ± S.E.M. ($n = 3$–5). $^{*}p < 0.05$ as compared with control.

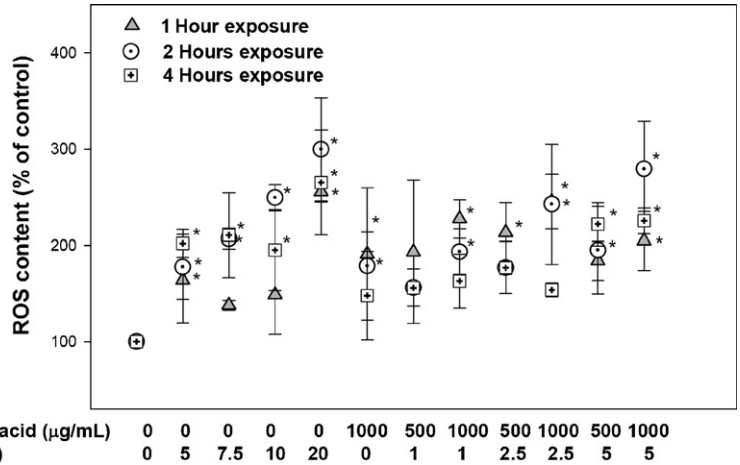

Fig. 5. Effect of HA and As on the intracellular oxidative property of HIT-T15 β cells. The cells were exposed to different concentrations of HA, As, or both of them for 1 h, 2 h, or 4 h. After removing of the supernatant, cells were washed two times with PBS, and them a new medium which contained 10 μM DCFDA was added and incubated for a further 20 min. The cytoplasmic fluorescence intensity was measured by a fluorescence spectrometer (Perkin-Elmer LS-30) with excitation and emission wavelengths of 405 and 520 nm, respectively. The values (%) are expressed in relation to control cells. Data are presented as mean ± S.E.M. ($n = 5$–7). $^{*}p < 0.05$ as compared with control.

3.7. Insulin release of HIT-T15 cells and the role of GSH

Insulin concentrations in the supernatant of HIT-T15 β cells, which were exposed to higher concentrations of HA, As, or combination of them for 4 h were significantly decreased than that of control group ($p < 0.05$, Fig. 7). As shown in Figs. 7 and 8, HIT-T15 β cells preincubated with NAC, a precursor of GSH, for 30 min, and then exposed to 300–500 μg/mL HA or co-exposed to HA and As for added 4 h could recover the function of insulin secretion ranging from 8% to 70%

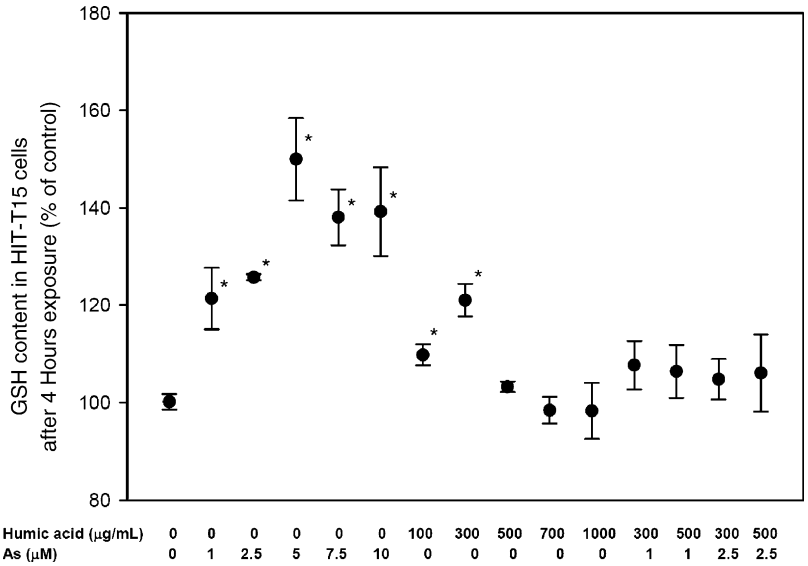

Fig. 6. Effect of HA and As on the intracellular GSH content of HIT-T15 β cells. The cells were exposed to different concentrations of HA, As, or both of them for 4 h. Cells were washed with PBS after treatment, and then a new medium which contained 60 μM mBCL, specified to GSH, was added and incubated for further 30 min. Cells were lysed in lysing buffer, and the mBCL-GSH related fluorescence in the cells was monitored by a fluorescence spectrometer (Perkin-Elmer LS-30) with excitation and emission wavelengths of 385 and 485 nm, respectively. The values (%) are expressed in relation to control cells. Data are presented as mean ± S.E.M. ($n = 5$–7). $^{*}p < 0.05$ as compared with control.

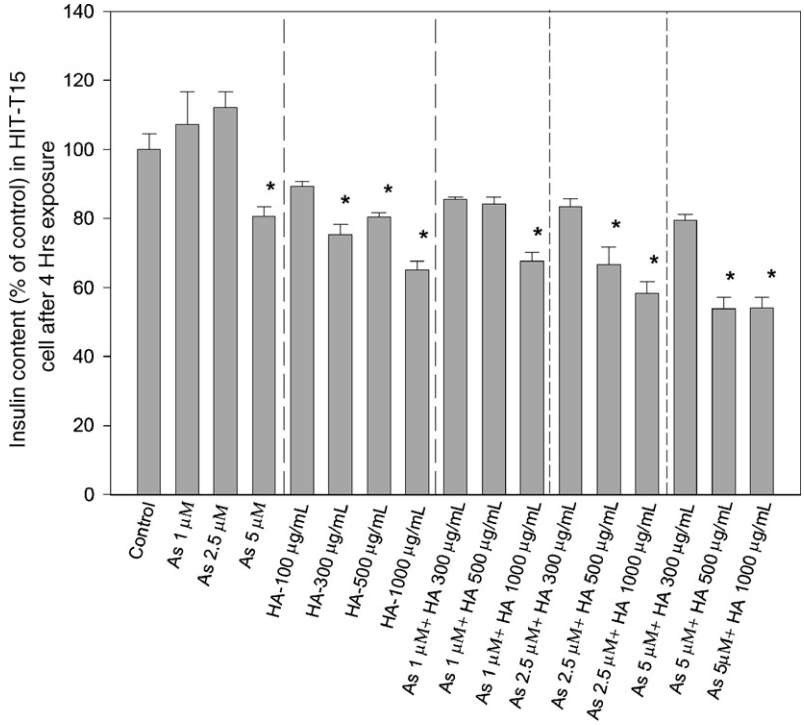

Fig. 7. Effect of HA and As on the insulin secretion of HIT-T15 β cells. The supernatant of cells, which was exposed to difference concentrations of HA and As or both of them for 4 h, were collected to examine the insulin release. The amount of insulin in the supernatant was assay by according to the manufacturer's instruction of rat insulin ELISA kit. The values (%) are expressed in relation to control cells. Data are presented as mean ± S.E.M. ($n = 5$–7). $^{*}p < 0.05$ as compared with control.

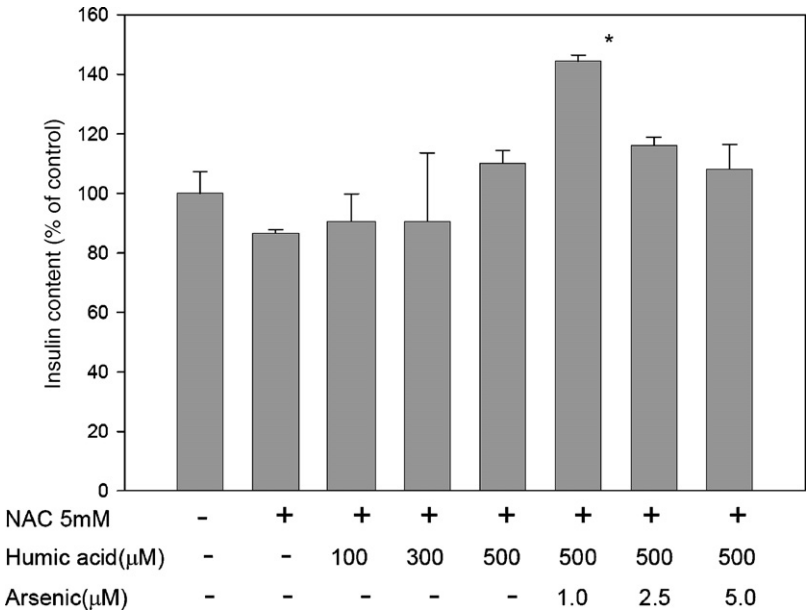

Fig. 8. The role of GSH in the insulin release functions of HIT-T15 β cells. HIT-T15 cells were preincubated for 30 min in a medium with 5 mM NAC, which is a precursor of GSH. The supernatant of cells after incubated for 4 h with HA, As, or both of them were collected to examine the insulin release. The amount of insulin in the supernatant was assayed according to the manufacturer's instruction of rat insulin ELISA kit. The values (%) are expressed in relation to control cells. Data are presented as mean ± S.E.M. ($n = 5$–7). $^{*}p < 0.05$ as compared with control.

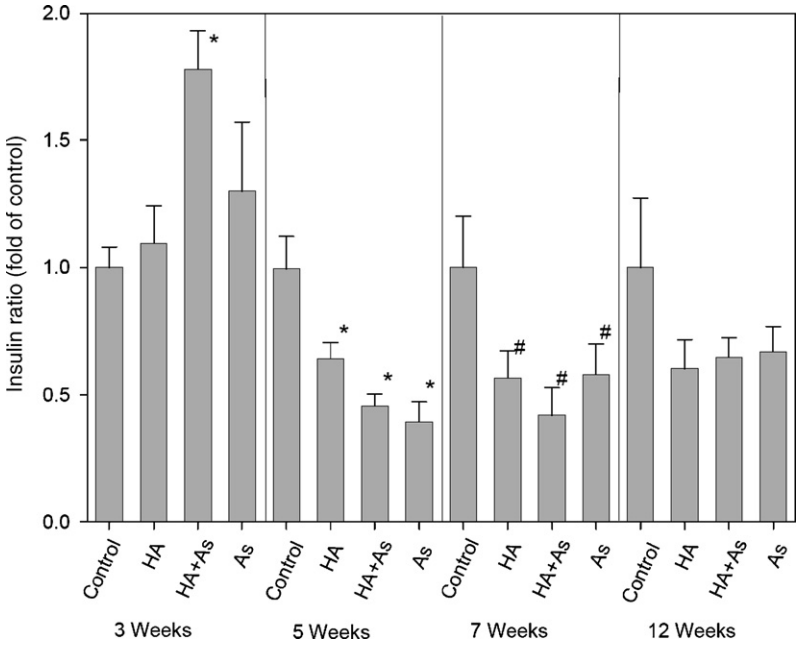

Fig. 9. Effect of HA and As on the insulin secretive function of ICR mice. The animals were received 500 mg/L humic acid, 10 mg/L As_2O_3, or both of them in drinking water for 12 weeks. Blood was collected from orbital vascular plexus at 3, 5, 7, and 12 weeks, and then the amount of insulin in the plasma was assayed according to the manufacturer's instruction of rat insulin ELISA kit. The values (fold of control) are expressed in relation to control animals. Data are presented as mean ± S.E.M. ($n = 8$). $^{*}p < 0.05$ as compared with control; $^{\#}p < 0.1$ as compared with control.

compared to the cells which were non-pretreated with NAC.

3.8. Plasma insulin contents of ICR mice

The average amount of water consumption of one mouse in each group was about 5.4 ± 0.6 mL/day, and there was no differences between each group of mice (data not shown). Fig. 9 shows plasma insulin was decreased in the 5, 7, and 12 weeks after exposure of HA, As, or combination of them. But plasma insulin was increased ranging from 1.11 to 1.78-fold of the control group at early stage of exposure (3 weeks).

3.9. Pathology lesions in pancreas of ICR mice

The tissue slices were examined blind by the pathologist in order to evaluate whether HA and As could induced pathological lesions in pancreas after 5 weeks exposure. As shown in Fig. 10, HA and As exposure could induce minimal to mild inflammatory cells (lymphocytes) infiltration within the interstitial and periductal areas in about 30–50% of slices which were reported as sub-acute peri-pancreatitis or pancreatitis. In addition, acinar cells atrophy or hyperplastic pancreatic islet was also observed in some slices of HA or As exposure group.

4. Discussion

According to the prospective study carried out in the black foot disease hyperendemic villages of Taiwan by Tseng et al. (2000), all of the diabetes patients were similar to type 2 diabetes mellitus (T2DM). Insulin resistance occurs during the early stage of T2DM, which could maintain normal blood glucose level by hyperinsulinemia. But at the time of diagnosis of T2DM, β cell dysfunction was always present with the progression of T2DM, especially when long-term glycermic control was not adequate, pancreatic β cell dysfunction could be severe that insulin injection was necessary (Kahn, 2003).

A high concentration of humic acid (approximately 200 ppm), and arsenic (approximately 0.05–1.98 ppm) are present in the artesian well water from BFD-endemic area (Huang et al., 1994; Tseng et al., 1961). Tseng et al. (2000, 2002) demonstrated that As exposure from the artesian well water has a dose–response relation with the incidence of diabetes mellitus. On the other hand, Navas-Acien et al. (2006) performed a systemic review of the evidence on the association of arsenic and T2DM from in vitro and in vivo studies, and the current available evidence is inadequate to establish a causal role of arsenic in diabetes, which required further confirmation.

The aim of this study is to analyze the diabetogenic effect of the combination of HA and AS on a HIT-T15 β

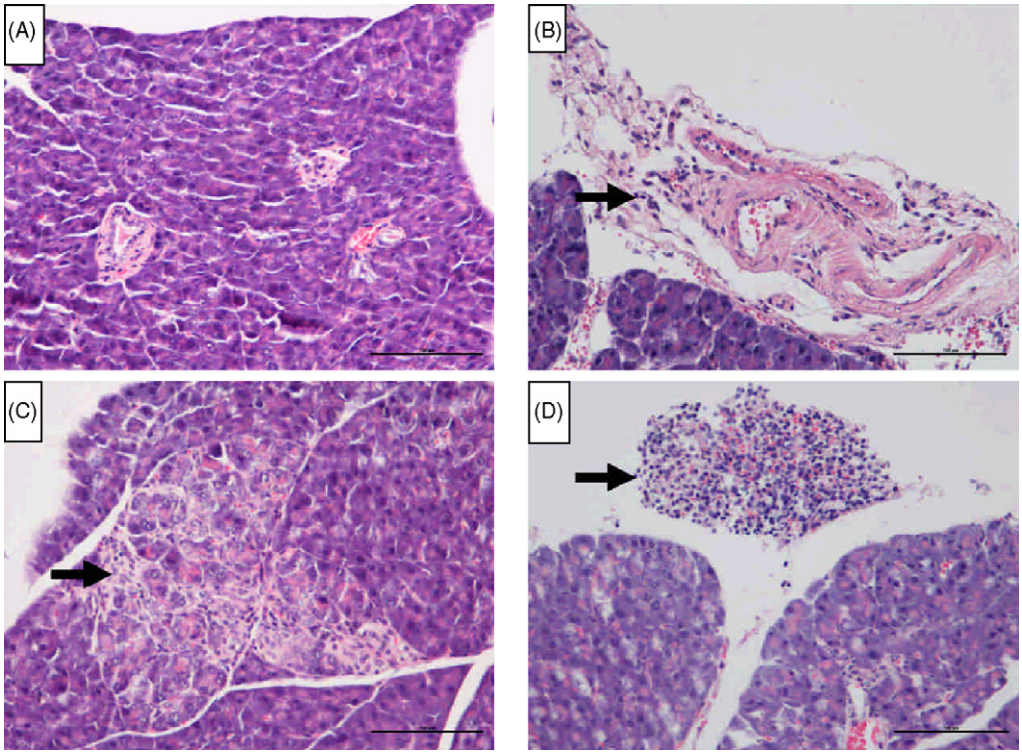

Fig. 10. Photomicrographs of the pancreas of ICR mice after HA and As exposure. The animals were received 500 mg/L humic acid, 10 mg/L As_2O_3, or both of them in drinking water for 5 weeks. A portion of the pancreas was fixed overnight in a 10% neural formaldehyde solution and embedded in paraffin. Tissue slices were subjected to hematoxylin and eosin staining and histological study by light microscopy (200×). (A) Control group, no pathological lesions were found. (B) HA group, there were mild lymphocytes and plasma cells within the interstitial area (denoted by black arrow). (C) As group, there were mild accumulation of inflammatory cells into the periductal and interstitial areas and acinar cells atrophy and loss (denoted by black arrow). (D) HA + As group, there were mild accumulation of inflammatory cells into the peri-pancreatic area (denoted by black arrow).

cell line, and ICR mice. The in vivo and in vitro results reported herein revealed that HA and As exert β cell dysfunction and inhibited insulin secretive effects. Not only the combination of higher concentration HA and As, but also HA or As along could exert antiproliferative action and growth inhibition in cultured HIT-T15 β cells (Fig. 1). The results of that cytoxicity were decreased by the co-exposure of lower concentrations HA and As are similar to the study of Hseu and Yang (2002) which the HA could bind As(V) and inhibit the enhancement effects of HA on the hemolysis. The mechanism may be mediated via chelating of As by HA leading to less available As and decrease HA functional groups which may lead to high reactivity and oxidative damage to RBC (Cheng et al., 1999). On the other hand, the effects of HA on shortening prothrombin time would become more apparent in the present of As (Lu et al., 1994). Based on these observations, the toxicokinetic processes of HA and As complex are more complicated than single exposure, which are needed further information of the toxicity mechanism under our progressive studies.

Furthermore, 6-CFDA and AnnCy3 positive stained cells and the activation of caspase 3 exhibited the characteristic of apoptosis (Figs. 2 and 3). The caspase activation is an essential step in these complex apoptosis pathways (Thornberry and Lazebnik, 1998). Recently, many papers have reported that HA can induce apoptosis in HL-60 cells (Yang et al., 2004), human primary fibroblasts and endothelial cells (Cheng et al., 2003). Our data provide an important evidence that HA and As induced β cell death was apoptosis related. On the other hand, co-exposure of difference concentrations HA and 2.5 μM As significantly increased caspase 3 activity than control, but which was less potency than 2.5 μM As along. The mechanism may be mediated via chelating of As by HA leading to less available As and decrease HA functional groups as described above.

ATP was significantly decreased after exposure to higher concentration of HA, As, or both of them for 2–24 h ($p < 0.05$, Fig. 4). ATP contents remained over 30% of control except the exposure of higher concentration of As and HA for 24 h, which can support

the minimum energy requirement of apoptosis pathway (Leist et al., 1999). In addition, glucose transport into the β cell can initiate insulin secretion by the depolarization of ATP-sensitive potassium channel and open the voltage-dependent calcium channel. The calcium flux through the opened channel releases insulin (MacDonald and Wheeler, 2003). It is possible that the ATP-dependent insulin secretion could be impaired in the absence of sufficient energy supply (Tseng, 2004).

A growing number of reports have suggested a link between increased ROS production or oxidative stress and the development of insulin resistance and β cell dysfunction in humans (Tseng et al., 2004). The presence of free radicals in HA has been revealed using ESR spectroscopy (Lu et al., 1988). HA-induced oxidative injury in rabbit articular chondrocytes may be mediated by superoxide anion production (Liang et al., 1998). HA-induced echinocyte transformation occurs via oxidative generation in human erythrocytes (Hseu et al., 2000). In addition, As is well known for its ability to induce the production of superoxide (Barchowsky et al., 1999; Lynn et al., 2000). On the other hand, pancreatic β cells are most vulnerable to damages caused by oxidative stress because they are low in free radical quenching enzymes such as catalase and SOD (Tiedge et al., 1997). We used DCFDA as a free radicals probe to study the intracellular oxidation in HIT-T15 cells which were treated for 1 h, 2 h, or 4 h with HA or As, could significantly induce oxidative stress in the cells ($p < 0.05$, Fig. 5). Based on our data, the increased oxidative stress induced by HA and As could cause damage to the pancreatic islets cells, impairment in insulin secretion, and pathological lesions in pancreas (Matsumura et al., 2001). In addition, ROS was significantly increased in HIT-T15 cells which exposed to HA or co-exposure of HA and lower concentration of As for 1 h which was early than As along. Recently, Latch and McNeill (2006) observed that irradiated humic acid solutions can product singlet oxygen in aquatic systems which can react with proteins, DNA, and other bimolecular which are also reported in our previous studies (Lu et al., 1988; Cheng et al., 1999, 2003). It seems that HA could be a faster free radicals donor when compared with As in this study.

By increasing the concentration of As in the medium, the cellular GSH content rose to 110–150% of control (Fig. 6), which may be caused by up regulation of GSH synthesis (Li and Chou, 1992) and decreased the ROS contents in the β cells when compared with 2 h exposure (Fig. 5). On the other hand, higher concentration HA would consume the cellular GSH pool which was agreed with the finding of Cheng et al. (1999, 2003). HA and As exert β cells dysfunction and inhibited insulin secretive effects in the in vitro studies (Fig. 7). Furthermore, the inhibition effects of insulin secretion in HIT-T15 cells could be restored by pretreatment of NAC, a precursor of GSH and an antioxidant (Fig. 8). Elevation of GSH in response to HA and As exposure may reflect a self-protective mechanism against cellular injury (Li and Chou, 1992).

ICR mice were divided into four groups and received different content of HA and As in drinking water for 12 weeks. Fig. 9 shows the plasma insulin was decreased in the 5, 7, and 12 weeks after exposure of 500 mg HA/L or 10 mg/L As_2O_3. On the contrary, plasma insulin was increased ranging from 1.11 to 1.78-fold of control group at early stage of exposure (3 weeks). This phenomenon of hyperinsulinemia in early stage of exposure and hypoinsulinemia in later stage of exposure is more likely to that of T2DM, which is also demonstrated by the prospective study carried out in the BFD-hyperendemic villages in Taiwan (Tseng et al., 2000). The results of microscopy shown the sub-acute peri-pancreatitis and islet damage caused by the infiltration of inflammatory cells in pancreas after exposure of HA and As in drinking water for 5 weeks (Fig. 10). Matsumura et al. (2001) suggest that oxidative stress is primary rather than secondary to infiltration of inflammatory cells in the pancreas which is more vulnerable to prolonged oxidative stress compared with the liver and kidney. Based on these observations, evaluation of ROS may be the important pathogenic factors of pancreatitis. It is clearly demonstrated that further studies are needed to better characterized the molecular mechanisms involved in the interactions with GSH, antioxidation enzymes, ROS, and pathological lesions in the cells and tissues, which will provide further information of the diabetogenic effect of the HA and AS under our progressive studies.

5. Conclusion

A high concentration of humic acid (approximately 200 ppm), and arsenic (approximately 0.05–1.98 ppm) are present in the artesian well water from BFD-endemic area (Huang et al., 1994; Tseng et al., 1961). The in vivo and in vitro results reported herein reveal that HA and As exert β cell dysfunction, inhibited insulin secretive effects, and pathological lesions of pancreas. Based on our data, the increased oxidative stress induced by HA and As could cause damage to the pancreatic islets cells and impairment in insulin secretion. On the other hand, the inhibition effects of insulin secretion in HIT-T15 cells could be restored by pretreatment of NAC, a precursor of GSH and an antioxidant. Elevation of GSH in response

to HA and As exposure may reflect a self-protective mechanism against cellular injury (Li and Chou, 1992).

The plasma insulin of mice, which were exposed to HA and As in drinking water for 12 weeks, was decreased in the 5, 7, and 12 weeks. On the contrary, plasma insulin was increased ranging from 111% to 178% compared to that of control group at early stage of exposure (3 weeks). This phenomenon of hyperinsulinemia in early stage of exposure and hypoinsulinemia in later stage of exposure is more likely to that of T2DM. In addition, the sub-acute peri-pancreatitis and islet damage caused by the infiltration of inflammatory cells after exposure of HA and As in drinking water for 5 weeks. Our study has important implications in the diabetogenic effect of the HA and AS which may be mediated by ROS and further information of the toxicity mechanisms will provide under our progressive studies.

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