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Oxidatively damaged DNA induced by humic acid and arsenic in maternal and neonatal mice

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ABSTRACT

We measured the levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), a useful biomarker of oxidative injury, in liver (or urine) of maternal mice and their offspring, that were treated with humic acid (HA) and arsenic (As) alone, or their combination during pregnancy. A highly sensitive and specific isotope-dilution LC–MS/MS method was used for 8-oxodGuo quantification. Total arsenic accumulated in the offspring was also measured using ICP–MS. This study demonstrated, for the first time in an animal model, that HA alone treatment led to a significant increase of 8-oxodGuo levels both in liver and urine of maternal mice. No enhanced effect was observed when HA was combined with As, compared with the As alone treatment. With regard to the associated offspring, elevated levels of 8-oxodGuo and total arsenic were observed in offspring only when mother mice were treated with As and its combination with HA, but not for the HA-treated alone. It was worthy to note that the offspring from maternal combined treatment with HA and As had a significantly lower 8-oxodGuo than those of maternal treatment with As alone. This could be explained by that part of As formed complexes with HA and these macromolecules of HA–As complexes may not readily cross the placenta to the fetus, as evidenced by the lower accumulated total As observed in the offspring livers. Overall, it seems that HA may be detrimental to the maternal mice, but in the meantime it can be beneficial to the offspring by reducing free As.

1. Introduction

Arsenic (As) poisoning has become one of the major environmental concerns in the world as millions of people have been exposed to excessive arsenic through contaminated drinking water (McLellan, 2002; Schoen et al., 2004). Arsenic is a known human carcinogen that has been associated with cancers of the skin, lung, urinary bladder, and possibly liver, kidney and prostate in humans (IARC, 2004).

In south-western Taiwan, long-term arsenic exposure has been found to induce blackfoot disease (BFD), a unique peripheral arterial occlusive disease. The clinical symptoms include numbness or coldness of one or more extremities, resulting in black discoloration, ulceration or gangrenous changes to the extremities (Tseng, 1989). Although numerous epidemiological studies have shown a causal association between drinking-well water containing arsenic and the occurrence of BFD, the exact pathological mechanisms of BFD is still unclear (Tseng, 2005).

In addition to arsenic, Lu (1990a) reported that artesian-well water of the area of Taiwan to which BFD is endemic also contained a high level of fluorescent compounds (approximately 200 mg L^{-1}). These fluorescent compounds purified from well water were found to induce blackening of the tail and feet of experimental mice (BFD-like symptoms), and has been implicated as a possible etiological factor for BFD (Lu and Liu, 1986; Lu, 1990a). These fluorescent compounds were further identified as humic acid (HA) by infrared (IR) and atomic absorption spectrometry (Lu et al., 1988; Lu, 1990b). HA is a group of high molecular weight polymers that are products of the decomposition of organic matter, particularly dead plants, and exists abundantly in peat, soil, well water and other sources. Chemical and IR spectroscopic analyses

Abbreviations: 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; HA, humic acid; ROS, reactive oxygen species; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ICP–MS, inductively coupled plasma–mass spectrometry.

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reveal that HA consists of an aromatic phenol core with aliphatic side chains, with functional groups such as carboxyl, carbonyl, phenolic hydroxyl, hydroxyl and methoxyl attached to the benzene rings and the side chains of the polymeric phenolic structure. Previously, HA has been shown to generate reactive oxygen species (ROS) such as superoxide anion (Vaughan and Ord, 1982) and found to cause depletion of glutathione and several antioxidant enzymes as well as sister chromatid exchanges in cell cultures (Liang et al., 1998; Cheng et al., 1999; Hseu et al., 2008). Despite considerable evidence indicating that HA causes oxidative injury in *in vitro* studies, little information has been available on the relationship between oxidatively damaged DNA and HA exposure in animal models as well as its interaction with arsenic.

Inorganic arsenic has been extensively studied as a teratogen in animals. Data from animal studies have demonstrated that arsenic is readily transferred to the fetus and produces developmental toxicity and cancers in rodents (Golub et al., 1998; Waalkes et al., 2007). Meanwhile, the potential adverse effect of HA on developmental outcome has yet to be fully examined in offspring. In the present study, we therefore investigated the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), a useful biomarker of oxidatively damaged DNA, in maternal mice as well as their offspring, that were treated with HA and As alone or their combination during pregnancy. A highly sensitive quantitative method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) was applied to measure 8-oxodGuo either in liver DNA or urine of mice. Total arsenic accumulated in offspring livers were also measured using inductively coupled plasma–mass spectrometry (ICP–MS).

2. Materials and methods

2.1. Chemicals

Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: As_2O_3, Triton X-100, sodium dodecyl sulfate (SDS), desferrioxamine mesylate salt (DFO), proteinase K, nuclease P1, unlabeled 8-oxodGuo and 2′-deoxyguanosine (dGuo) (Sigma–Aldrich); HNO_3 (J.T. Baker); RNase A, RNase T1 and alkaline phosphatase (Roche Diagnostic); $^{15}N_5$-2′-deoxyguanosine ($^{15}N_5$-dGuo) and $^{15}N_5$-8-oxo-7,8-dihydro-2′-deoxyguanosine ($^{15}N_5$-8-oxodGuo) (Cambridge Isotope Laboratories).

2.2. Humic acid (HA) purification

HA was prepared from a commercial available humate salt (Sigma–Aldrich). HA was purified as described (Yen et al., 2007). Briefly, HA was dissolved in 1 N NaOH solution (pH > 10), and any undissolved material was removed by filtration. The solution was then acidified with 1 N HCl to pH < 2.0 to precipitate the HA, and the resulting precipitate was collected by centrifugation at 3000*g* for 30 min and redissolved in 1 N NaOH. This precipitation and dissolution procedure was repeated three times to obtain highly purified HA as described by Schnitzers (1982). The final precipitate was dissolved in 0.1 N NaOH, and the pH of the solution was adjusted to 7.2–7.4, followed by freeze-drying to obtain a powdered product of HA.

2.3. Experimental design

Female CD-1 (ICR) mice were obtained from BLT (BioLASCO Taiwan CO. Ltd.) fed with MF-18 standard Chow (Oriental Yeast Co., Japan), and maintained at 22 ± 1 °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 in-house guidelines for the care and use of laboratory animals at the Chung Shan Medical University. The experimental design is shown in Fig. 1. Briefly, pregnant mice were divided randomly into four groups and were treated daily (from day 8 to 18 of gestation) by intragastric gavage with HA (0.125 mg g b w^{-1}) and As as arsenic trioxide (0.0125 mg g b w^{-1}) alone or their combination, and normal saline (0.3 mL) as a control group. After delivery, the maternal mice continuously received the treatment in drinking water during their breeding period; the doses were as followed: 500 mg L^{-1} for HA-treated group, 50 mg L^{-1} As_2O_3 for As-treated group and 500 mg L^{-1} HA + 50 mg L^{-1} As_2O_3 for the combined treatment group. The maternal mice were sacrificed after all the respective offspring had been killed and the experiment was terminated. The livers were collected and stored at −80 °C before analysis. The urine output from each maternal mouse was also collected daily using metabolic cages for consecutive 3 days prior to sacrifice (i.e. day 21–24 after the last group of offspring was weaned and sacrificed).

At birth, the offspring of each group were weighed and separately sacrificed at day 1, 5, 10 and 21, and the livers were collected and stored at −80 °C before analysis.

2.4. Mouse liver DNA isolation

Mouse liver DNA isolation was performed according to the procedure recommended by the European Standard Committee on Oxidative DNA Damage (ESCODD) (Gedik et al., 2005). Briefly, approximately 100 mg of liver tissue was homogenized with 3 mL of buffer A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.5, 0.1 mM DFO and 1% (v/v) Triton X-100). After homogenization, the sample was centrifuged at 1500*g* for 10 min. The resulting pellet was washed with 1.5 mL of buffer A and recovered by centrifugation (1500*g* for 10 min). A total of 600 μL of buffer B (10 mM Tris/HCl, pH 8, 5 mM EDTA-Na_2, 0.15 mM DFO) and 35 μL of 10% (w/v) SDS was added, and the sample agitated vigorously. After 30 μL of RNase A (1 mg mL^{-1}) in RNase buffers (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 2.5 mM DFO) and 8 μL of RNase T1 (1 U μL^{-1} in RNase buffer) were added, the samples were incubated at 37 °C for 1 h to remove contaminating RNA from the DNA. After the removal of RNA, 30 μL of proteinase K (20 mg mL^{-1}) was added and the samples were incubated at 37 °C for 1 h. Subsequently, 1.2 mL of NaI solution (7.6 M NaI, 40 mM Tris/HCl, pH 8.0, 20 mM EDTA-Na_2, 0.3 mM DFO) and 2 mL of 2-propanol were added. The sample was gently shaken until the DNA had precipitated completely and then centrifuged at 5000*g* for 15 min. The DNA pellet was washed with 1 mL of 40% (v/v) 2-propanol. After centrifugation (5000*g* for 15 min) the DNA pellet was washed with 1 mL of 70% (v/v) ethanol. Finally, DNA pellet was collected by centrifugation and dissolved in 0.1 mM DFO overnight. DNA concentration was measured by the absorbance at 260 nm. Routinely, 150–300 μg DNA was obtained per 100 mg of liver tissue. Protein contamination was checked using the absorbance ratio A260/A280; an absorbance ratio over 1.6 was acceptable.

2.5. Enzymatic hydrolysis of DNA

DNA hydrolysis was performed as described by Chao et al. (2008). Briefly, DNA samples (20 μg) from mouse liver were spiked with 2.82 pmol of $^{15}N_5$-8-oxodGuo and 84.3 pmol of $^{15}N_5$-dGuo. The DNA solutions were then added 10 μL of 1U μL^{-1} nuclease P1 (in 300 mM sodium acetate and 1 mM $ZnSO_4$, pH 5.3) and the DNA was incubated at 37 °C for 2 h. Thereafter, 10 μL of 10 × alkaline phosphatase buffer (500 mM Tris/HCl, pH 8, 1 mM EDTA) together with 0.2 μL of alkaline phosphatase were added and the incubation was pursued at 37 °C for 2 h. Subsequently, 10 μL of 0.1 M HCl was added to neutralize the solution. The neutralized DNA hydrolysates were then directly measured by LC–MS/MS.

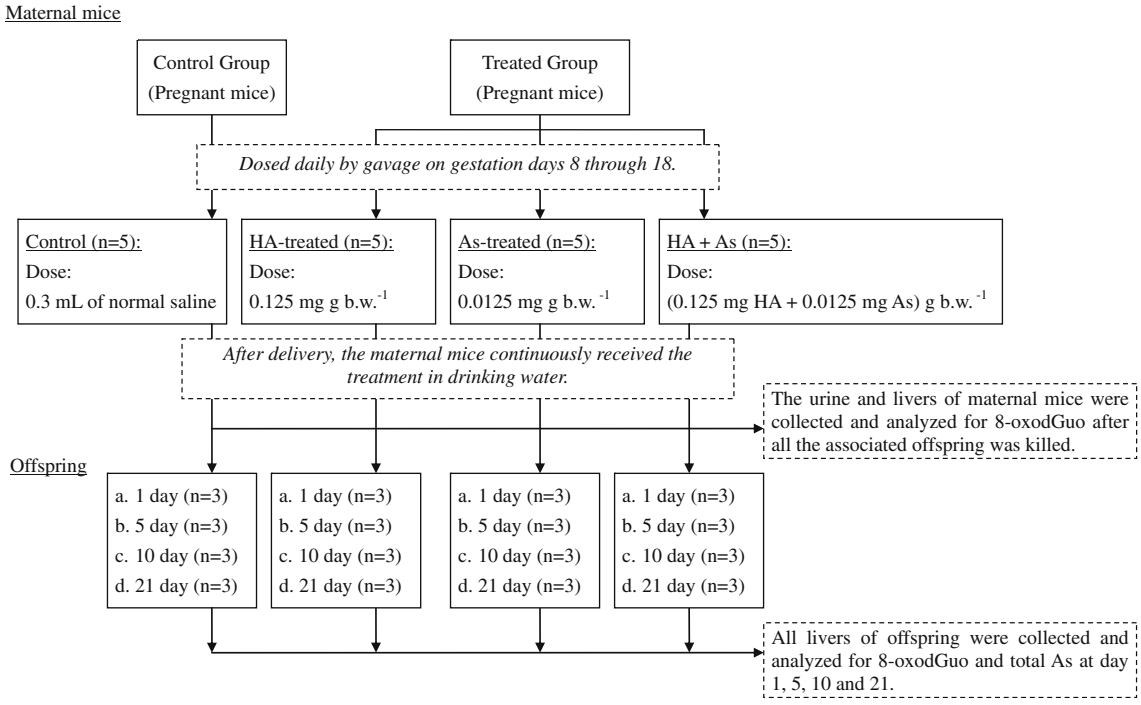

Fig. 1. Experimental scheme.

2.6. Analysis of 8-oxodGuo in tissue DNA and urine of mice

8-OxodGuo concentrations in liver DNA were measured using a validated method of LC–MS/MS with on-line solid-phase extraction (SPE) as recently reported (Chao et al., 2008). Briefly, after automatic sample cleanup, LC–MS/MS analysis was performed using a PE Series 200 HPLC system interfaced with a PE Sciex API 3000 triple quadrupole mass spectrometer with electrospray ion source (ESI). Detection was performed in the positive ion multiple reaction monitoring (MRM) mode for simultaneous quantitation of 8-oxodGuo and dGuo, and the transitions of the precursors to the product ions were as follows: 8-oxodGuo (m/z 284 → 168), $^{15}N_5$-8-oxodGuo (m/z 289 → 173), dGuo (m/z 268 → 152), and $^{15}N_5$-dGuo (m/z 273 → 157). With the use of isotopic internal standards and on-line SPE, this method exhibited a low limit of detection (LOD) of 1.8 fmol for 8-oxodGuo, which corresponds to 0.13 adducts/10^6 dGuo when using 20 μg of DNA per analysis.

Urinary 8-oxodGuo concentrations in mice were also measured using a validated method of LC–MS/MS with on-line SPE as previously reported (Hu et al., 2006). 20 μL of urine was diluted 10-fold with 5% methanol containing 0.1% formic acid. After addition of 40 μL of $^{15}N_5$-8-oxodGuo solution (20 μg L^{-1} in 5% methanol/0.1% formic acid) as internal standard, a 100 μL of prepared urine sample was directly analyzed using the same LC–MS/MS as described above. The samples were analyzed in the positive ion MRM mode and the transitions of the precursors to the product ions were as follows: m/z 284 → 168 (quantifier ion) and 284 → 140 (qualifier ion) for 8-oxodGuo, m/z 289 → 173 (quantifier ion) and 289 → 145 (qualifier ion) for $^{15}N_5$-8-oxodGuo. With the use of isotopic internal standards and on-line SPE, this method had a high sensitivity with LOD of 5.7 ng L^{-1} (2.0 fmol) on column.

2.7. Analysis of total arsenic in mice liver

Total arsenic analysis was performed as described by Kozul et al. (2008) with several modifications. Briefly, approximately 0.1–0.2*g* of mice livers was weighed into a Teflon microwave digestion vessel and 10 mL of concentrated HNO_3 was added. The samples were microwave digested at 210 °C with a ramp time and hold time of 5 min each (MARS5 CEM Corporation). The digested samples were made up to a volume of 25 mL with H_2O and analyzed for total arsenic concentrations using an Elan 5000 inductively coupled plasma mass spectrometer (Perkin–Elmer). Method accuracy was evaluated by a recovery test using two known amounts of As (0.02 and 0.04 μg) spiked into mice liver prior to digestion. The recovery of the present method obtained was 96–105%.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation. Data from the treated and control groups were compared using the Student's *t*-test. All comparisons were considered significantly different when $p < 0.05$.

3. Results

3.1. Animal body weights and growth rates

Body weights of pregnant mice were monitored throughout the pregnancy period and the results were shown in Fig. 2. There were no significant differences between the HA- or As-treated mice compared to controls during pregnancy. However, when mice were treated with the combination of HA and As, the body weights were apparently lower than other groups at day 17–18 of gestation. The growth rates of mice were further estimated using the ratios between the body weights at day 18 and day 8. The mean ratios of weights between day 18 and day 8 were 1.73 ± 0.04, 1.76 ± 0.10, 1.71 ± 0.03 and 1.52 ± 0.11 for control, HA, As and HA + As treatment groups, respectively. The combined treatment group had a significantly lower growth rate than the control group ($p < 0.005$ by two-tailed *t*-test). Although the growth rates differed, the fur of all animals remained smooth and shiny throughout the experiment and no observable behavioral changes (grooming, gait, etc.) were noted.

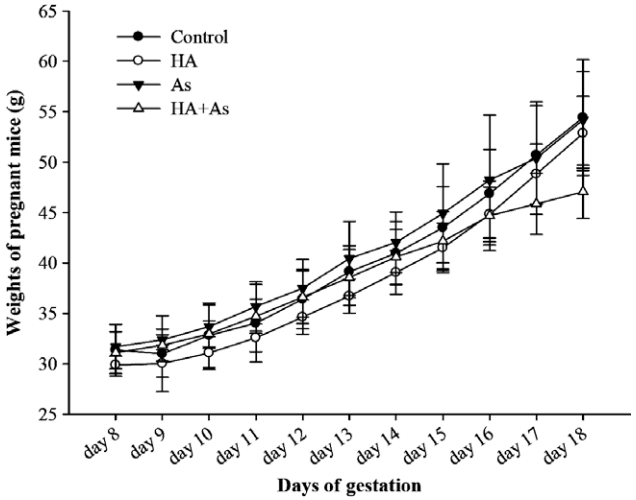

Fig. 2. Mean body weights of maternal mice during day 8–18 of gestation.

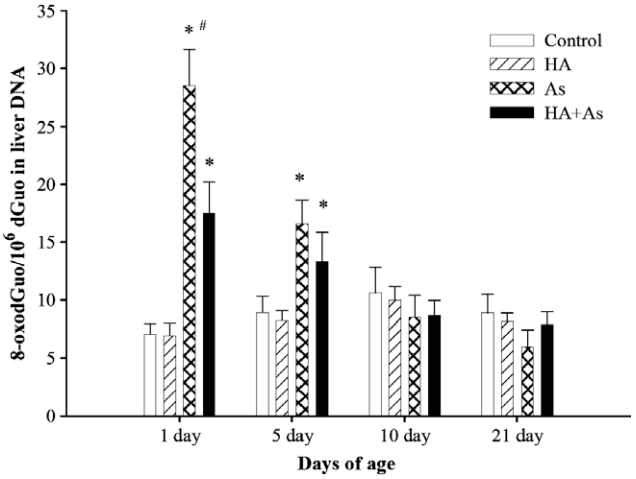

Fig. 3. Mean liver 8-oxodGuo levels in offspring at age day 1, 5, 10 and 21. $^{*}p < 0.05$ compared with controls. $^{\#}p < 0.05$ compared between those born to HA + As-treated and those born to As-treated alone.

3.2. 8-OxodGuo concentrations in liver DNA and urine of maternal mice

After weaning, the maternal mice were killed, and the urine and liver tissues were collected and analyzed for 8-oxodGuo. The 8-oxodGuo levels of maternal mice were summarized in Table 1. When mice were treated with HA, As or their combination by daily gavage, the liver levels of 8-oxodGuo were significantly increased in comparison with that of controls ($p < 0.05$ by two-tailed t-test). Although the HA + As-treated group had the highest level of 8-oxodGuo in liver, it did not significantly differ from that of As-treated alone maternal mice. Similarly, the urinary 8-oxodGuo concentrations of treated groups were found to be significantly higher than that of control group ($p < 0.05$) and no significant between-group differences were observed among the treated groups.

3.3. 8-OxodGuo concentrations in liver DNA of offspring

At birth, the offspring of each group were separately sacrificed at day 1, 5, 10 and 21, and the livers were collected and analyzed for 8-oxodGuo. The results were shown in Fig. 3. At day 1, it was noted that the maternal treatment with HA did not cause increased 8-oxodGuo levels in offspring liver as compared to that of control. The elevated levels of 8-oxodGuo were only observed in offspring that were born to the mothers treated with As alone or its combination with HA, when compared with that of controls. Meanwhile, the offspring that were born to As-treated mothers had significantly higher 8-oxodGuo than those born to As + HA-treated mothers (mean ± SD: 28.5 ± 3.2 vs. 17.5 ± 2.7 8-oxodGuo/10^6 dGuo, $p < 0.05$). At day 5, the 8-oxodGuo levels markedly decreased by 42% (16.6 ± 2.7 8-oxodGuo/10^6 dGuo) from day 1 in offspring that were born to As-treated pregnant mice, but still remained significantly higher in comparison with the control group ($p < 0.05$). Meanwhile, the 8-oxodGuo levels in offspring that were born to As + HA-treated mothers had slightly decreased (13.3 ± 2.5 8-oxodGuo/10^6 dGuo) at day 5 and remained significantly higher in comparison with the control group ($p < 0.05$). By day 10, the 8-oxodGuo levels in both offspring that were born to As- and As + HA-treated mothers had significantly decreased (As-treated: 8.5 ± 1.9 and As + HA-treated: 8.6 ± 1.3 8-oxodGuo/10^6 dGuo) and no significant difference in liver levels of 8-oxodGuo were observed between the offspring that were born to treated and control pregnant mice. Similarly, no significant difference was found between the treated and control groups in day 21 with the levels around 6.0–8.9 8-oxodGuo/10^6 dGuo.

3.4. Total arsenic concentrations in livers of offspring

The mean concentration of total As in livers of offspring were determined as shown in Fig. 4. At day 1, the greatest total As concentration (548.9 ± 41.5 ng g^{-1}) was observed in offspring that were born to the mothers treated with As alone, and was significantly higher than those born to mothers treated with the combination of As and HA (As + HA-treated: 294.9 ± 32.3 ng g^{-1}, $p < 0.05$). For the maternal treatment with HA only, the mean total As concentrations in offspring livers did not differ significantly to that of controls. At day 5, the elevated As concentrations were dramatically decreased by 83% (94.6 ± 27.8 ng g^{-1}) from day 1 in offspring that were born to As-treated maternal mice, but still remained significantly higher than the control group ($p < 0.05$). Similarly, the mean total As concentration in offspring that were born to As + HA-treated mothers was also highly decreased by 87% (39.0 ± 11.6 ng g^{-1}) at day 5, and remained significantly higher than the control group ($p < 0.05$). By day 10, the mean concentration of total As in both offspring that were born to As- and As + HA-treated mothers were continuously decreased and no significant differences were observed between the offspring that were born to treated and control maternal mice.

4. Discussion

In the present study, we investigated the oxidatively damaged DNA induced by HA with or without arsenic using an animal

Table 1
Mean 8-oxodGuo concentrations in liver DNA and urine of maternal mice.

Group	Control ($n = 5$)	HA ($n = 5$)	As ($n = 5$)	HA + As ($n = 5$)
Liver (8-oxodGuo/10^6 dGuo)	7.3 ± 1.0	11.2 ± 1.4[a]	15.6 ± 3.7[a]	17.1 ± 2.5[a]
Urine (ng mL^{-1})	2.15 ± 1.59	6.59 ± 2.33[a]	9.04 ± 2.91[a]	7.00 ± 4.77[a]

[a] Statistically different from controls ($p < 0.05$).

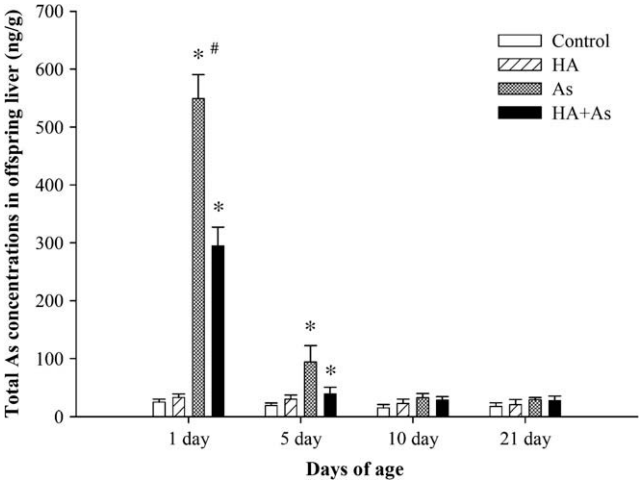

Fig. 4. Mean total As concentrations in offspring livers at age day 1, 5, 10 and 21.*$p < 0.05$ compared with controls. #$p < 0.05$ compared between those born to HA + As-treated and those born to As-treated alone.

model. We measured 8-oxodGuo levels in liver DNA (or urine) of maternal mice and the associated offspring, that were treated with HA, As or their combination during pregnancy. Formation of 8-oxodGuo was targeted in this study because of its abundance and mutagenic potential generating G–T transversion, and its concentration could be a good indicator of reactive oxygen species and a potential biomarker of carcinogenesis *in vivo* (Cooke et al., 2006). Moreover, since there is evidence that substantial oxidation of dGuo occurs during the protracted treatment of DNA samples in preparation for chromatographic analysis (ESCODD, 2003), we used the protocols suggested by ESCODD (Gedik et al., 2005) for liver DNA extraction and hydrolysis to minimize/eliminate the artifactual formation of 8-oxodGuo. In this study, the highly sensitive and specific isotope-dilution LC–MS/MS method with on-line SPE (Chao et al., 2008) was adapted to quantify the 8-oxodGuo levels in small amounts of liver DNA.

It is well established that ROS generation is one of several proposed mechanisms of action for arsenic-induced carcinogenesis (Schoen et al., 2004; Liu and Waalkes, 2008). A previous animal study has shown that arsenic exposures through drinking water (100 mg L^{-1} arsenite) increased the ROS level in blood and tissues and decreased the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (Mittal and Flora, 2006). It has also been observed that arsenic treated mice (2 mg L^{-1} arsenite in drinking water) had significantly increased cellular 8-oxodGuo in brain (up to ~22 8-oxodGuo/10^6 dGuo) (Piao et al., 2005). Our results for arsenic are in agreement with previous studies.

HA is a group of high molecular weight macromolecules consisting of complex polymeric aromatic structures. HA have been shown to exist in the gastrointestinal tract of humans and animals and could circulate in the blood (Visser, 1973; Klockling, 1994). They are also known to be metabolized by liver extracts (Sato et al., 1986). Although several *in vitro* studies have shown that the pretreatment with HA induced ROS and DNA damage in a dose- and time-dependent manner in cultured human cells (Gau et al., 2001; Hseu et al., 2008), minimal *in vivo* data are available. Only one *in vivo* study demonstrated that HA induced both structural and numerical chromosome abnormalities in intestinal cells of mice after oral administration of 0.1 mg g b w^{-1} of an aqueous solution of HA (Bernacchi et al., 1996). In the present study, it was of interest that oral treatment with HA was found to significantly increase 8-oxodGuo in mice liver DNA as well as in urine (see Table 1). To our best knowledge, this is the first study showing that HA exposure significantly induced oxidative DNA damage *in vivo* by measuring 8-oxodGuo levels. It is generally accepted that the 8-oxodGuo levels in cellular DNA represents the steady-state level of 8-oxodGuo resulting from a dynamic balance between the rate of DNA repair and the continued oxidative damage to DNA induced by the toxicants present in the tissue, while the 8-oxodGuo levels in urine could be attributed to DNA repair and possibly the hydrolysis of 8-oxodGTP in the nucleotide pool (Cooke et al., 2008). In this study the elevated 8-oxodGuo levels both observed in liver and urine (Table 1) could further substantiate the occurrence of severely oxidative injury induced by HA (and As) exposure.

Furthermore, HA has a strong affinity towards metal cations and can exist as an organometallic complex with As, Fe, Cu, Cr, Pb, Cd, Zn, etc. (Lu et al., 1988; Lind and Glynn, 1999; Paciolla et al., 1999; Buschmann et al., 2006). Because of its complexing properties, HA has been studied for the possible role in detoxification of heavy metals (Klockling, 1994; Tsiridis et al., 2006). However, some contrary results were also reported demonstrating that HA-metal complexes could be more potent than metal ions alone (Paciolla et al., 1999; Hseu et al., 2001; Hseu and Yang, 2002). Interestingly, in this study, the 8-oxodGuo levels in liver DNA (and urine) of the combined treatment maternal mice did not differ significantly from that of As-treated maternal mice (Table 1), implying no enhanced effect was observed in the present animal model, although it still remained significantly higher than that of controls. Nevertheless, a significant reduction in body weight gain was observed for the combined treatment mice (see Fig. 2) and needs further investigation.

Inorganic arsenic or its metabolites can cross the placenta to fetus and produce developmental toxicity in embryo culture or in animal experiments (Golub et al., 1998; Devesa et al., 2006; Jin et al., 2006). Indeed, this study demonstrated significantly high As concentrations accumulated in the livers of new born mice following maternal treatment with arsenite (see Fig. 4, day 1). We further found that maternal oral treatment with As (0.0125 mg g b w^{-1} by daily gavage) significantly increased the liver levels of 8-oxodGuo in newborn mice up to 4-times higher than control (see Fig. 3, day 1). Our data may help to address the involvement of ROS in the developmental toxicity induced by arsenic. On the contrary, it was found that the maternal treatment with HA did not increase the cellar levels of 8-oxodGuo in the offspring (see Fig. 3). This could be due to the fact that HA is a group of polymers with high molecular weights and may not be able to cross the placenta to cause oxidatively damaged DNA in offspring.

Though the maternal treatment with the combination of HA and As has significantly increased the 8-oxodGuo levels in their offspring when compared with controls, its 8-oxodGuo levels induced was significantly lower than maternal treatment with As alone (see Fig. 3, day 1). This finding could be explained by that part of arsenite which formed complexes with HA may not readily cross the placenta. The resulting 8-oxodGuo caused by free arsenic that was able to cross the placenta, was thus restrained in fetus. It has been reported that approximately 10–40% of arsenite is bound to soluble HA to form HA–As complexes in natural system (Bissen and Frimmel, 2003; Ko et al., 2004; Buschmann et al., 2006). Such phenomenon could be also evidenced by a relatively lower total As concentration accumulated in the livers of offspring that were born to As + HA-treated maternal mice, compared to that of As-treated alone maternal mice (day 1, see Fig. 4). Our study suggested that HA may have adverse effects to the maternal mice but in the meantime it also protected the offspring from the As-induced damage.

The elevated levels of 8-oxodGuo in offspring liver at day 1 gradually decreased with time as a result of DNA repair processes (see Fig. 3) and returned to the background levels within 10 days. In previous animal experiments, Zastawny et al. (1996) and

Hamilton et al. (2001) reported that the 8-oxodGuo was rapidly repaired in livers of rats and mice after γ-irradiation, in which 8-oxodGuo levels were shown to return to background levels within 1 h. Thaiparambil et al. (2007) also demonstrated that the increased levels of 8-oxodGuo in mice lung following exposure to cigarette smoke was significantly removed by 16–20 h after the cessation of smoke exposure. Apparently, a longer persistence of As-induced 8-oxodGuo was observed in the offspring in the present study. This could be explained by that it takes time to metabolize/eliminate the arsenic in offspring after cessation of exposure. Indeed, arsenicals have been suggested to be cleared from tissues with half times of a few days in mice (Hughes et al., 2003; Waalkes et al., 2007), and the residual arsenic present in the offspring may have continuously induced ROS, resulting in the persistent 8-oxodGuo generated in liver. The elimination rate (i.e. $t_{1/2}$) of arsenic in offspring in this study was estimated to be of ~48 h, which was similar to a previous study with an estimated $t_{1/2}$ of 45 h (Hughes et al., 2003).

Meanwhile, a continuous exposure of arsenic to offspring through breast milk may have also accounted for the long persistence of 8-oxodGuo. Concha et al. (1998) and Fängström et al. (2008) have reported that low levels of arsenic are detected in human breast milk of mothers exposed to high concentrations of arsenic in the drinking water. However, this is less likely in our case since the total As concentration in offspring livers eventually returned to the background levels within 10 days even with continuous exposure over 21 days (see Fig. 4).

In conclusion, this study has demonstrated for the first time that HA could induce oxidative stress in mice by increasing the 8-oxodGuo levels in liver DNA as well as urine. However, the combined treatment of HA and As in our study failed to show the enhanced effect on oxidative damage. With regard to the potential adverse effect of maternal exposure on their offspring, we demonstrated that although HA may have significantly induced oxidatively damaged DNA in maternal mice, it certainly protected their offspring from the As-induced oxidative damage by complexation with As. However, some limitations of this study should be mentioned for consideration in future research. One is that the additional measurements of HA in tissues are required to provide more information on the actual internal exposure of maternal mice as well as the offspring. Nevertheless, to date, the exact structure for HA is unknown. Though the HA has been previously measured by fluorescence spectrophotometer or by using the total organic carbon (TOC) as a surrogate in environmental studies (Buschmann et al., 2006; Subbiah and Mishra, 2008), the measurement of HA has been problematic *in vivo* because of the interferences of biological matrix. Secondly, more evidence in terms of antioxidant defense measurement, other markers of oxidative stress, metabolism or repair activity would be helpful to substantiate our present findings on roles of HA- and/or As-induced oxidative stress.

5. Declaration of interest

The authors report no conflicts of interest.

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