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Single Cell Gel Electrophoretic Analysis of 2-Aminoanthracene Exposed F-344 Rats

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ABSTRACT

To assess DNA damage from exposure to 2-Aminoanthracene (2AA), Single Cell Gel Electrophoresis (SGCE) was employed to examine single strand breaks in blood of Fisher-344 rats. Although subcellular injury due to 2-AA exposure has been noted in the past, there is yet to be a direct demonstration of genetic alterations due to 2-AA intoxication. In the current study, alkaline comet assay was used to evaluate the type of DNA damage that is on-going in the blood samples of F-344 animals. The animals were fed control (0 mg kg^{-1}), LD (50 mg kg^{-1}), MD (75 mg kg^{-1}) and HD (100 mg kg^{-1}) 2-AA diet for two and four weeks. At the end of each exposure period (14 or 28 days), rats were euthanized with CO_2 and blood was collected by cardiac puncture. Twenty micro liters of whole blood samples were added to 1 mL of Hanks Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+}) and subsequently snapped frozen in liquid nitrogen. Following comet assay analysis, single strand break were assessed via tail moment, tail length, tail intensity and head intensity scoring of comets along with cell viability. There seems to be an apparent dose response. That is 50, 75 and 100 mg kg^{-1} diet 2-AA rats when compared with the control demonstrated significant ($p<0.05$) damage as measured by tail length and tail moment values. Head and tail intensity values for intoxicated animals were also significant at 2 and 4 weeks relative to the controls, although there were no dramatic shifts between exposure time.

Key words: 2-Aminoanthracene (2-AA), comet assay, single strand break, DNA damage

INTRODUCTION

Arylamines are known carcinogens that occur both naturally and synthetic form. They are employed in the manufacture of dyes, drugs, inks, rubber antioxidants, plastics and agricultural chemicals. These aromatic amines are also used as curing agents in synthesizing epoxy resins and polyurethanes and are found in road tars and synthetic fuels. 2-Amino anthracene is the benchmark aromatic amine for toxicity studies. It is a model aryl amine because relatively, 2-Amino anthracene is potent direct-acting carcinogen and induces mutations in eukaryotic and prokaryotic cells (Boudreau *et al*., 2006; Snyderwine *et al*., 1992; Zhu *et al*., 1995).

It is reported that 2-Aminoanthracene is a typical aromatic amine. In order for 2-AA to become toxic, it is biotransformed via drug metabolizing enzymes such as N-acetyltransferases (NAT) and cytochrome P450 (P450) primarily in the liver. The process involves an initial oxidation in the liver by P450 enzymes to a more reactive N-hydroxylamine followed by NAT enzymes metabolism to produce highly reactive O-substituted N-hydroxylamine intermediates. This electrophilic reactive

metabolites form DNA-adducts (So *et al.*, 2008; Sugamori *et al.*, 2006; Watson *et al.*, 1995). Previous research has inferred subcellular injury due to 2-AA exposure, there is yet to be a direct demonstration of genetic alterations. It is therefore the goal of this study to evaluate what type of DNA damage that is on-going. We employ alkaline Single Cell Gel Electrophoresis (SCGE) to analyze DNA break in Fisher-344 rats. The SCGE is normally referred to as the Comet assay.

The Fisher 344 rat inbred strain was developed in 1920 to address the lack of reproducible animal model for cancer research. In 1970, the National Cancer Institute selected Fisher 344 rat as a replacement for the Osborne-Mendel rat model in cancer bioassay program because tumor latency due to chemical exposure is relatively short whilst maintaining good survivability. Fairly recent literature has indication that F-344 rats are prone to exhibit inflammatory effects and mononuclear cell leukemia due to exposure of a range of chemicals and pharmaceuticals. Nevertheless, this animal model has been employed in as many cancer, toxicological, aging, neurological, organ transplant, heart disease etc., studies in the literature (Cameron *et al.*, 1985; Caldwell, 1999; Uchida *et al.*, 2008; Gemma *et al.*, 2004).

Single cell gel electrophoresis analysis also referred to as the comet assay is a simple biochemical method that has been used to measure DNA strand breaks in eukaryotic cells. Compared with other genotoxicity assays, the comet assay is sensitive because it can detect low levels of DNA damage, is able to employ small number of cells per sample, is flexible, low cost, ease of use and a short duration required to complete a study. The most common version uses alkaline pH>13 for unwinding and electrophoresis. At this pH, a broad spectrum of DNA damage can be detected and as such, is the first step in cases of regulatory compliance. Briefly, the assay involves; a cell suspension is obtained followed by microscopic slides being layered with cells in agarose. Then cells are lysed to liberate DNA and exposed to alkali buffer (pH>13) in order to obtain single-stranded DNA as well as show alkali-labile sites as single stranded breaks. Electrophoresis is undertaken under alkaline conditions after which samples are neutralized, nucleotides stained and comet visualized and scored (Tice *et al.*, 2000; Hartmann *et al.*, 2003a; Collins, 2004; Nirmala *et al.*, 2007).

In this study, single strand DNA damage was assessed via alkaline single cell gel electrophoresis analysis, otherwise known as comet assay in F-344 rats exposed to various concentration of 2-AA.

MATERIALS AND METHODS

Diet preparation: 2-AA (CAS# 613-13-8) [98+% Pure] was obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. A kilogram of rat diet supplied by PMI Nutrition International, LLC (Brentwood, MO) was immersed in 1 L molecular grade ethyl alcohol containing the mass of 2-AA necessary to yield the target dose concentrations in the diets and the ethyl alcohol evaporated under the hood with periodic thorough mixing to assure homogeneity. The diet was stored in the freezer and protected from light until given to the animals.

Experimental design: Twenty-four post-weaning 3-4 week old Fisher 344 rats were purchased from Harlan Laboratories and randomly assigned to one of four dietary dose regimens of 0 mg kg^{-1}-diet (control), 50 mg kg^{-1}-diet (low dose), 75 mg kg^{-1}-diet (medium dose) and 100 mg kg^{-1}-diet (high dose) 2-aminoanthracene (2-AA) for either 14 or 28 days. Rats were provided distilled water *ad libitum*. Rats were housed at the Southern Illinois University Animal Facility. The animals were treated according to the principles outlined in the National Institutes

of Health (NIH) and Southern Illinois University Guide for the Care and Use of Laboratory Animals. At the end of each exposure period (14 or 28 days), rats were euthanized with CO_2 and blood was collected by cardiac puncture. Twenty micro liters of whole blood samples were added to 1 mL of Hanks Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+}) and subsequently snapped frozen in liquid nitrogen. The feeding study was carried out between 2009 and 2010 whereas the comet assay was done in 2010.

Comet assay: DNA damage was assessed by single cell gel electrophoresis (Comet assay) analysis under alkaline conditions (Tice *et al*., 2000) with slight changes. Blood samples in HBSS were incubated for 60 min at 37°C. At the end of the incubation period, cells were pelleted, supernatant removed and re-suspended in HBSS with an accompanying 500 uL set aside for cell viability assessment. While the sample was being incubated, lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base, 1% Triton-X 100 and 10% dimethyl sulfoxide (DMSO) was prepared and refrigerated for at least 30 min prior to slide addition. The cells for single cell gel electrophoresis analysis were pelleted and much of the supernatant removed. Cells were resuspended in 0.5% low melting agarose in Phosphate Buffered Saline (PBS) and spread on precoated slides (CometSlide, Trevigen Gaithersburg MD). The slides were covered with coverslip and placed on cold tray to solidify after which the coverslips were removed and slides transferred into cold (4°C) lysing solution for 1 h. Slides were then transferred to a horizontal electrophoresis chamber containing alkaline buffer (300 mM NaOH, 1 mM Na_2EDTA, pH >13) for 25 min and subsequently electrophoresed for 25 min at 25 V (300 mA). Slides were then rinsed thrice for 5 min using Tris-HCl buffer (0.4 M, pH 7.5) and stained with 100 uL 1X ethidium bromide (10X stock-20 μg mL^{-1}). Stained nucleotides were assessed via Allied Vision Technologies (AVT) CCD camera attached to a trinocular microscope, using Perceptive Instruments Comet Assay IV software, Suffolk, UK. For each sample, 100 cells were randomly selected and analyzed for DNA damage using the Olive Tail Moment (percent of DNA in tail x distance between the center of gravity of DNA in the tail and the center of gravity of DNA in the head). All samples were run in triplicates.

Cell viability assessment: We employed a method developed by Strauss (1991) to assess viability of cells. The 500 μL of cells set aside for cell viability assessment was reduced to 40 μL of at least 10^6 cells mL^{-1}. To this sample was added 10 μL of dual stain (5-6 carboxyfluorescein diacetate:ethidium bromide; 1:1 ratio) for 5 min at 37°C. Excess stain from cells was washed with 1 mL of PBS twice followed by mixing of cells in a drop fluid (10 μL). The drop fluid was placed on a slide, covered with coverslip and observed with a fluorescent microscope (FITC filter). Approximately 100 cells were scored for each sample, viable cells exhibited green fluorescence in cytoplasm.

Data analysis: Scored cells were imported into Microsoft Excel. Significant differences in tail moment, tail length, tail and head intensity following scoring of comets were analyzed by one-way Analysis of Variance (ANOVA) and were expressed as Mean±SE.

RESULTS AND DISCUSSION

DNA damage from alkaline comet assay was scored using Perceptive Instruments Comet Assay IV software. We employ the most commonly used parameters for comets; such as tail moment, tail length, relative tail and head fluorescent intensity (Collins, 2004; Polasa *et al*., 2006; Banerjee *et al*., 2010). Figure 1a and b is a representation of tail moment values as a result of

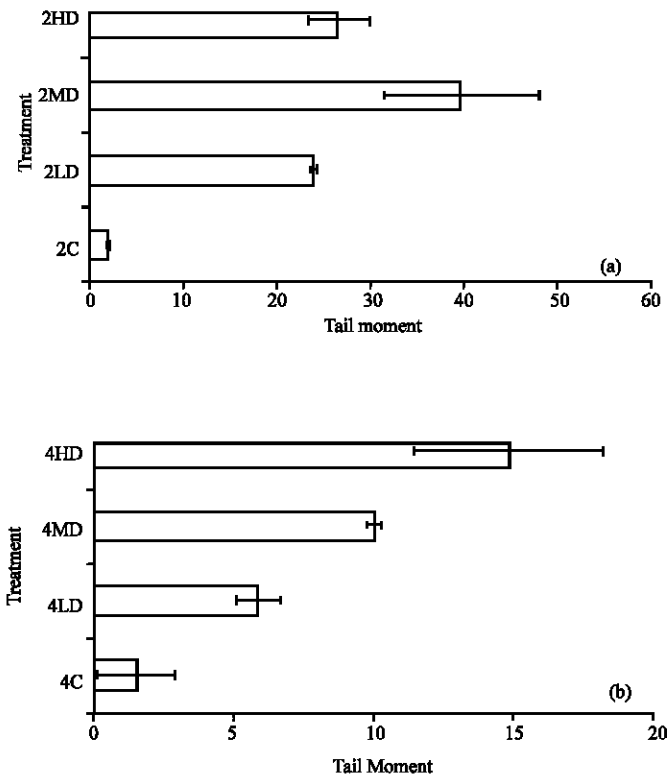

Fig. 1: The effects of 2-AA consumption on F-344 rat dietary intake via the measurement of Tail Moment. Each data point is the mean of 3 F-344 male rats given untreated (C-0 mg kg^{-1}-diet) and treated (LD-50 mg kg^{-1}-diet, MD-75 mg kg^{-1}-diet and HD-100 mg kg^{-1}-diet) 2-AA diet. Mean tail moment was significantly increased at $p<0.05$ for both time points in the low, medium and high dose treatments when compared with the controls. (a) 14-day treatment group and (b) 28-day time frame

consumption of 2-AA diet by F-344 animals. The tail moment which, measures the percent DNA in tail relative to the DNA center of gravity in the head and tail. was observed to be greater for the 2-week study than the four-week treatment period. Medium dose treatment group for the short-term study showed greater DNA damage as observed in tail moment values. There was no significant difference between the low dose and high dose tail moment for this treatment time frame. With respect to the four week treatment period, the high dose group had greater tail moment figures followed by the medium dose and low dose in that order.

DNA damage observed via tail length, tail intensity and head intensity was similar to the trend seen when tail moment was the measuring parameter. In all these cases, there were significant increases ($p<0.05$) relative to the control. Illustrated in Fig. 2 a and b is the mean tail length of F-344 rats fed 2-AA diet. Similar to Fig. 1, the 12-day study ($p<0.01$) showed slightly greater tail length than the 28-day group ($p<0.05$). Within the 2 week subgroup, high dose animals had slightly less tail length than the medium dose rats. Low dose tail length was less than high dose and medium dose. On the contrary, tail intensity values for both time points were quite similar. In Fig. 3a and b, tail intensity values of treated animals range between 25-33 and 19-36 arbitrary units for two and four weeks study respectively. The head intensity plot shown in Fig. 4a and b is

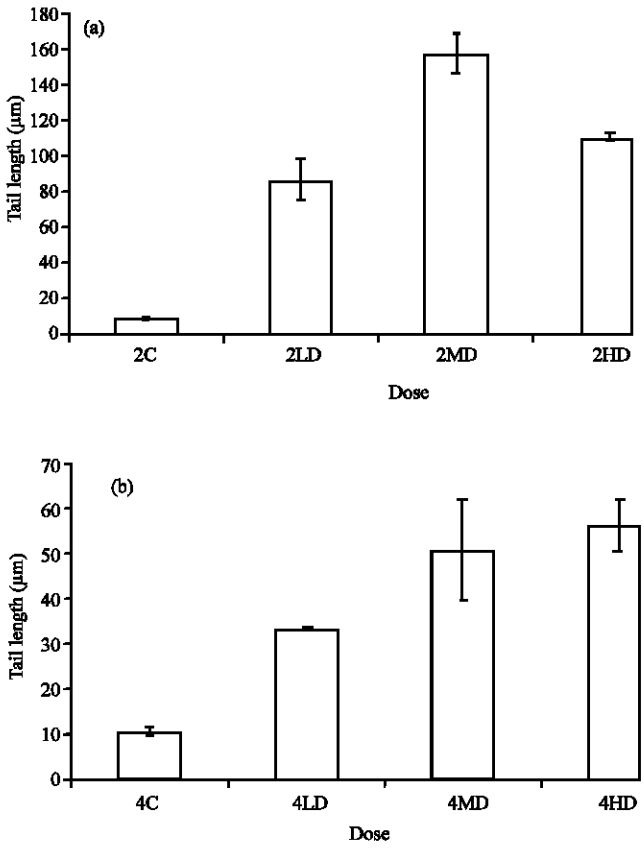

Fig. 2: Mean tail length (µm) from blood of F-344 rats fed 2-AA diet for (a) 14 days ($p<0.01$) and (b) 28 days($p<0.05$)

similar in trend observed for Fig. 3. In this case, the intensity values ranged between 15 and 30 for treated animals during 14 and 28-day exposure times. When compared with the control, the head intensity values increased significantly ($p<0.01$). A significant decrease ($p<0.01$) in cell viability was observed for both time points between the treated and control animals. Result was shown in Fig. 5.

We evaluated the ability of 2-AA to directly cause DNA breakage in F-344 rats using the alkaline comet assay which measures single strand breaks. Parameters scored were the tail moment, tail length, tail intensity and head intensity along with the cell viability assessment. Collins (2004) noted that the most commonly used parameters in the assessment of DNA by comet assay were tail length, tail intensity, head intensity and tail moments. Head and tail intensities refer to relative fluorescence of head and tail that is normally expressed as a percent DNA within the tail (Collins, 2004). Tail length measures distance of DNA migration from the body of the nuclear core. This parameter is sometimes referred to as tail extent.

Both time points show apparent dose response. That is the increasing 2-AA concentration resulted in greater apparent single strand DNA breaks. Also, the short-term treatment group seemed to be show greater strand breaks than the four week exposure group in tail and head intensity scoring parameters. This observation is similar in pattern to an earlier study that examined F-344 rat's response to 2-AA intoxication using gene expression analysis. There was an

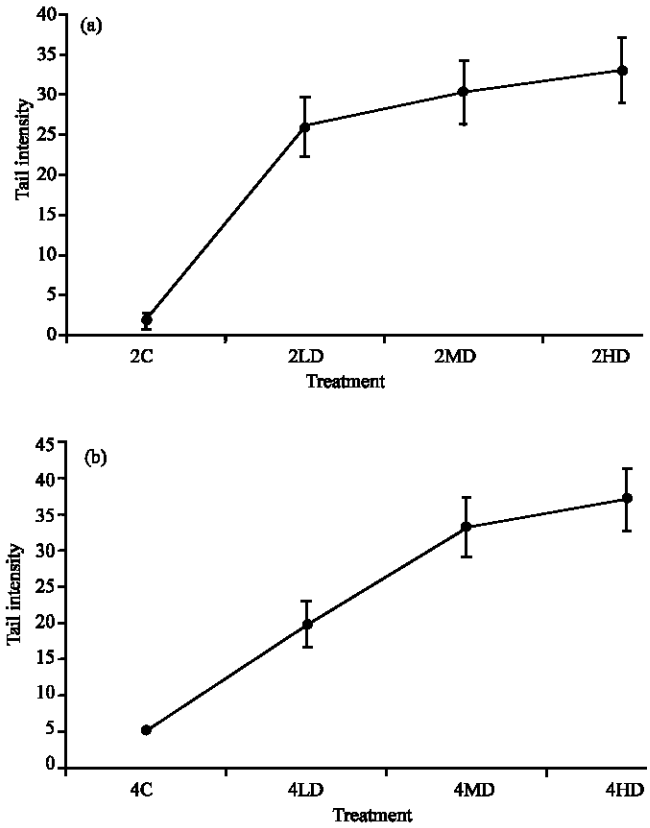

Fig. 3: Mean tail intensity of blood samples derived from F-344 rats that consumed 2-AA diets of C (0 mg kg^{-1}), LD (51 mg kg^{-1}), MD (75 mg kg^{-1}) and HD (100 mg kg^{-1}). The animals were treated for (a) 14 days and (b) 28 days. Each data point is a Mean of three replicates

apparent dose-response where a large number of genes in 2 weeks group were over-expressed in comparison to the 4 weeks animals. For a short-term exposure, animals show a stress response while showing adaptive recovery for longer exposure times (Gato and Means, 2011). It is quite interesting to observe a significant change in all the parameters analyzed for the low dose group in either time points. In a recent study (Gato and Means, 2011), we reported that body weight gain in low dose animals in comparison to controls were not significantly altered in response to 2-AA consumption of 2-AA adulterated diet. which does not necessarily mean that there no injurious effects at such low dose. The current finding supports the notion that effects are rather intrinsic and continuously affecting the animal during its entire life at the tissue and cellular function level.

The results obtained from the current study were consistent with previous reports that employed 2-AA as a positive control in genotoxicity assessment by comet assay (Hartmann *et al.*, 2001; Basaran *et al.*, 1996) which found DNA damage as due to 2-AA toxicity. For instance Kawamoto *et al.* (2010) reported DNA migration in paramecia (unicellular protozoa) exposed to 10 μM 2-AA to range from 104-115 μm between 1 and 24 h. In this study, the authors measured DNA migration by subtracting the diameter (diameter of head) from length (length of whole comet). A similar finding was reported by Hartmann *et al.* (2003b). They noted mean tail length of V79 incubated with 23 μM 2-AA for 3 h as 31 μM. Also determination of cell viability using bioluminescent measurement of ATP in S9 rat liver homogenate was 59% (Suter *et al.*, 2004).

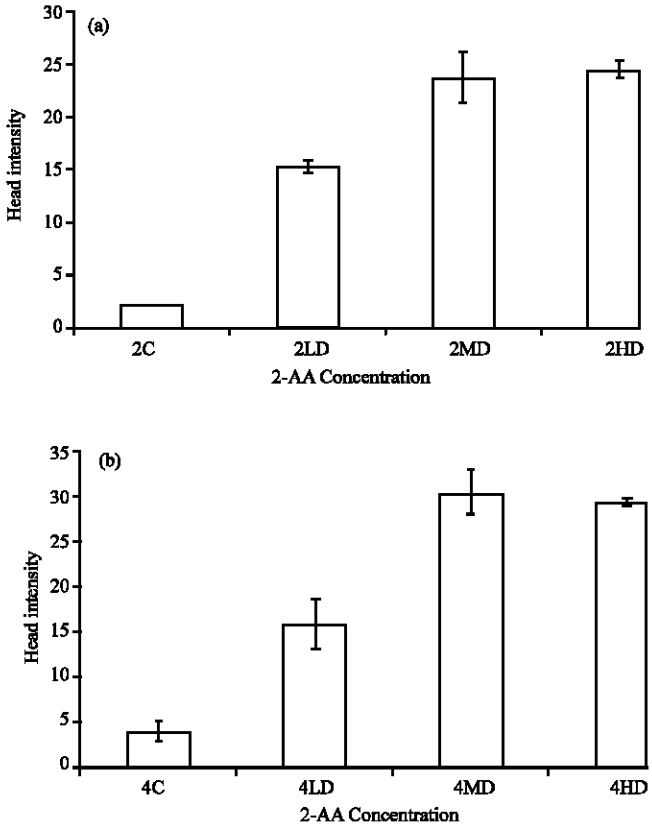

Fig. 4: DNA damage in F-344 rats that consumed 2-AA diet was examined using Comet Assay. Mean head intensity for 0, 50, 75 and 100 mg kg^{-1} diet is shown for (a) 14 days treatment period and (b) 28 days exposure time

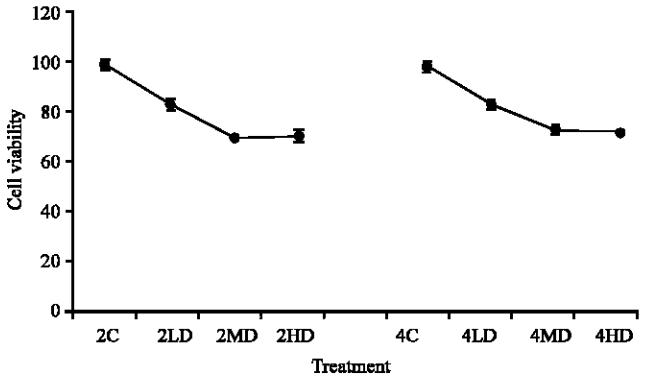

Fig. 5: Cell viability assessment of F-344 rats fed 2-AA diets of C-0, LD-50, MD-75 and HD-100 mg kg^{-1} for 2 and 4 weeks. Cell viability of 2-AA exposed animals was significantly reduced relative to controls ($p<0.01$)

Clearly the values reported from present study are somewhat greater especially for the medium and high dose. These differences can be attributed to study methodology as well as duration of exposure to 2-AA.

Single strand DNA break was measured in order to identify DNA damage in rats treated to 2-AA. Analyzed comet parameters such as tail moment, tail length, tail intensity and head intensity values demonstrated significant increases relative to control groups at varying degrees. Other researchers observed DNA strand break due to 2-AA exposure in paramecia and V79 cells that seem to support the findings of the current study. There are ongoing experiments to determine double stranded breaks as well as alkali-labile AP-sites and intermediate in base- or nucleotide-excision repair.

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