Journal of Toxicology and Environmental Health Sciences

Volume 6 Number 8, October 2014
ISSN 2006 - 9820

Academic Journals

ABOUT JTEHS

The **Journal of Toxicology and Environmental Health Sciences (JTEHS)** is published monthly (one volume per year) by Academic Journals.

The **Journal of Toxicology and Environmental Health Sciences (JTEHS)** is an open access journal that provides rapid publication (monthly) of articles in all areas of the subject such as toxicogenomics, enzyme inhibition, drug overdose, Children's Environmental Exposure Research Study etc.

The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JTEHS are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: jtehs@academicjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The Journal of Toxicology and Environmental Health Sciences (JTEHS) will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the JPP to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The **Title** should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The **Abstract** should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The **Introduction** should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b;Tristan, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anti coagulation therapy management. Ann. Pharmacother. 31:604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds), Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International. pp 181-190.

Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50: 91-95

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences:

(1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the Journal of Toxicology and Environmental Health Sciences (JTEHS) is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Journal of Toxicology and Environmental Health Sciences

Table of Contents: Volume 6 Number 8 October 2014

ARTICLES

Research Articles

academicJournals

Vol. 6(8), pp. 147-153, October, 2014
DOI: 10.5897/JTEHS2014.0313
Article Number: F26537547667
ISSN 2006-9820

http://www.academicjournals.org/JTEHS

Journal of Toxicology and Environmental Health Sciences

Full Length Research Paper

Determination of the effects of some artificial sweeteners on human peripheral lymphocytes using the comet assay

Zeynep Fındıklı and Şifa Türkoğlu*

Department of Biology, Faculty of Arts and Science, Cumhuriyet University, Sivas, Turkey.

Received 02 August 2014; Accepted 11 September 2014

In this study, the genotoxicity of the artificial sweeteners acesulfame potassium, aspartame, saccharin, and sorbitol, which are used in food industry and by patients with diabetes, was investigated in human peripheral lymphocyte cells using the single-cell gel electrophoresis (comet) technique. Human lymphocyte cells were treated with the substances for three hour at each of the three dosages (1.25, 2.5, and 5 ppm). The chemical additives were studied, and the related DNA damages in the study group were compared to the control group for each of the treatment dosages. The DNA breakages observed in the comet assay were assessed in terms of tail moment and tail DNA percent using the comet parameters. The statistical and photographic analyses were performed using SPSS 15 and BAB BS 200 Pro software, respectively. Based on the results for the short-term in vitro treatments, the 4 different food flavorings were found to have genotoxic effects.

Key words: Comet assay, DNA damages, artificial sweeteners, human peripheral lymphocytes.

INTRODUCTION

Sugar-free food products are sweetened by sugar substitutes that are commonly referred to as non-nutritive sweeteners, low calorie sweeteners, artificial sweeteners, or alternative sweeteners. Irrespective of their name, all sugar substitutes taste similar to sugar, but contain few to no calories and produce a low glycemic response. These sweeteners are widely used in processed foods, including baked goods, carbonated beverages, powdered drink mixes, candy, puddings, canned foods, jams, jellies, and dairy products.

Artificial sweeteners have been the subject of intense scrutiny for decades. Critics of artificial sweeteners maintain that sweeteners cause a variety of health problems, including cancer. Their arguments are based on studies dating to the 1970s, which linked saccharin to bladder cancer in laboratory rats. Because of these studies, saccharin once had a warning label stating that the product might be hazardous to human health.

Rapid increases in the consumption of sugar and sugar-containing foods have led to the emergence of certain health problems. High sugar consumption is associated with dental caries, obesity, and cardiovascular

*Corresponding author. E-mail: turkoglu@cumhuriyet.edu.tr. Tel: +90 346 2191010. Fax: +90 346 2191186.

disease, which occur because of a rapid increase in blood sugar levels and cause deleterious effects, especially in patients with diabetes (Howard and Wylie-Rosett, 2002). Considering the effect of sugar on diabetes patients, the most effective management, aside from medical treatment, involves a diet with limited quantities of sugar and sugary foods. Therefore, it is undesirable to use natural sweeteners to replace sugar.

The most popular artificial sweeteners are acesulfame potassium, aspartame, sorbitol, cyclamate, saccharin, sucralose, alitame, neotame, and neohesperidin dihydrochalcone. The latter is a semi-synthetic sweetener. In this study, the genotoxic effects of acesulfame potassium, aspartame, saccharin, and sorbitol were investigated.

Acesulfame potassium is a high-intensity, non-nutritive sweetener that is 200 times sweeter than sucrose. It is non-carcinogenic and stable under high temperatures and has an excellent shelf life. Acesulfame potassium is used as a sweetener in many foods, including chewing gums, baked goods, dessert and dairy products, alcoholic beverages, canned foods, candies, and over 4000 other products sold in approximately 90 countries, including Turkey, Australia, Canada, and Germany (Calorie Control Council, 2006). Acesulfame potassium is not metabolized or stored in the body. It is quickly absorbed and then excreted without undergoing modification. The results of several long-term animal studies that tested significantly higher amounts of acesulfame potassium than normally consumed by humans found no evidence of the development of cancers or tumors. Acesulfame potassium contains the chemical methylene chloride, a known carcinogen. Long-term exposure to methylene chloride can cause headaches, depression, nausea, mental confusion, liver and kidney effects, and cancer in humans (ATSDR, 1998; HSDB, 1993, Graves et al., 1994). Another one of the byproducts of acesulfame potassium's breakdown in the body is acetoacetamide, which is toxic at high doses. Center for Science in the Public Interest (CSPI) notes that acetoacetamide has been shown to cause tumor growth in the thyroid gland in rats, rabbits, and dogs after administration of only 1% acetoacetamide in the diet for three months (http://www.cspinet.org/reports/asekquot.html).

Aspartame has a sugar-like taste that is used to enhance fruit and citrus flavors. It can be safely heated to high temperatures with some loss of sweetness, and it is non-carcinogenic. In addition, aspartame is approximately 200 times sweeter than sucrose. Since its approval, aspartame has been used in over 6000 different types of products, including soft drinks, dessert mixes, frozen desserts and yogurt, chewable multi-vitamins, breakfast cereals, table top sweeteners, and pharmaceuticals (Rencüzoğulları et al., 2004). It is consumed by millions of people around the world (Butchko et al., 2002; Fry, 1999). Upon digestion, aspartame breaks down into small amounts of methanol and the amino acids aspartic acid and phenylalanine. Chronic exposure to aspartame has been reported to cause the following symptoms: headaches, blurred vision, epileptic fits, brain tumors, eye problems, numbness, insomnia, memory loss, nausea, slurred speech, loss of energy, hyperactivity, hearing problems, neurological problems, and behavioral disturbances (Humphries et al., 2008).

Saccharin is an artificial sweetener that has been used for over a century to sweeten foods and beverages without adding calories or carbohydrates. It is found in food such as soft drinks, baked goods, chewing gum, canned fruit, salad dressings, cosmetic products, and pharmaceuticals. Saccharin has been approved for use in more than 100 countries. After ingestion, saccharin is neither absorbed nor metabolized; instead, it is excreted unmodified via the kidneys. Because saccharin is not metabolized, the Food and Drug Administration (FDA) of the USA considers it safe.

Sorbitol, also known as glucitol, is a sugar alcohol that is slowly metabolized by the human body. It is often used in diet foods, mints, cough syrups, and sugar-free chewing gum. Sorbitol contains fewer calories than sugar and has minimal effects on blood sugar levels. However, sorbitol consumption is associated with side effects, especially when ingested in large quantities. One common side effect of sorbitol is diarrhea. Johannes et al. (1992) reported that sorbitol induced DNA fragmentation in Chinese hamster ovary cells.

Consistent ingestion of food additives has been reported to induce toxic, genotoxic, and carcinogenic effects (Demir et al., 2010; Hobbs et al., 2012; Güngörmüş and Kılıç, 2012; Jeffrey and Williams, 2000; Kumar and Srivastava, 2011; Saad et al., 2014 Zengin et al., 2011). The DNA damage induced by food additives depends on their transport across cellular/nuclear membranes, the activation and deactivation of intracellular enzymatic processes, the levels of radical scavengers, and the repair mechanisms in the target cell population.

The comet assay has been used to determine the effects of these cellular processes on the amount of DNA damage induced (Kasamatsu et al., 1996; Szeto et al., 2002; Tice et al., 2000).

This assay is a powerful tool for determining genotoxicity, because it is simple and highly sensitive, has a short response time, and requires a relatively small number of cells and test substances (Adegoke et al., 2012; Benedetti et al., 2013; Čabarkapa et al., 2014; Fabiani et al., 2012; Liman et al., 2011; Severin et al., 2010).

Food sweeteners are widely used in food, but little is known about their genotoxic effects. Thus, the purpose of this study was to evaluate the potential genotoxic effects of food sweeteners, such as acesulfame potassium, aspartame, saccharin, and sorbitol, on isolated human lymphocytes using the comet assay.

MATERIALS AND METHODS

Acesulfame potassium (CAS No: 55589-62-3), aspartame (CAS No: 22839-47-0), saccharin (CAS No: 81-07-2), and sorbitol (CAS No: 50-70-4) were purchased from Sigma Chemical Co.

On each day of the analysis, fresh human peripheral blood was obtained by venipuncture from three healthy male human volunteers and placed into BD Vacutainer collection tubes containing heparin as an anticoagulant. The volunteers were non-smokers, unmedicated, and had an average age of 23 ± 1 years. Fresh blood (2 ml) was diluted with an equal volume of PBS, and 2 ml of the diluted blood was layered gently over 2 ml of Histopaque and incubated for 25 min. The tube was centrifuged at 1000 rpm for 40 min, and the buffy coat was aspirated into 4 ml of PBS. The resulting mixture was centrifuged at 1000 rpm for 10 min. The supernatant was then discarded, and the lymphocyte pellet was resuspended at a concentration of 10^6 cells/ml in RPMI-1640 medium. The isolated lymphocytes were incubated with various concentrations of the different food sweeteners at 37°C for 3 h.

The concentrations of the four sweeteners were selected on the basis of permissible sweetener levels (Turkish Food Codex, 2011). The following food sweetener concentrations were used: 1.25, 2.5, and 5 ppm. The samples prepared were incubated at 37°C for 3 h. After incubation, the lymphocytes were harvested by centrifugation at 2000 rpm for 10 min, and the cell pellet was resuspended in PBS.

The comet assay was performed according to the methods of Singh et al. (1989), Tice et al. (1991), and Ghosh et al. (2010) with slight modifications. To visualize DNA damage, slides were examined at 400× magnification using a fluorescence microscope, and 100 cells were randomly selected for analysis in each sample (BAB Bs200ProP/BsComet DNA Comet Assay). The tail DNA (%) and tail moment were used to measure DNA damage because they give the most meaningful results in genotoxicity studies (Kumaravel and Jha, 2006). This study was performed in accordance with the Declaration of Helsinki and with the approval of the local ethics committee (No. 2010-04/04).

Statistical analysis was performed using SPSS (version 15.0). The percentage of DNA in the tail (% DNA tail) and tail moment (μm) were measured as comet parameters. All data were presented as arithmetic mean ± standard error. The statistical approach was analysis of variance (ANOVA), which was used to evaluate the significance of the difference in DNA damage between the control and treated cells. Results were considered statistically significant at $p < 0.05$.

RESULTS

The results of the comet assay are as shown in Tables 1 to 4. The percent tail DNA and tail moment for human lymphocytes exposed to different doses of acesulfame potassium or to distilled water (control group) are compared as shown in Table 1. Acesulfame potassium at 2.5 and 5 ppm increased the tail moment to 7.87 ± 1.30 and 17.60 ± 1.58, respectively. The same concentrations of acesulfame potassium increased the tail DNA to 9.21 ± 1.02% and 19.01 ± 0.63%, respectively. Thus, these concentrations of acesulfame potassium increased DNA damage.

Exposure to 1.25, 2.5, and 5 ppm of aspartame increased the tail moment and tail DNA (Table 2). Among these doses, the highest DNA damage was observed after treatment with 2.5 ppm of aspartame (tail moment: 17.62 ± 1.00, tail DNA: 18.92 ± 1.87%).

All of the saccharin concentrations tested increased DNA damage relative to that observed in the control group (Table 3). The group treated with 2.5 ppm of saccharin exhibited the greatest DNA damage.

Table 4 summarizes the comet assay data, which are expressed as the tail moment and tail DNA (%), for human lymphocytes exposed for 3 h to different concentrations of sorbitol. Cells treated with 2.5 ppm of sorbitol exhibited a significant increase in single-strand breaks when measured by the tail moment (25.85 ± 1.64) and tail DNA (27.44% ± 2.17%).

DISCUSSION

Acesulfame potassium, aspartame, saccharin, and sorbitol are commonly used as sweeteners in the food and pharmaceutical industries. These sweeteners are low in calories and provide sweetness with little to no intake of food energy. According to a 2007 survey published by the Calorie Control Council, 86% of Americans consume low-calorie, reduced sugar, or sugar-free foods and beverages (Calorie Control Council, 2007).

Food additives are widely used in factory-made foods. Therefore, they must be completely safe for human consumption. Nevertheless, scientific studies on these additives have yielded unfavorable results, especially in gene toxicity and carcinogenicity tests. Genotoxicity pertains to all types of DNA damage. Agents that interact with DNA and/or associated cellular components (e.g., the spindle apparatus) or enzymes (e.g., topoisomerases) are considered genotoxins (Dearfield et al., 2002; Jouyban and Parsa, 2012; Robinson, 2010).

Acesulfame potassium, aspartame, saccharin, and sorbitol are used as sweeteners in food products and pharmaceuticals. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Union's Scientific Committee have found these sweeteners to be safe for use in food. However, using the comet assay, we determined that these sweeteners were genotoxic.

Reports on the genotoxicity of acesulfame potassium, aspartame, saccharin, and sorbitol are inconsistent. Very little research has been done on acesulfame potassium, which was considered once under scrutiny by the FDA for being a potentially dangerous compound with adverse side effects. The few studies on acesulfame potassium were incomplete and inconclusive, but found the compound to be genotoxic and clastogenic in mice (Mukherjee and Chakrabarti, 1997), non-mutagenic in mammalian cells (Marquardt, 1978), and non-cytotoxic and non-genotoxic in *in vivo* and *in vitro* experiments (Baeder et al., 1977). Bandyopadhyay et al. (2008) used the comet assay to determine the mutagenic activity of acesulfame potassium. Additional research on the effects

Table 1. Effect of acesulfame potassium on DNA damage measured as comet percent tail DNA and tail moment (mean± SE).

Dose (ppm)	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25	2.96±1.55[a]	2.08±0.92[a]
2.50	9.21±1.02[b]	7.87±1.36[b]
5.00	19.01±0.63[c]	17.60±1.58[c]

*Means with the same letters do not significantly differ at 0.05 level.

Table 2. Effect of aspartame on DNA damage measured as comet percent tail DNA and tail moment (mean±SE).

Dose (ppm)	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25	13.88±1.58[b]	12.55±1.76[b]
2.50	18.92±1.87[c]	17.62±1.00[c]
5.00	10.94±1.23[d]	9.99±1.15[d]

*Means with the same letters do not significantly differ at 0.05 level.

Table 3. Effect of saccharin on DNA damage measured as comet percent tail DNA and tail moment (mean±SE).

Dose	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25 ppm	16.63±1.52[b]	15.49±1.85[b]
2.50 ppm	21.96±3.03[c]	20.48±1.74[c]
5.00 ppm	13.00±2.27[d]	11.61±2.46[d]

*Means with the same letters do not significantly differ at 0.05 level.

Table 4. Effect of sorbitol on DNA damage measured as comet % tail DNA and tail moment (mean±SE).

Dose	Tail DNA* (%)	Tail moment*
Control	3.05±0.14[a]	2.19±1.16[a]
1.25	4.63±1.54[b]	3.39±1.11[a]
2.50	27.44±2.17[c]	25.85±1.64[b]
5.00	14.22±1.06[d]	12.35±1.35[c]

*Means with the same letters do not significantly differ at 0.05 level.

of acesulfame potassium on mice revealed chronic use over a period of 40 weeks resulted in a moderate but limited effect on neurometabolic function. These results suggest chronic usage of acesulfame potassium may alter neurological function (Cong et al., 2013).

Creppy et al. (1998) suggested that aspartame had antigenotoxic activity. According to Trocho et al. (1998), aspartame consumption might be hazardous because of its contribution to the formation of formaldehyde adducts. Furthermore, Soffritti et al. (2006, 2007, 2010, 2014) have demonstrated the carcinogenic potential of this compound. The safety of aspartame and its metabolic breakdown products (phenylalanine, aspartic acid and methanol) was investigated *in vivo* using chromosomal aberration (CA) test and sister chromatid exchange (SCE) test in the bone marrow cells of mice. Treatment

with aspartame induced dose dependently chromosome aberrations at all concentrations while it did not induce sister chromatid exchanges. On the other hand, aspartame did not decrease the mitotic index (MI). However, statistical analysis of the results show that aspartame is not significantly genotoxic at low concentration (AlSuhaibani, 2010). Mukhopadhyay et al. (2000) reported that blends of aspartame and acesulfame potassium did not increase chromosomal aberrations in the bone marrow of Swiss albino mice. Sasaki et al. (2002) examined the *in vivo* genotoxicity of aspartame in eight mouse organs after 3 and 24 h using the comet assay. Chromosome aberrations and the results of a micronucleus test performed on human lymphocytes indicated that aspartame had genotoxic effects. However, the Ames/*Salmonella*/microsome test detected no mutagenic effects (Rencüzoğulları et al., 2004). Aspartame has also been administered orally to pregnant rats, and cytogenetic effects were observed in the mother rats and their offspring (Abd El Fatah et al., 2012). In addition, mutagenicity studies on acesulfame potassium (Mukherjee and Chakrabarti, 1997) indicated that when mice were administered doses within the acceptable daily intake of 15 mg/kg body weight, the number of chromosomal aberrations was not statistically different from the number in control mice. However, at high doses, acesulfame potassium was clastogenic and genotoxic. Taken together, these studies show that, depending on the dose, acesulfame potassium interacts with DNA and causes genetic damage.

Saccharin has also been the subject of extensive scientific research and debate. It is one of the most studied food ingredients. Although studies indicate that saccharin is safe for human consumption; there has been controversy over its safety. Bladder tumors have been reported in some male rats fed high doses of saccharin (Arnold et al., 1980; Schoenig et al., 1985). In an *in vitro* clastogenicity assay, high doses of saccharin induced chromosomal aberrations and sister chromatid exchange in Chinese hamster ovary and lung cells (IARC, 1999). Furthermore, Sasaki et al. (2002) used the comet assay to assess the ability of 39 currently used food additives, including saccharin and sodium saccharin, to induce DNA damage in mice. They observed that DNA damage in the colon increased a statistically significant amount 3 h after saccharin exposure. Sorbitol is a sugar alcohol that is slowly metabolized by the human body. After it is absorbed in the body, sorbitol oxidizes to fructose, which is subsequently metabolized to fructose-1-phosphate. Sorbitol has been shown to induce DNA damage in Chinese hamster ovary (CHO) cells (Johannes et al., 1992). However, to our knowledge, the genotoxicity of sorbitol in human lymphocytes has not been reported.

In this study, comet assay was used to determine whether four sweeteners induced DNA damage in human peripheral lymphocytes. This assay has been used as a rapid and sensitive tool to assess chemically induced DNA damage. The results showed that all selected concentrations of the four sweeteners significantly increased the level of DNA damage ($p < 0.05$ vs. control). Especially, cells treated with the highest concentration of acesulfame potassium showed a significant increase of the percent tail DNA values compared to the control and other food sweeteners. Acesulfame potassium is stable in foods, beverages and cosmetic preparations under normal storage conditions. Under extreme conditions of pH and temperature, detectable decomposition may occur leading to the formation of acetone, CO_2, and ammonium hydrogen sulfate, or amido-sulfate, as final decomposition products; under acid (pH 2.5) conditions, minute quantities of acetoacetamide and acetoacetamide N-sulfonic acid are formed as unstable intermediate decomposition products, while under alkaline (pH 3-10.5) conditions, acetoacetic acid and acetoacetamide N-sulfonic acid can be detected (http://www.inchem.org/documents/jecfa/jecmono/v16je02.htm). These degradation products may cause DNA strand breaks. Another reason of observed high damage rate on 5 ppm dosage of acesulfame potassium may be smaller size of fractures and consequently faster movement in electrophoresis.

The food sweeteners induced a concentration-dependent increase in DNA single-strand breaks, as evident by comet formation. The DNA damage detected in this study may have originated from DNA single-strand breaks, DNA double-strands breaks, DNA adducts formations, and DNA-DNA and DNA-protein cross-links (Mitchelmore and Chipman, 1998) that result from the interactions of sweeteners or their metabolites with DNA. The mechanism by which acesulfame potassium, aspartame, saccharin, and sorbitol exposure induces DNA strand breaks which is poorly understood, and little is known about these sweeteners or the metabolites that are responsible for DNA strand breaks.

Studies on the interactions between small molecules and DNA will be valuable for the development of disease treatments and the prevention of disease, because DNA is a major drug target and can be damaged by harmful chemicals. The DNA damage caused by sweeteners may be associated with the generation of free radicals (reactive oxygen species), which cause DNA strand breaks and irreversible damage to proteins involved in DNA replication, repair, recombination, and transcription (Lin et al., 2007).

Conclusion

This study demonstrated that acesulfame potassium, aspartame, saccharin, and sorbitol cause DNA damage. Commonly used food sweeteners may be toxic at high concentrations in the long term. Therefore, the effects of sweeteners on human health should be extensively investigated, especially when used at high concentrations

in foods and beverages.

Conflicts of İnterest

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by the Cumhuriyet University Coordination Unit of Scientific Research Projects (Project Code: F-325). The authors are grateful to Dr. Serpil Ünver Saraydın (for the fluorescence microscope) and Babacan Uğuz (for the BAB Bs200ProP/BsComet DNA Comet Assay).

Abbreviations: ADI, Acceptable Daily Intake; **FDA,** Food and Drug Administration; **JECFA,** Joint FAO/WHO Expert Committee on Food Additives (JECFA).

REFERENCES

Abd El Fatah AAM, Ghal IS, Hanafy SM (2012). Cytotoxic effect of aspartame (diet sweet) on the histological and genetic structures of female albino rats and their offspring. Pak. J Biol. Sci. 15, 904-918.

Adegoke OA, Kyup JK, Mukherjee A (2012). *In vitro* genotoxicity evaluation of 4-carboxyl-2,6-dinitrophenylazohydroxynaphthalenes using human lymphocytes. Food Chem. Toxicol. 50(3-4):936-941.

AlSuhaibani ES (2010). *In vivo* cytogenetic studies on aspartame. Int. J. Genom. Comp. Funct. Genom. 1-5.

Arnold DL, Moodie CA, Grice MC, Charbonneau SM, Stavric B, Collins BT, McGuire PF, Zawidzka ZZ, Munro LC (1980). Long-term toxicity of ortho-toluenesulfonamide and sodium-saccharin in the rat. Toxicol. Appl. Pharmacol. (52):113-152.

ATSDR: Agency for Toxic Substances and Disease Registry (1998). Toxicological Profile for Methylene Chloride (Update). Draft for Public Comment. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

Baeder GH, Weigand W (1997). Oral mutagenicity study (micronucleus test) of Acesulfam in NMRI mice. Report No. 591/77. Unpublished report submitted by Hoechst, A.G.

Bandyopadhyay A, Sarbani Ghoshal S, Mukherjee A (2008). Genotoxicity testing of low-calorie sweeteners: aspartame, acesulfame-K, and saccharin. Drug Chem. Toxicol. 31:447-457.

Benedetti D, Nunes E, Sarmento M, Porto C, dos Santos CEI, Dias JF, da Silva J (2013). Genetic damage in soybean workers exposed to pesticides: Evaluation with the comet and buccal micronucleus cytome assays. Mutat. Res. 752(1-2):28-33.

Butchko HH, Stargel WW, Comer CP, Mayhew DA, Benninger C, Blackburn GL, de Sonneville LM, Geha RS, Hertelendy Z, Koestner A, Leon AS, Liepa GU, McMartin KE, Mendenhall CL, Munro IC, Novotny EJ, Renwick AG, Schiffman SS, Schomer DL, Shaywitz BA, Spiers PA, Tephly TR, Thomas J, Trefz FK (2002). Aspartame: review of safety. Regul. Toxicol. Pharmacol. 35(2 Pt 2):S1-93.

Čabarkapa A, Živković L, Žukovec D, Djelić N, Bajić V, Dekanski D, Spremo-Potparević B (2014). Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using in vitro comet assay with human peripheral leukocytes. Toxicol. *In Vitro* 28:451-456.

Calorie Control Council (2006). Sweet choices- Questions and Answers about sweeteners in Low calorie Foods and beverages. http://www.caloriecontrol.org/sites/default/files/pdfs/lcs_broch4_1.pdf

Calorie Control Council (2007). Saccharin: how sweet it is. Retrieved April 23, 2008. From from www.saccharin.org/facts_policy.html

Cong WN, Wang R, Cai H, Daimon CM, Scheibye-Knudsen M (2013). Long-Term Artificial Sweetener Acesulfame Potassium Treatment Alters Neurometabolic Functions in C57BL/6J Mice. PLoS One 8 (8):e70257.

Creppy EE, Baudrimont I, Anne-Marie W (1998). How aspartame prevents the toxicity of ochratoxin A. J. Toxicol. Sci. 23:165-72.

Dearfield KL, Cimino MC, McCarroll NE, Mauer I, Valcovic LR (2002). Genotoxicity risk assessment: a proposed classification strategy. Mutat. Res. 521:121-135.

Demir E, Kocaoğlu S, Kaya B (2010). Assessment of genotoxic effects of benzyl derivatives by the comet assay. Food Chem. Toxicol. 48:1239-1242.

Fabiani R, Rosignoli P, De Bartolomeo A, Fuccelli R, Morozzi G (2012). Genotoxicity of alkene epoxides in human peripheral blood mononuclear cells and HL60 leukaemia cells evaluated with the comet assay. Mutat. Res. 747:1-6.

Fry J (1999). The world market for intense sweeteners. World Rev. Nutr. Diet 85:201-211.

Ghosh M, Bandyopadhyay M, Mukherjee A (2010). Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes. Chemosphere 81:1253-62.

Graves RJ, Callander RD, Green T (1994). The role of formaldehyde and S-chloromethylglutathione in the bacterial mutagenicity of methylene chloride. Mutat. Res. 320:235-243.

Güngörmüş C, Kılıç A (2012). The safety assessment of food additives by reproductive and developmental toxicity studies. Food Additive, InTech (2012).

Hobbs CA, Swartz C, Maronpot R, Davis J, Recio L, Hayashi S (2012). Evaluation of the genotoxicity of the food additive, gum ghatti. Food Chem. Toxicol. 50:854-860.

Howard BV, Wylie-Rosett J (2002). Sugar and cardiovascular disease. Circulation 106:523-527.

HSDB (1993). U.S. Department of Health and Human Services. Hazardous Substances Data Bank (HSDB, online database). National Toxicology Information Program, National Library of Medicine, Bethesda, MD.

Humphries P, Pretorius E, Naudé H (2008). Direct and indirect cellular effects of aspartame on the brain. Eur. J. Clin. Nutr. 62:451-462;

International Agency for Research on Cancer (1999). Some chemicals that cause renal or urinary bladder tumours in rodents, and some other substances. Saccharin and its salts. IARC Monogr. Eval. Carcinogen. Risk Chem. Hum. 73:517-624.

Jeffrey AM, Williams GM (2000). Lack of DNA-damaging activity of five non-nutritive sweeteners in the rat hepatocyte/DNA repair assay. Food Chem. Toxicol. 38:(4)335-338.

Johannes C, Boes R, Obe G (1992). Uptake of the restriction endonuclease AluI by Chinese hamster ovary cells measured by frequencies of induced chromosomal aberrations: effect of hypertonic concentrations of glycerol and sorbitol. Mutagenesis 7:225-32.

Jouyban A, Parsa H (2012). Genotoxic impurities in pharmaceuticals. In: Acree Jr. WE (Ed.), Toxicity and Drug Testing. InTech, Rijeka. pp. 387-414.

Kasamatsu T, Kohda K, Kawazoe Y (1996). Comparison of chemically induced DNA breakage in cellular and subcellular systems using the comet assay. Mutat. Res. 369:1-6.

Kumar G, Srivastava N (2011). Genotoxic effects of two commonly used food additives of boric acid and sunset yellow in root meristems of *Trigonella foenum-graecum*. Iran. J. Environ. Health. Sci. Eng. 8:361-366.

Kumaravel TS, Jha AN (2006). Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat Res. 16:7-16.

Liman R, Ciğerci İH, Akyıl D, Eren Y, Konuk M (2011). Determination of genotoxicity of Fenaminosulf by *Allium* and Comet tests. Pestic. Biochem. Phys. 99-61-64.

Lin AJ, Zhang X, Chen M, Cao Q (2007). Oxidative stress and DNA damages induced by cadmium accumulation. J. Environ. Sci. (China) 19:596-602.

Marquardt HWJ (1978). Mutagenicity and oncogenicity of Acetosulfam-K. Studies with mammalian cells in vitro. Unpublished report of the Memorial Sloan-Kettering Cancer Center, New York. Submitted to WHO by Hoechst, A.G.

Mitchelmore CL, Chipman JK (1998). DNA strand breakage in aquatic organisms and the potential values of the comet assay in environmental

monitoring. Mutat. Res. 399:135-147.
Mukherjee A, Chakrabarti J (1997). In vivo cytogenetic studies on mice exposed to acesulfame-K--a non-nutritive sweetener. Food Chem. Toxicol. 35:1177-9.
Mukhopadhyay M, Mukherjee AJ (2000). In vivo cytogenetic studies on blends of aspartame and acesulfame-K. Food Chem. Toxicol. 38:75-77.
Rencüzoğulları E, Tüylü BA, Topaktaş M, İla HB, Kayraldız A, Arslan M, Diler SB (2004). Genotoxicity of aspartame. Drug Chem. Toxicol. 27:257-268.
Robinson DI (2010). Control of genotoxic impurities in active pharmaceutical ingredients: a review and perspective. Org. Process Res. Dev. 14:946-959.
Saad A, Khan FA, Hayee A, Nazir MS (2014). A review on potential toxicity of artificial sweetners vs safety of stevia: A natural bio-sweetner. Journal of Biology, Agriculture and Healthcare 4(15):1-12.
Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K (2002). The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat. Res. 519:103-19.
Schoenig GP, Goldenthal EI, Geil RG, Frith CH, Richter WR, Carlborg FW (1985). Evaluation of the dose response and in utero exposure to saccharin in the rat. Food Chem. Toxicol. 23:475-490.
Severin I, Dumont C, Jondeau-Cabaton A, Graillot V, Chagnon M-C (2010). Genotoxic activities of the food contaminant 5-hydroxymethylfurfural using different in vitro bioassays. Toxicol. Lett. 192:189-194.
Singh NP, McCoy MT, Tice RR, Schneider EL (1998). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175:184-191.
Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L, Tibaldi E, Rigano A (2006). First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. Environ. Health Perspect. 114:379-385.
Soffritti M, Padovani M, Tibaldi E, Falcioni L, Manservisi F and Belpoggi F (2014). The carcinogenic effects of aspartame: The urgent need for regulatory re-evaluation. American Journal of Industrial Medicine. 57(4):383-397.
Soffritti M, Belpoggi F, Manservigi M, Tibaldi E, Lauriola M, Falcioni L, Bua L (2010). Aspartame administered in feed, beginning prenatally through life span, induces cancers of the liver and lung in male Swiss mice. Am. J. Ind. Med. 53: 1197-1206.
Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M (2007). Life-span exposure to low doses of aspartame beginning during prenatal life increases cancer effects in rats. Environ. Health Perspect. 115:1293-1297.
Szeto YT, Collins AR, Benzie IFF (2002). Effects of dietary antioxidants on DNA damage in lysed cells using a modified comet assay procedure. Mutat. Res. 500:31-38.
Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35:206-221.
Trocho C, Pardo R, Rafecas I, Virgili J, Remesar X, Fernández-López JA, Alemany M (1998). Formaldehyde derived from dietary aspartame binds to tissue components in vivo. Life Sci. 63(5):337-349.
Turkish Food Codex (2011). Sweeteners for use in foods notification. Notification No: 2006-45. The Official Newspaper 21.09.2006-26296.
Zengin N, Yüzbaşıoğlu D, Ünal F, Yılmaz S, Aksoy H, (2011). The evaluation of the genotoxicity of two food preservatives: sodium benzoate and potassium benzoate. Food Chem. Toxicol. 49:763-769.

academicJournals

Vol. 6(8), pp. 154-160, October, 2014
DOI: 10.5897/JTEHS2014.0310
Article Number: 9E0A08347674
ISSN 2006-9820

http://www.academicjournals.org/JTEHS

Journal of Toxicology and Environmental Health Sciences

Full Length Research Paper

Bioaccumulation of heavy metals in cane rat (*Thryonomys swinderianus*) in Ogun State, Nigeria

Durojaye A. Soewu[1]*, Olufemi M. Agbolade[2], Rasheed Y. Oladunjoye[2] and Ibukun A. Ayodele[3]

[1]Department of Fisheries and Wildlife Management, College of Agriculture, Osun State University, P.M.B.4494, Osogbo, Osun State, Nigeria.
[2]Department of Plant Science and Applied Zoology, Faculty of Science, Olabisi Onabanjo University, P.M.B. 2002, Ago- Iwoye, Ogun State, Nigeria.
[3]Department of Wildlife and Ecotourism Management, Faculty of Agriculture and Forestry, University of Ibadan, Ibadan, Oyo State, Nigeria.

Received 7 June, 2014; Accepted 26 August, 2014

Wild animals have provided complimentary protein for human populations across the world over the centuries. This study investigated on bioaccumulation of heavy metals in different organs of cane rat (*Thryonomys swinderianus*) and the health implications of its consumption. Four carcasses were collected from each of the four ecotomes (Mosinmi, Agbara, Omo forest reserve and Ibese) and concentrations of 7 heavy metals (Fe, Cu, Cd, Pb, Mn, Cr, Zn) were examined in four organs (skin, liver, lung and kidney) from each specimen used by Atomic Absorption Spectrophotometer. Analysis of variance revealed no significant difference (P>0.05) in the concentration of metals in the animal except Fe and Cu, while significant variation exists when specimens were compared across different ecotomes. Total mean concentrations were Fe (400.512±60.0107), Cu (8.569±1.0396), Cd (0.06±0.040), Pb (0.3156±0.1175), Mn (9.4200±1.0383), Cr (1.3013±0.2739) and Zn (72.771±10.5672). Average mean concentration for all the metals in the study area was found to be higher than the recommended level which suggests that consumption of animals from this ecotomes are hazardous to human health and no single organ is completely safe for human consumption.

Key words: Bioaccumulation, *Thryonomys swinderianus*, cane rat, bush meat, heavy metals, wildlife consumption, animal toxicity.

INTRODUCTION

Heavy metals are natural components of earth's crust which cannot be degraded or destroyed. Living organisms especially human, require varying amounts of heavy metals such as Fe, Co, Cu, Mn, Mo, and Zn, but exceeding

*Corresponding author. E-mail: durosoewu@hotmail.com.

exceeding these levels can be dangerous to the organisms. Some heavy metals (e.g. Cu, Sc Zn) are essential to maintain metabolism in human body and can also lead to poisoning at higher concentration (Thomas et al., 1997). Ingestion of metals such as Pb, Cd, Hg, As, Ba and Cr may pose great risks to human health as they accumulate in living things any time they are taken up and stored faster than they are broken down, that is, metabolized or excreted (Smith et al.,1997).

Other heavy metals like Hg and Pb are toxic metals that have no known beneficial effects on organisms whereas their accumulation over time in the body can cause serious illness. Certain elements that are normally toxic could be beneficial for some organisms under certain conditions e.g., vanadium, tungsten and cadmium (Lane et al., 2005).

Heavy metal pollution in the environment arises from many sources, but the most common are purification of metals like smelting of copper and electroplating in chromium and cadmium. Through precipitation of their compounds or by ion exchange in soils and muds, heavy metals can localize or lay dormant. Unlike organic pollutants, they do not decay and thus pose a different kind of challenge for remediation (Lane and Morel, 2000). Their uptake and accumulation can be active (energy-dependent), passive (energy-independent) or both.

For instance, heavy metals poisoning could occur from drinking-water contamination (lead pipes), high ambient air concentrations near emission sources and intake via the food chain (Bilos et al., 2001). Their presence in the atmosphere, soil and water, can cause serious problems to all organisms even in traces and bioaccumulation in the food chain can be highly dangerous to human health (Cambra et al., 1999). Heavy metals intake by human populations through food chain has been reported in many countries (Damek-Poprawa and Sawicka-Kapusta, 2003). They usually enter human body through two routes: inhalation of dust and ingestion which is the main route of exposure to these elements in human population via consumption of food plants grown in metal-contaminated soil (Cambra et al., 1999).

Vegetables take up metals by absorption from contaminated soils and deposits on parts exposed to polluted environments (Zurera-Cosano et al., 1989). Metal contamination of garden soils may be widespread in urban areas due to continuous industrial activities and the use of fossil fuels (Chronopoulos et al., 1997). Several studies have shown that the concentrations of heavy metals in vegetation and natural rodent populations are generally correlated with environmental pollution (Schleich et al., 2010; Hawley, 1985; Sánchez-Camazano et al., 1994; Sterrett et al., 1996; Van Lune, 1987). Heavy metals which usually got transferred unto man and have highly adverse effects are lead, cadmium, copper, chromium, selenium and mercury (Wong, 1996). Long term exposure to lead in human can cause acute or chronic damage to the nervous system (Dudka and Miller, 1999). Human exposure to cadmium for a long period has been associated with renal dysfunctions and obstructive lung diseases which have been linked to lung cancer and damage to respiratory systems (WHO, 1984). Copper is an essential substance to human life, but in higher doses, it can cause anemia, intestinal irritation, stomach, liver and kidney damage. Mercury causes damages to the brain, central nervous system and psychological disorder. Normally, mercury is a toxic substance which has no known beneficial functionsin human biochemistry and makes developmental changes in children (WHO, 1984; Department of Environment, Food and Rural Affairs (DEFRA), 1999).

Chromium is used in metal alloys, pigments for paint, cement, paper, rubber and other materials. Low-level exposure can cause skin irritation and ulceration, while long-term exposure can cause damages to the kidney, liver, circulatory system and nerve tissues. Chromium often accumulate in aquatic life, this increases the danger of fish consumption, while selenium causes damage to circulatory tissue and more severe damages to the nervous system (Dudka and Miller, 1999).

Bush meat or wild meat is the term commonly used for meat of wild animals, killed for subsistence or commercial purposes throughout the humid tropics of the Americas, Asia and Africa. Wild animals are efficient users of native vegetation and can also adapt to man-modified habitats. In the past, the main populations who demanded for bush meat were indigenous people, rural communities and migratory workers. These people were averagely poor and bush meat consumption is an important aspect of their livelihoods. This situation is gradually changing as consumption of bush meat is now a common practice in sub-urban and urban communities by virtually all classes of the economy (Mathew, 2008; Wilkie and Godoy, 2001; Soewu et al., 2012). Many terrestrial ecosystems, which include wild populations of small mammals, are usually contaminated with potentially toxic trace elements from the accumulation of agricultural pesticides, fertilizers, industrial effluents and wastes disposal. These wastes are usually high in heavy metals which can be absorbed by plants and later found in high concentrations in animal tissues, and finally humans (Schleich et al., 2010). Grass cutters or cane rats (*Thryonomys* species) are widely distributed and valuable animal source for protein supplements in West and Central Africa.

The plant taxonomy is as follows: Kingdom, Animalia; Phylum, Chordata; Class, Mammalia; Order, Rodentia; Suborder, Hystricomorpha; Family, Thryonomyidae; Genus, *Thryonomys*; Species, *Thryonomys swinderianus* (Temminck, 1827). Cane rats can be found in cultivated forest regions, in sugar cane plantation and field where groundnut, maize, rice and cassava are grown. With regards to diet, they are monogastric herbivores, make good use of roughages, very fond of sweet and salty foods, but are wasteful feeders. They readily adapt to a

Table 1. Result for Omo Forest Reserve ecotome.

Organ/Metal (mg/kg)	Pb	Fe	Cu	Zn	Cd	Cr	Mn
Skin	0.42	205.80	4.15	123.5	0.00	0.00	11.04
Liver	0.77	438.90	8.55	103.72	0.00	0.77	6.16
Lung	0.90	337.15	3.75	79.56	0.00	2.20	5.28
Kidney	1.45	182.80	13.50	93.20	0.00	1.44	5.76

variety of diets which includes grasses (elephant grass, guinea grass, sugar cane), leguminous fodder (Centrocema, *Pueraria phaseoloides*), roots, coconut palms, fruits (pawpaw, pineapple, mango), tubers (cassava, sweet potatoes) and food crops (groundnut, rice, maize and grain legumes) (Mathew, 2008; Wilkie and Godoy, 2001).

Grasscutter meat is regarded as delicacy in Nigerian diet. Every part of the animal except the hair is consumed and its' nutritive value is relatively about 22.7% compared to 20.7% for rabbit meat and 19.25% for chicken meat. Their hairs on the other hand are used as a lotion to treat wounds after burning and the pancreas is believed to have medicinal properties employed in local preparations for diabetes treatment (Mathew, 2008). There is no known religious discrimination against their meat consumption and no competition with man for food as they feed mostly on grasses, leguminous plants (groundnuts, millets, cassava) among others (Mathew, 2008; Wilkie and Godoy, 2001).

This study was carried out mainly to assess the differential accumulation of heavy metals in the organs of grass cutter, as the eventual consumption of this animal may lead to passage of the metals to humans, thereby causing bioaccumulation. It also attempts to determine the safety level for continuous consumption of this delicacy with due reference to globally accepted standards (WHO, 1984).

MATERIALS AND METHODS

Ogun State is entirely in the tropics. Located in the southwest zone of Nigeria with a total land area of 16,409.26 km^2, it is bounded on the west by the Benin Republic, on the south by Lagos State and the Atlantic Ocean, on the east by Ondo State and on the north by Oyo and Osun States. It is situated between Latitude 6.2°N and 7.8°N and Longitude 3.0°E and 5.0°E. The state has estimated population of 3,486,683 people for the year 2005 (Soewu et al., 2012; Martiniaková et al., 2010).

The four ecotomes used represent different levels of anthropogenic activities and consequent exposure to pollution by heavy metals. Omo Forest Reserve is a relatively undisturbed ecotome with minimal human influence on the environmental parameters and Ewekoro host a large cement producing factory which is exposed to continuous injection of effluents with heavy metals constituents into the ecotome. Mosinmiecotome is directly in the heart of a very vast conglomerate of oil and gas processing industries and Agbara is the main industrial axis of t he state where various industries and production activities are cited. Four wild grass cutters carcass were obtained from each of the identified four ecotomes. Each carcass was a daily fresh-killed grass cutter and the organs were removed immediately so as to avoid autolysis. The abdominal part of the samples was carefully macerated to remove the kidneys and liver, while lung was removed from the thoraxic cavity. Internal skin was also cut and the procedures were repeated for all the sampled ecotomes accordingly. Each set of organs was put in a separate glass container and labeled accordingly. All the samples were digested and analysed by Atomic Absorption Spectrophotometer (VGB 210 Bulk Scientific) using the standard procedure (Schleich et al., 2010). It is known that bioaccumulation of heavy metals vary according the sex, size and/or age of the animals (W HO, 1984). Therefore, adult males that weighed around 4.5 to 4.7 kg were used to avoid differences that may be caused by any of these factors. The data obtained were subjected to statistical analysis using SPSS (version 16.0) which analysed for descriptive statistics and one-way analysis of variance (ANOVA) to find out the significant difference of heavy metal in each organ. Mean values were separated using Duncan Multiple Range Test (DMRT) of variance to determine variations due to sampling errors and differences in mean values were determined and accepted as being significantly different if $P< 0.05$.

RESULTS AND DISCUSSION

Results showed that Fe and Zn were more abundant in quantity, while Pb and Cd occurred as a trace metals. Also, the mean concentration of heavy metals in the skin and liver were significantly higher than other organs studied. There is a generally low/no Pb accumulation in the carcass from ecotomes three and four (Tables 3 and 4) as no Pb load was detected in the specimens. Fe was the most accumulated metal compared to others, especially in specimens from ecotome two (Table 2). Cu was detected in relatively low quantity as against Zn concentration that was high (Tables 1 to 4). Cd had little/no accumulation with Cr and Mn occurring in small quantities. Table 5 presents the level of heavy metals in the samples between the group and within the group. Analysis of variance ($P>0.05$) showed that there was no significant variability in the concentration of the studied metals except for Fe and Cu in the samples. However, apparent and significant variation exists when the samples were compared. Fe is a major component of haemoglobin which is responsible for the transport of oxygen in the body, but it may also be toxic to animals and man when ingested in large amount. Fe had the highest mean levels among the metals examined with 400.512+60.0107 (Table 5) and the highest level was found in the lung with 578.300±141.1273, followed

Table 2. Result for Mosinmi ecotome.

Organ/Metal (mg/kg)	Pb	Fe	Cu	Zn	Cd	Cr	Mn
Skin	0.55	240.50	6.90	92.36	0.00	0.78	19.76
Liver	0.00	657.00	8.05	87.08	0.58	0.00	16.20
Lung	0.00	983.95	4.05	71.52	0.00	0.00	8.80
Kidney	0.96	661.20	13.85	95.68	0.00	1.08	11.04

Table 3. Result for Ibese ecotome.

Organ/Metal (mg/kg)	Pb	Fe	Cu	Zn	Cd	Cr	Mn
Skin	0.00	114.97	6.85	103.32	0.00	0.00	10.08
Liver	0.00	402.97	11.90	77.12	0.00	2.16	8.64
Lung	0.00	533.25	4.60	80.36	0.00	3.78	6.48
Kidney	0.00	284.70	13.90	86.40	0.00	2.04	6.72

Table 4. Result for Agbara ecotome.

Organ/Metal (mg/kg)	Pb	Fe	Cu	Zn	Cd	Cr	Mn
Skin	0.00	121.25	5.60	119.76	0.00	1.14	12.16
Liver	0.00	592.00	11.90	114.12	0.00	0.80	11.52
Lung	0.00	458.85	4.30	53.36	0.00	2.47	5.32
Kidney	0.00	192.20	15.25	97.00	0.32	2.16	5.76

by the Liver with 522.718±60.6882, while the skin had the lowest with 170.705±31.2212. 15.0 ppm of Fe was reported in fruit juice in Spain (Contreraslopez et al., 1987), while a study that analyzed the levels of Fe in fruit drink using ICP-MS and reported concentration range of iron to be 4.49 to 8.25 ppm, and considered this to be unsafe for human consumption (Adraiano, 1984). The mean level of Fe in this study is higher than the values recorded in most previous reports.

Cu is an essential element for growth, but when present in some beverages such as milk products and fruit juices tends to impair the shelf life or keeping the quality of such products, so it is expected that fruit juice and milk products should contain relatively low levels of copper. A concentration range of 0.87 to 0.97 ppm in fruit drink was reported in Italy (Paolo and Maurizio, 1978), mean levels of 5.00 ppm were reported for Spain (Contreraslopez et al., 1987) and mean levels 1.41 to 7.19 ppm was recorded for Nigeria (Chukwujindu et al., 2008). The levels of Cu found in this study are higher than that of concentrations found in selected heavy metals in bones and femoral bone structure of polluted biotopes in Slovakia (Martiniaková et al., 2010). However, the kidney and liver (with the kidney having the highest value) have the mean level exceeding reported values of trace metals in the terrestrial environment (Contreraslopez et al., 1987) (14.125±0.3854 and 10.100±1.0442), respectively.

The concentration of Pb in the sampled organs follow the order: kidney (0.6025±0.3195) > skin (0.2425±0.1425) > lung (0.2250±0.2250) > liver (0.1925±0.1925). Pb levels of 0.01 ppm was reported for beverage drink in Canada (Adraiano, 1984), 0.38 ppm was recorded for food in Italy (Paolo and Maurizio, 1978), 0.15 ppm was reported for human consumption in Spain (Contreraslopez et al., 1987), while 0.6 to 1.93 ppm was reported for canned drinks in Nigeria (Chukwujindu et al., 2008). The mean levels of Pb in the various organs studied conform to the levels reported by previous researches except in kidney with 0.6025±0.3195. The Pb concentration in the lung, liver and skin conform to the standard set for human consumption (Paolo and Maurizio, 1978).

The highest concentration of Mn was found in the skin part of the specimen with 13.6200±2.2079 while the lung has the lowest concentration. The order of concentration of Mn in the examined organs follow the order: skin (13.2600±2.2079) > liver (10.6300±2.15557) > kidney (7.3200±1.26048) > lung (6.4700±0.8501). The level of Cd found in this study was generally low compared to any other metal examined and the highest mean levels was detected in the liver (0.15±0.145). Apart from the lung and skin, all other organs have mean concentrations of Cd exceeding the WHO permissible limit for human consumption (WHO, 1984) Table 6. The means concentration pattern of Zn follows the order: skin (88.385±27.5144)

Table 5. Analysis showing the concentration of the metals and the significance variance.

Heavy metal	Parameter	Sum of square	df	Mean square	f	Significance
Pb	Between groups	0.444	3	0.148	0.619	0.616
	Within group	2.868	12	0.239		
	Total	3.312	15			
Ni	Between groups	0.000	3	0.000	-	-
	Within group	0.000	12	0.000		
	Total	0.000	15			
Fe	Between groups	417120.9	3	139040.306	3.731	0.042
	Within groups	447188.6	12	37265.717		
	Total	864309.5	15			
Cu	Between groups	239.112	3	79.704	47.087	0.000
	Within groups	20.313	12	1.693		
	Total	259.424	15			
Zn	Between groups	2370.759	3	790.253	0.388	0.764
	Within groups	24429.130	12	2035.761		
	Total	26799.889	15			
Cd	Between groups	0.059	3	0.020	0.718	0.560
	Within groups	0.329	12	0.027		
	Total	0.388	15			
Cr	Between groups	6.448	3	2.149	2.231	0.137
	Within groups	11.563	12	0.964		
	Total	18.011	15			

Table 6. Accepted level of metal concentration for human consumption (WHO, 1987).

Heavy metal	Standard concentration (safe limit)
Fe	4.49 – 15.0 ppm
Cu	0.87 – 5.0 ppm
Zn	0.41 – 5.0 ppm
Mn	Yet to be affirmed
Cd	0.003 ppm
Cr	Yet to be affirmed
Pb	0.01 – 0.38 ppm

> liver (75.753±23.8742) > kidney (72.613±19.7098) > lung (54.332±17.8958). 5.0 ppm Zn was reported in foods in Spain (Contreraslopez et al., 1987), while 0.41 ppm was reported in drinks in Italy (Paolo and Maurizio, 1978). The levels of Zn found in this study were higher and greater than mean levels reported in metal content of apple juice for cider in Asturia (Contreraslopez et al., 1987) and 0.69 to 1.25 ppm recorded for drinks in Nigeria (Chukwujindu et al., 2008). Lungs has higher mean concentration of Cr (2.1125±0.78415) followed by the kidney (1.6800±0.2545) while the liver and skin which both has value of 0.9325±0.444910 and 0.4800±0.28671, respectively. In the analysis test for variance, Fe has the highest total means of 400.512±60.0107, F=3.731 and is significant (p 0.042<0.05). The Zn sequence then follows with total means of 72.771±10.5672, F=0.388 and not significant (p 0.0764 > 0.05). Cu mean concentrations remain significant

(p 0.00<0.05) and the total mean concentrations of 8.569+1.0397 and F=47.087. Other heavy metals studied were insignificant (P>0.05) with total mean concentrations of 0.3156±0.11747 (F=0.619, p 0.616), 0.06±0.0040 (F=0.718, p 0.560) and 1.3013±0.27394 (F=2.231, p 0.137) for the Pb, Cd and Cr, respectively.

This also agreed with some previous studies on some other mammals. The mean concentrations of Cu and Zn in animals captured in Malawi were higher in the liver compared to muscle while very low concentrations of Pb and Cd indicate no health risk connected with local consumption of silvery mole-rats in the study area (Figure 1) (Martiniaková et al., 2010). In the report, Cu has the highest accumulation record in both the body and stomach content of voles with very few exceptions where Ni and Pb had the highest figure (Martiniaková et al., 2010). On the overall, there is no single part (or organ) of the cane rat studied that is completely safe from all the metals, but some organs such as lung and skin are both Cu and Cd safe, while all parts except kidney is Pb safe from accumulation. Further statistical analysis shows that lung and skin are significantly less affected parts compared to other organs.

Generally, man should avoid consuming much of grass cutter from the study area as the heavy metals bio-accumulate and if it should be consumed at all, liver and the kidney should be avoided at all the time. Also, the study shows that the concentrations of heavy metals studied in the grasscutter found in the study areas were generally above safe limits for human consumption. The levels of Pb are above the guideline value for Pb in foods and drinking water except in the liver, while the level of Cd found in the various organs was higher than the value recommended (WHO, 1984; Adraiano, 1984), except for the lung and the skin which remain Cd undetected and safe for consumption. The data reported herein will be valuable in complementing available food composition and estimating dietary intakes of heavy metals in Nigeria.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the authorities of Omo Forest Reserve, Area J4, Ogun State for allowing field assistants into the reserve. DAS conceived of the research, participated in the laboratory analysis and prepared the final manuscript; OAM and IAA participated in the literature search and preparation of the report; RYO coordinated the procurement and laboratory analysis of the specimen.

Conflicts of interest

No competing interests exist.

REFERENCES

Adraiano D (1984). Trace Metals in the Terrestrial Environment. 1st Ed., VerlagSpiegler, New York.

Bilos C, Colombo JC, Skorupka CN, Rodriguez-Presa MJ (2001). Sources, distribution and variability of airborne trace metals in La Plata City area, Argentina. Environ. Pollut. 111(1):149-158.

Cambra K, Martínez T, Urzelai A, Alonso E (1999). Risk analysis of a farm area near a lead and cadmium contaminated industrial site. J. Soil Contam. 8(5):527-540.

Chronopoulos J, Haidouti C, Chronopoulou-Sereli A, Massas I (1997). Variations in plant and soil lead and cadmium content in urban parks in Athens, Greece. Sci. Total Environ. 196(1):91-98.

Chukwujindu M A I, Nwozo SO, Ossai EK, Nwajei GE (2008). Heavy Metal Composition of Some Imported Canned Fruit Drinks in Nigeria. Am. J. Food Technol. 3:220-223.

Contreraslopez A, Llanaza CA, Santamaria DP (1987). Metal content of apple juice for cider in Asturia (Spain). Afinidad 44:501-503.

Schleich CE, Beltrame MO, Carlos DA (2010). Heavy metals accumulation in the subterranean rodent Ctenomys talarum (Rodentia: Ctenomyidae) from areas with different risk of contamination. Folia Zool. 59(2):108-114.

Damek-Poprawa M, Sawicka-Kapusta K (2003). Damage to liver, kidney and teeth with reference to burden of heavy metals in yellow-necked mice from areas around steelworks and zinc smelters in Poland. Toxicology, 186(1):1-10.

Department of Environment, Food and Rural Affairs, (DEFRA) (1999). Total Diet Study of Aluminium, Arsenic, Cadmium, Chromium, Copper, Lead, Mercury, Nickel, Selenium, Tin and Zinc. The Stationery Office: London.

Dudka S, Miller W P (1999). Permissible concentrations of arsenic and lead in soils based on risk assessment. Water Air Soil. Pollut. 113(1/4):127-132.

Hawley JK (1985). Assessment of health risk from exposure to contaminated soil. Risk Anal. 5(4):289-302.

Köck M, Schaffler R, Sixl W, Pichler-Semmelrock FP, Kosmus W, Marth E (1989). Accumulation of heavy metals in animal parts: Heavy metal contamination in small mammals at a waste disposal site. pp. 855-1857.

Lane TW, Morel FMM (2000). A biological function for cadmium in marine diatoms. Proc. Natl. Acad. Sci. 97(9): 4627-4631.

Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, Morel FM (2005). Biochemistry: A cadmium enzyme from a marine diatom. Nature 435(7038):42-42.

Martiniaková M, Omelka R, Jančová A, Stawarz R, Formicki G (2010). Concentrations of Selected Heavy Metals in Bones and Femoral Bone Structure of Bank (*Myodesglareolus*) and Common (*Microtusarvalis*) Voles from Different Polluted Biotopes in Slovakia. Arch. Environ. Contam. Toxicol. 60(3):524-532.

Mathew J (2008). The value of Grasscutter. World Ark. January-February, pp. 23-24.

Paolo B, Maurizio C (1978). Simultaneous determination of copper, lead and zinc in wine by differential pulse polarography. Analyst 107:271-280.

Rushton J, Viscarra RE (2004). Livestock Production Systems in South America-Analysis and Trends. Report for a study on livestock production systems in South America for FAO. Rome, Italy.

Sánchez-Camazano M, Sánchez-Martín MJ, Lorenzo LF (1994). Lead and cadmium in soils and vegetables from urban gardens of Salamanca (Spain). Sci. Total Environ. 146(147):163-168.

Smith TB, Robert KW, Derek JG, Michael WB (1997). A role for ecotones in generating rainforest Biodiversity. Science 276:1855-1857.

Soewu DA, Bakare OK, Ayodele IA (2012). Trade in Wild Mammalian Species for Traditional Medicine in Ogun State, Nigeria. Global J. Med. Res. 12(3):6-21.

Sterrett SB, Chaney RL, Gifford CH, Meilke HW (1996). Influence of fertilizer and sewage sludge compost on yield of heavy metal accumulation by lettuce grown in urban soils. Environ. Geochem. Health 18(4):135:142.

Van Lune P (1987). Cadmium and lead in soils and crops from allotment gardens in the Netherlands. Netherlands J. Agric. Sci. 35:207-210.

Wilkie DS, Godoy RA (2001). Income and price elasticity of bush meat demand in Lowland Amerindian Societies. Conserv. Biol. 15(3):761-

769.
Wong JWC (1996). Heavy metal contents in vegetables and market garden soils in Hong Kong. Environ. Technol. 17(4):407-414.
World Health Organization (WHO) (1984). Guidelines for Drinking Water Quality: Health Criteria and Other Supporting Information. Vol. 2, WHO, Geneva.
Zurera-Cosano G, Moreno-Rojas R, Salmeron-Egea J, Pozo LR (1989). Heavy metal uptake from greenhouse border soils for edible vegetables. J. Sci. Food Agric. 49(3)307-314.

academicJournals

Vol. 6(8), pp. 161-169, October, 2014
DOI: 10.5897/JTEHS2014.0314
Article Number: 111C3E847679
ISSN 2006-9820

http://www.academicjournals.org/JTEHS

Journal of Toxicology and Environmental Health Sciences

Full Length Research Paper

Effect of dipentyl phthalate in 3-dimensional *in vitro* testis co-culture is attenuated by cyclooxygenase-2 inhibition

Susanna Wegner, Xiaozhong Yu*, Hee Yeon Kim, Sean Harris, William C. Griffith, Sungwoo Hong and Elaine M. Faustman

Institute for Risk Analysis and Risk Communication, School of Public Health, University of Washington, Washington DC, USA.

Received 16 August, 2014; Accepted 15 September, 2014

Exposure to phthalate esters is associated with changes in steroidogenesis, leading to the hypothesis that this is a primary mechanism of phthalate reproductive toxicity. However, some phthalate-induced male reproductive toxicity has been demonstrated in the absence of changes to testosterone production, suggesting additional mechanisms of action. There is evidence that phthalate exposure increases expression of the inflammatory enzyme cyclooxygenase 2 (cox-2). Furthermore, inhibition of cox-2 enhances expression of the steroidogenic acute regulatory protein (StAR), which mediates the rate-limiting step in steroidogenesis. This study hypothesized that phthalate-induced toxicity and testosterone perturbation are mediated in part by cox-2. A 3D *in vitro* rat testis co-culture to explore the role of cox-2 in phthalate toxicity was employed. Cells were treated with 100 µM dipentyl phthalate (DPP) with and without pre-treatment with the specific cox-2 inhibitor NS-398. Effects were evaluated after 8, 24, and 72 h. DPP exposure significantly increased cox-2 expression at 8 and 24 h ($p<0.01$) and resulted in significant, dose-dependent cytotoxicity. Pre-treatment with NS-398 significantly reduced the cytotoxicity of DPP at 8 and 24 h ($p<0.01$). NS-398 also mitigated the effects of DPP on testosterone regulation. Total testosterone concentrations in cell culture media were significantly increased following 8 and 24 hr of DPP exposure ($p<0.001$) and NS-398 reduced this effect ($p<0.05$). Simultaneously, DPP significantly decreased StAR protein expression after 8 h ($p<0.01$) and this effect was significantly attenuated by the presence of NS-398 ($p<0.01$). These results suggest that the DPP-induced changes in testosterone regulation observed in this experiment are mediated in part by an inflammatory response that is cox-2 dependent.

Key words: dipentyl phthalate, testosterone, cyclooxygenase 2, in vitro toxicology

INTRODUCTION

Phthalates are a class of chemicals widely used as plastic softeners and stabilizers in a range of products including adhesives, lubricants and cosmetics. Because they are not bound covalently to these materials, phthalates are prone to leaching out of common consumer products, raising concern over ongoing human

*Corresponding author. E-mail: faustman@uw.edu.

exposures. Monoester phthalate metabolites have been detected in maternal urine during pregnancy, amniotic fluid and breast milk, indicating that low levels of exposure can occur during critical periods of fetal development (Hauser and Calafat, 2005; Silva et al., 2004; Fromme et al., 2011).

A subset of structurally similar phthalate esters are widely recognized as male reproductive toxicants. Male rats exposed to developmentally toxic phthalates *in utero* exhibit reduced testes size, undescended testes, decreased (feminized) anogenital distance, and decreased sperm count (Mylchreest et al., 1998; Gray et al., 2000). Related effects have been documented in humans as well. Male infants whose mothers had high levels of phthalate metabolites (MEP, MBP, MBzP, and MiBP) in their urine during pregnancy had significantly shortened anogenital distance (Swan et al., 2005; Swan, 2008) and *in utero* exposures to diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP) metabolites were associated with decreased masculine play behavior in boys (Swan et al., 2010). In adult men, urinary concentrations of phthalate metabolites (MEHP, MINP, MBzP, MBP, and MEP) have been associated with a decline in sperm quality (Jurewicz et al., 2013). Dipentyl phthalate (DPP), a phthalate commonly used to soften PVC plastic, appears to have particularly potent effects on male reproductive development (Foster et al., 1980; Hannas et al., 2011).

Because fetal testosterone and insulin-like 3 peptide hormone are key regulators of male reproductive development, the decrease in these hormones observed in response to a range of phthalates (Howdeshell et al., 2008; David, 2006) is considered an important mechanism of male reproductive toxicity of phthalates. Indeed, exposure to DBP has been shown to alter expression of a range of key steroidogenic factors, including transcription factors (e.g. GATA4, C/EBPbeta), cholesterol transporters (e.g. the HDL receptor, steroidogenic acute regulatory protein) and enzymes involved in the conversion of cholesterol to testosterone (e.g. hydroxysteroid dehydrogenase) (David, 2006; Lehmann et al., 2004; Barlow et al., 2003). Altered expression of the steroidogenic acute regulatory protein (StAR) is of particular interest because StAR mediates the transfer of cholesterol across the inner membrane of the mitochondria, the rate-limiting process in steroidogeneis (David, 2006; Clark and Cochrum, 2007).

The mechanism of altered StAR expression and testosterone regulation in response to phthalate has not been fully elucidated. Characterization of upstream mechanisms that lead to reduced testosterone synthesis could offer a broader understanding of the full range of possible toxic responses to phthalate exposure. This is particularly important given that some aspects of the toxic responses to phthalate cannot be attributed to altered hormone levels. While the effects of phthalate on hormone level have been shown to be species-specific, phthalates have been shown to alter germ cell development independent of effects on testosterone in mice, rats and human tissue (Gaido et al., 2007; Alam et al., 2010; Lambrot et al., 2009; Heger et al., 2012; Johnson et al., 2012).

Several studies point to the inflammatory enzyme cyclooxygenase 2 (cox-2) as a negative regulator of StAR expression. The cyclooxygenases are a family of enzymes responsible for the conversion of fatty acids into prostaglandins and thromboxanes, mediators of inflammation and vasoconstriction. Cox inhibitors are a widely used class of anti-inflammatory drugs. In cultured mouse Leydig cells, specific inhibition of cox-2 has been shown to increase sensitivity to cAMP stimulation. This results in enhanced expression of both the StAR gene and StAR protein, promoting steroid production (Wang et al., 2003). Conversely, an age-related increase in cox-2 protein in Leydig cells has been associated with an inhibitory effect on StAR-mediated testosterone production (Wang et al., 2005). Furthermore, there is evidence that mono (2-ethylhexyl) phthalate exposure increases expression of cox-2 as well as mitochondrial expression of the redox protein peroxiredoxin 3 in spermatocytes (Onorato et al., 2008). However, the increase in cox-2 following phthalate exposure has not yet been directly linked to subsequent changes in StAR expression or testosterone synthesis. It was hypothesize that the change in StAR gene expression and testosterone production typically observed in response to phthalate exposure is mediated in part by cox-2.

In this study, 3-dimensional *in vitro* testes-co-culture model was employed (Yu et al., 2005) to evaluate the role of cox-2 in mediating DPP-induced testicular toxicity. DPP was selected as a model phthalate due to its potency and selected treatment concentrations that had previously been demonstrated to alter gene expression in the absence of cytotoxicity (Yu et al., 2009). Specifically, the effects of DPP exposure on cox-2 protein expression, StAR protein expression and testosterone concentrations in the presence or absence of a specific cox-2 inhibitor was compared.

MATERIALS AND METHODS

Testis cell co-culture

Male Sprague-Dawley rat pups were obtained on postnatal day 4 (Harlan). On postnatal day 5, testis tissue was isolated and digested for cell culture as described previously (Wegner et al., 2013; Yu et al., 2005). Briefly, testes were removed and dissected to isolate seminiferous tubules, which were digested in a series of enzyme cocktails. Once dissociated, testis cells were suspended in serum-free Eagle's Minimal Essential Medium (Invitrogen) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 3 mM sodium lactate, 1% ITS culture supplement (BD Biosciences) and plated in 35 mm culture dishes at a density of 1.6×10^6 cells/plate. Ice-cold Matrigel (BD Biosciences) extracellular matrix overlay (30 µl, for a final concentration of 200 µg/ml) was immediately added to the center of each dish to provide a 3-dimensional

scaffold.

Phthalate treatment

After plating (48 h), medium was removed and replaced with fresh medium containing 100 µM of the developmentally toxic phthalate DPP (Sigma Aldrich # 80154, 99% purity) or a DMSO (Sigma Aldrich) vehicle control. Pre-treatment with the cox-2 inhibitor NS-398 (EMD Chemicals Inc.) was done 30 min prior to treatment with DPP. Each treatment was repeated in a minimum of three plates for each experiment. Phthalate treatment concentration was selected based on a previous assessment in our co-culture that found less than a 15% reduction in cell viability measured by neutral red uptake assay (Yu et al., 2009).

Microscopy

Following 8, 24 or 72 h of treatment, each three-dimensional co-culture was visualized using an Olympus microscope with phase-contrast optics at 20X magnification. Images were captured and digitized with a Coolsnap camera (Roper Scientific, Inc.).

Lactate dehydrogenase cytotoxicity assay

Following 8, 24, or 72 h of phthalate exposure, 50 µl media was collected from each culture plate for a lactate dehydrogenase (LDH) cytotoxicity assay (Promega Corporation), performed according to the kit protocol. Briefly, prior to harvesting cell lysates, 50 µl of media from each plate was added to a 96-well plate in triplicate. Samples were incubated with 50 µl substrate mix in the dark. After 30 min, reactions were stopped with stop solution and absorbance at 490 nm was read with a plate reader. OD values were normalized to blanks and presented relative to LDH positive controls (media from control plates killed by a freeze/thaw cycle). Data reflect at least 3 independent experiments.

Western blotting

Following 8, 24, or 72 h of treatment, cells were harvested in lysis buffer (Cell Signaling Technology) and protein was isolated by a series of freeze-thaw steps followed by centrifugation. Protein concentration was determined using a commercially available protein assay kit (Protein Assay kit, Bio-Rad Laboratories) and protein samples were diluted with sample buffer, reducing agent and buffer so that all samples contained equal protein concentrations. Samples were loaded in 4 to 12% Bis-Tris NuPage precast minigels (Invitrogen) and separated by running at 200 V for approximately 45 min in running buffer containing 500 µl antioxidant (Invitrogen). Protein was then transferred to polyvinylidene difluoride nylon membranes (Bio-Rad Laboratories) for immunoblotting. Efficiency of transfer was confirmed by commassie stain of the gel after the transfer was complete. Membranes were rinsed in tris-buffered saline (TBS) pH 7.6 then blocked with 5% non-fat dry milk in TBS with 0.1% Tween 20 (TTBS) for 1 h. Membranes were rinsed with TTBS then incubated overnight with primary antibody and for 2 h with secondary antibody conjugated to horseradish peroxidase. Primary antibodies included StAR (a generous gift from Dr. Douglas M Stocco, Texas Tech University), cox-2 (Cayman Chemical), actin (Sigma), anti-rabbit secondary antibody (Cell Signaling Technology Inc.), and anti-mouse secondary antibody (BD Pharminogen). After antibody incubations, membranes were washed 5 times for 5 min with TTBS and incubated with enhanced chemiluminescence detection reagent (GE Lifescience) and exposed to X-ray films (GeneMate). Western blots were analyzed by densitometry using ImageJ (NIH). The intensity of each band of interest was normalized to corresponding actin loading controls. Data presented here reflect at least 3 independent experiments.

Testosterone assay

Media was collected from cells under each treatment condition at 8 and 24 h following treatment. Testosterone concentrations in each medium sample were determined by an ELISA assay for total testosterone according to kit protocol (Neogen Corporation #402510). Testosterone concentrations measured in media were normalized to the protein content of corresponding cell lysate (determined as described earlier). Data on testosterone production are presented in terms of fold change in testosterone/protein relative to control. Data presented here reflect at least 3 independent experiments.

Statistical analysis

Statistical significance was determined by ANOVA using a mixed effects model in R and results are presented as the mean and 95% confidence intervals of the mean. Random effects are the variations between digesting at least three different sets of rat pup testes.

RESULTS

Morphology

Phase-contrast images illustrate the three-dimensional complexity provided by Matrigel overlay in these testicular co-cultures (Figure 1). These images also provide initial qualitative evidence of the effects of phthalate treatment on the 3D co-cultures (indicated by the presence of condensed nuclei and some floating cells) and the prevention of these effects in cells pre-treated with NS-398, a specific cox-2 inhibitor.

Cytotoxicity

In order to evaluate the cytotoxic response to DPP in the presence or absence of NS-398 in the testicular co-culture, a lactate dehydrogenase (LDH) release assay was used. It was found that treatment with DPP resulted in significant cytotoxicity at all time points. Pre-treatment with NS-398 significantly decreased the cytotoxicity of DPP treatment at 8 and 24 h (Figure 2). By 72 h, cox-2 inhibition was less effective in preventing cytotoxicity (data not shown). In order to ensure that NS-398, which has a slightly yellow color, did not interfere with the calorimetric LDH assay reading, an additional set of LDH assays using medium from a single untreated plate was run. No difference was found in LDH reading with and without the addition of NS-398 (data not shown).

Cox-2 protein expression

To further demonstrate that cox-2 is a plausible mediator of DPP-induced toxicity, Western blotting was used to

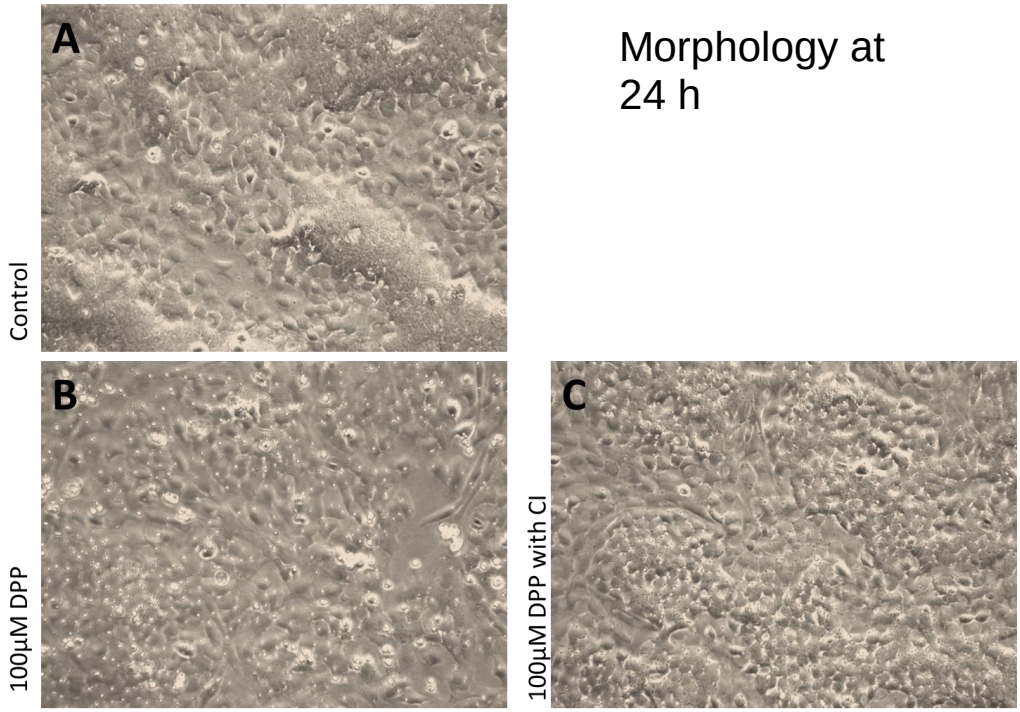

Figure 1. Morphological changes in response to DPP exposure with and without Cox2 inhibitor. (A) Representative morphology of control plates 24 h post treatment (72 h post plating). Cultures contain germ cells, Sertoli cells, Leydig cells in three-dimensional Matrigel matrix. (B) Representative morphology of 24 h 100 µM DPP exposed plates. Note the increase in dense nuclei, indicative of apoptotic cells. (C) Representative morphology of 24 h 100 µM DPP exposed plates with NS-398 cox2 inhibitor (CI) pre-treatment.

measure the effect of DPP exposure on cox-2 protein expression. Cox-2 protein was significantly increased in response to 100 µM DPP following 8 and 24 h of exposure (Figure 3). Interestingly, inhibition of cox-2 ultimately led to a dramatic increase in cox-2 expression by 72 h (data not shown), perhaps due to a feedback mechanism in response to the decrease in cox-2 activity.

Testosterone production

In order to determine the role of cox-2 in mediating DPP induced changes in testosterone concentration, an ELISA assay was used to measure total testosterone in the culture media relative to total protein content in the cell lysate. There was a significant, dose-dependent increase in cell culture media testosterone concentrations following 8 and 24 h of exposure to a cytotoxic concentration of DPP (100 µM). Pre-treatment with NS-398 significantly attenuated the DPP-induced increase in testosterone concentrations after 24 h ($p<0.05$) (Figure 4).

StAR protein expression

Western blotting was used to evaluate the effect of DPP exposure on expression of StAR protein, which mediates the rate limiting step in testosterone synthesis. In agreement with previous reports (Clark and Cochrum, 2007), it was found that exposure to 100 µM DPP significantly decreased expression of StAR protein relative to β-Actin loading control after 8 h of treatment (Figure 5). In order to evaluate the role of cox-2 in this observed decrease in StAR expression, the effect of cox-2 inhibition on the DPP-induced decrease in StAR expression was tested. Pretreatment of co-cultures with NS-398 prevented the DPP-induced decrease in StAR protein at 8 h (Figure 5). By 72 h of treatment, however, the preventive effect of NS-398 was done.

DISCUSSION

By attenuating the effects of DPP with cox-2 inhibitor pretreatment, cox-2 activity is involved in mediating DPP toxicity. Inhibition of cox-2 prevents DPP-induced cytotoxicity and reduces the effect of DPP on media testosterone concentrations and StAR protein expression.

The increase in testosterone concentrations and simultaneous decrease in StAR protein expression observed following DPP treatment in our co-culture suggests a temporarily dynamic response. While exposure to a range

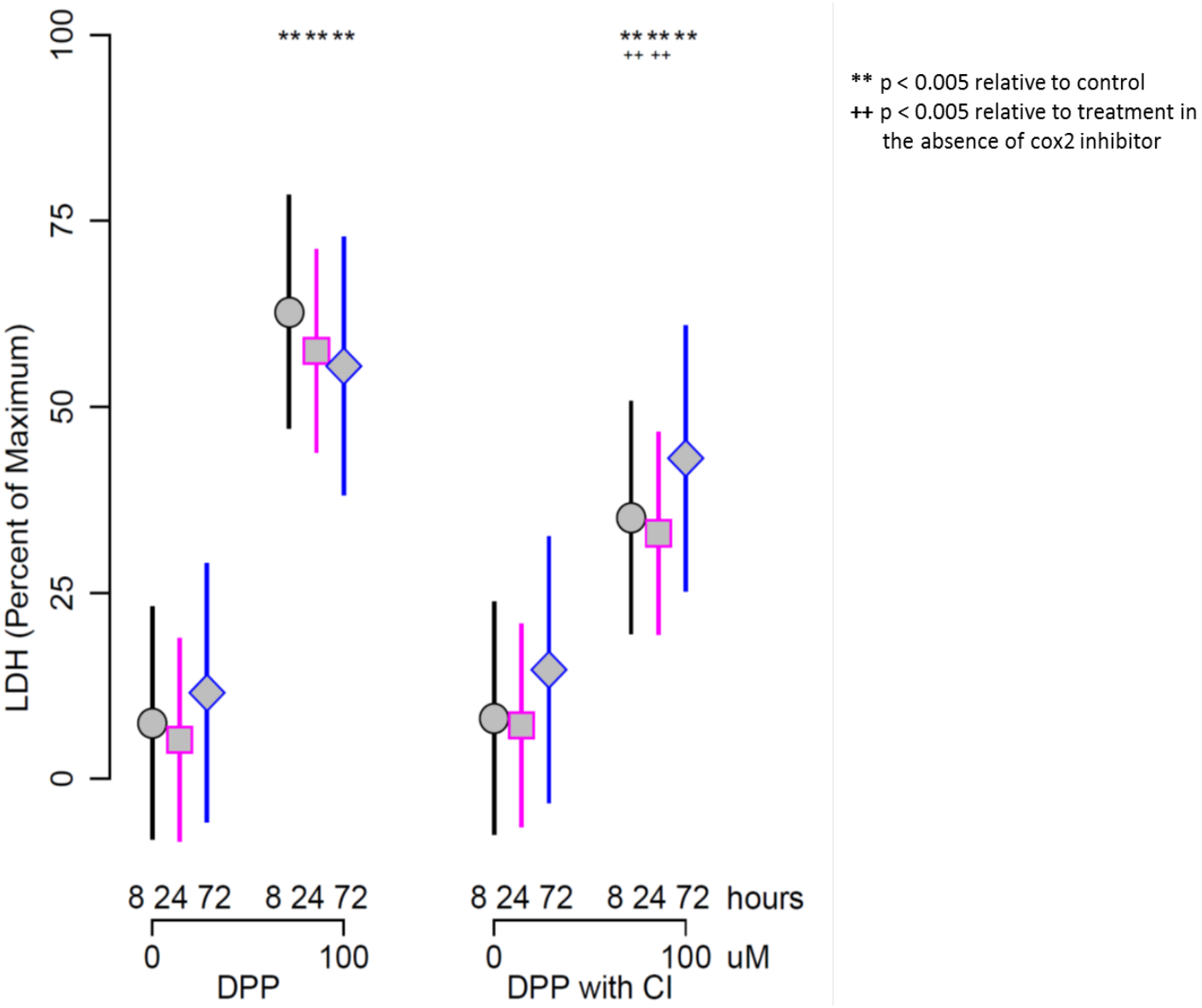

Figure 2. Effect of Cox2 inhibition on DPP Phthalate-Induced Cytotoxicity. After 8 or 24 h of treatment with 100 µM DPP with or without Cox2 inhibitor, medium was harvested and assayed for lactate dehydrogenase (LDH) as an indicator of cytotoxicity. 100 µM DPP dramatically increases cytotoxicity relative to controls. After 8 and 24 h of exposure, pre-treatment with NS-398 cox inhibitor (CI) significantly reduces cytotoxicity. Each data point represents results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by a mixed effect ANOVA.

of reproductively toxic phthalates is typically associated with a decrease in testosterone (David, 2006), an initial increase in testosterone concentrations in response to DPP at 8 and 24 h was seen. The simultaneous down regulation of StAR protein at these time points may foreshadow an eventual decrease in testosterone concentrations. Our previous research has demonstrated a dramatic increase in StAR gene expression following 24 h of exposure to several different reproductively toxic phthalates, including DPP (Yu et al., 2009). These observations are consistent with previous reports that the direction and magnitude of StAR gene expression responds to reproductively toxic phthalates in a time and dose-dependent manner (Lahousse et al., 2006). It still remains to be seen how cox-2 influences StAR protein expression and testosterone concentrations in seemingly contradictory ways. Our current studies do not reveal whether DPP acts on StAR directly, or indirectly via effects on other aspects of testosterone production and metabolism.

Upregulation of cox-2 protein by DPP may be due to feedback in response to direct inhibition by phthalate.

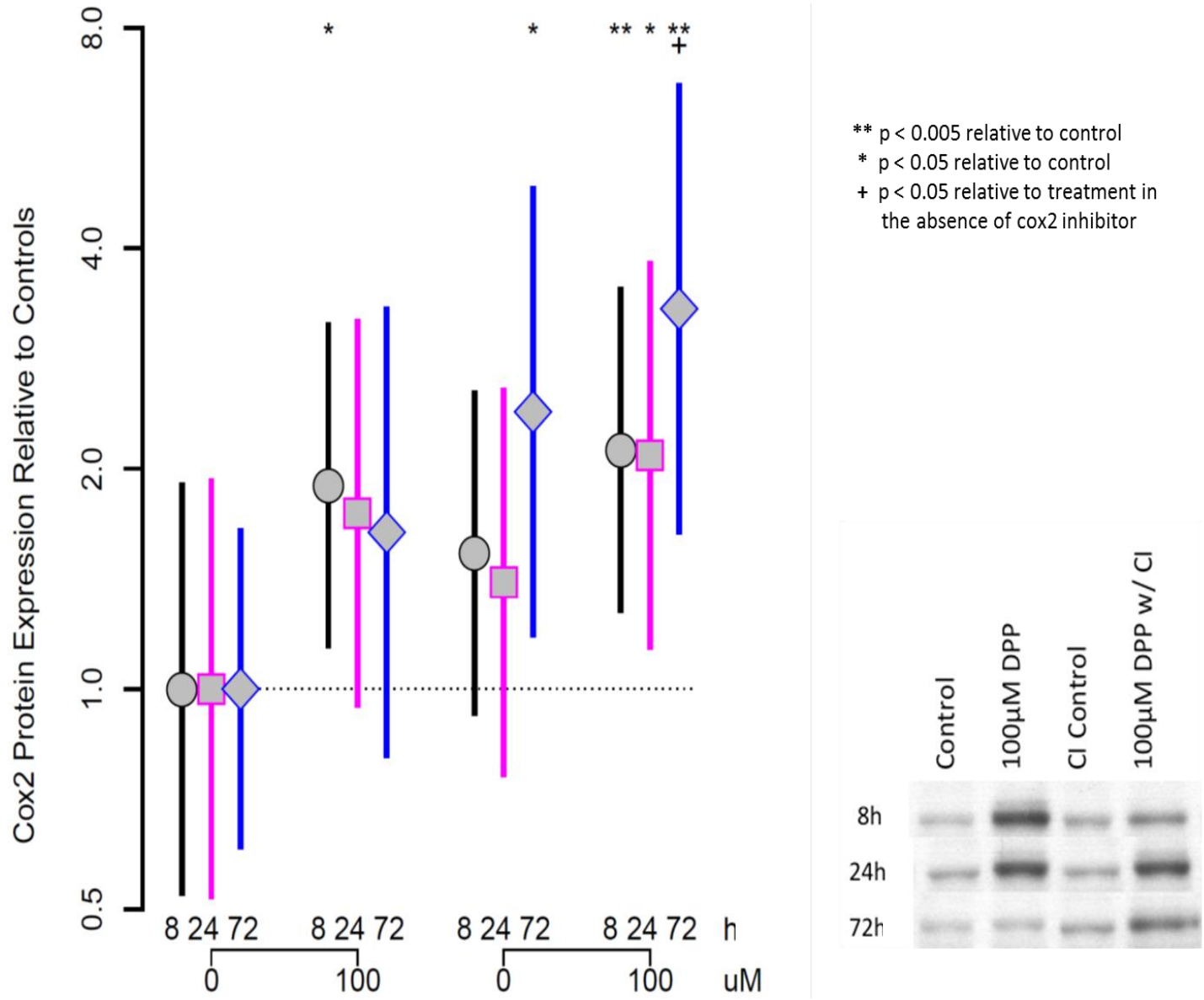

Figure 3. Effect of COX2 inhibition on DPP-induced changes in Cox2 protein expression. After 8 or 24 h of treatment with 100 µM DPP with or without Cox2 inhibitor, protein was harvested, run on western blot, and probed with an antibody to Cox2 protein. Cox2 protein expression was significantly increased in response to DPP phthalate after 8 and 24 h. Cox inhibition significantly increases Cox2 protein expression by 72 h. Each datapoint represents Western results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by a mixed effects ANOVA.

Indeed, several reproductively toxic phthalates have been shown to reduce prostaglandin synthesis following 24 h exposures *in vitro*, suggesting a decrease in cyclooxygenase activity. Computer modeling simulations indicate that several phthalates can directly inhibit cyclooxygenase enzymes by direct binding (Kristensen et al., 2011). Because cox-2 is known to suppress testosterone, such inhibition of cox-2 by phthalate could explain the higher levels of testosterone measured in the cell culture media following DPP exposure. DPP induced inhibition of cox-2 may underlie the ultimate upregulation of cox-2 observed in this study. Inflammatory signals like cox-2 can also be induced by cell death (Rock and Kono, 2008). Therefore, it was difficult to distinguish in our studies whether effects were also being seen on cox2 via cell death.

The effect of DPP on cox-2 expression has interesting implications. In addition to its effect on StAR expression, cox-2 contributes to prostaglandin and thromboxane synthesis and induces expression of other cytokines including IL1a and IL6 (Ishikawa et al., 2005). A phthalate-induced increase in cox-2 may therefore increase

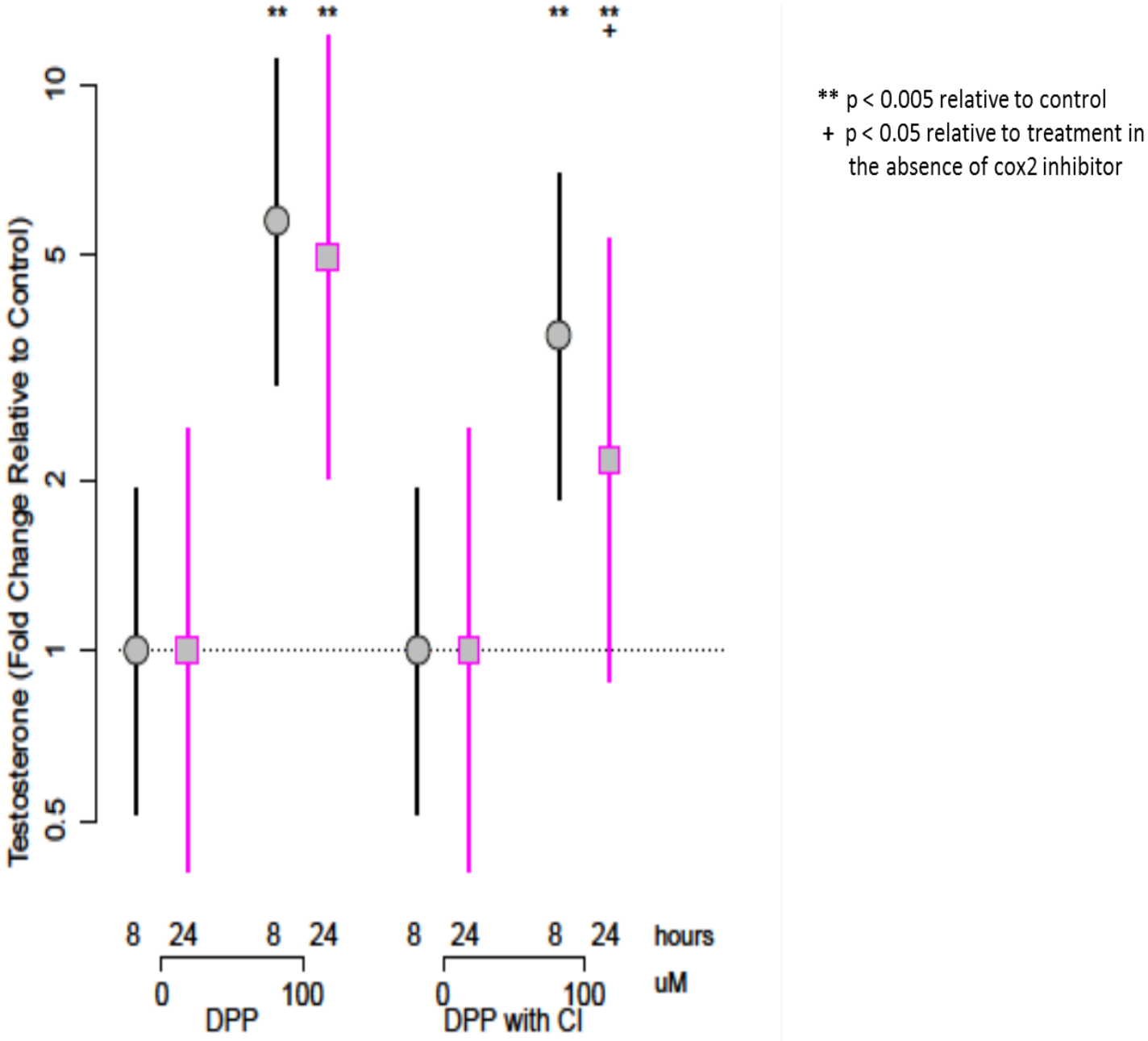

Figure 4. Effect of DPP on testosterone production. After 8 or 24 h of treatments with 100 µM DPP with or without Cox2 inhibitor, media was collected and assayed for testosterone concentration by ELISA assay. Testosterone concentrations were normalized to protein content and are presented here in terms of fold change relative to the control. Each datapoint represents Western results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by mixed effects ANOVA.

inflammatory secretions by Sertoli cells. Such paracrine signaling by Sertoli cells may be an important secondary mechanism of phthalate toxicity.

Further exploration of the upstream events that lead to altered StAR expression and subsequently altered testosterone concentrations may reveal common upstream mechanisms for other important pathways of phthalate toxicity. In particular, the role of inflammatory responses in mediating phthalate toxicity warrants further investigation. There is a growing body of evidence to suggest that inflammation plays an important role in phthalate toxicity. For example, DPP has been linked to inflammatory response in adult male rat testes (Granholm et al., 1992) and MEHP exposure in immature rats has been shown to lead to an increase in recruitment of immune cells into the testis, followed by an increase in germ cell apoptosis

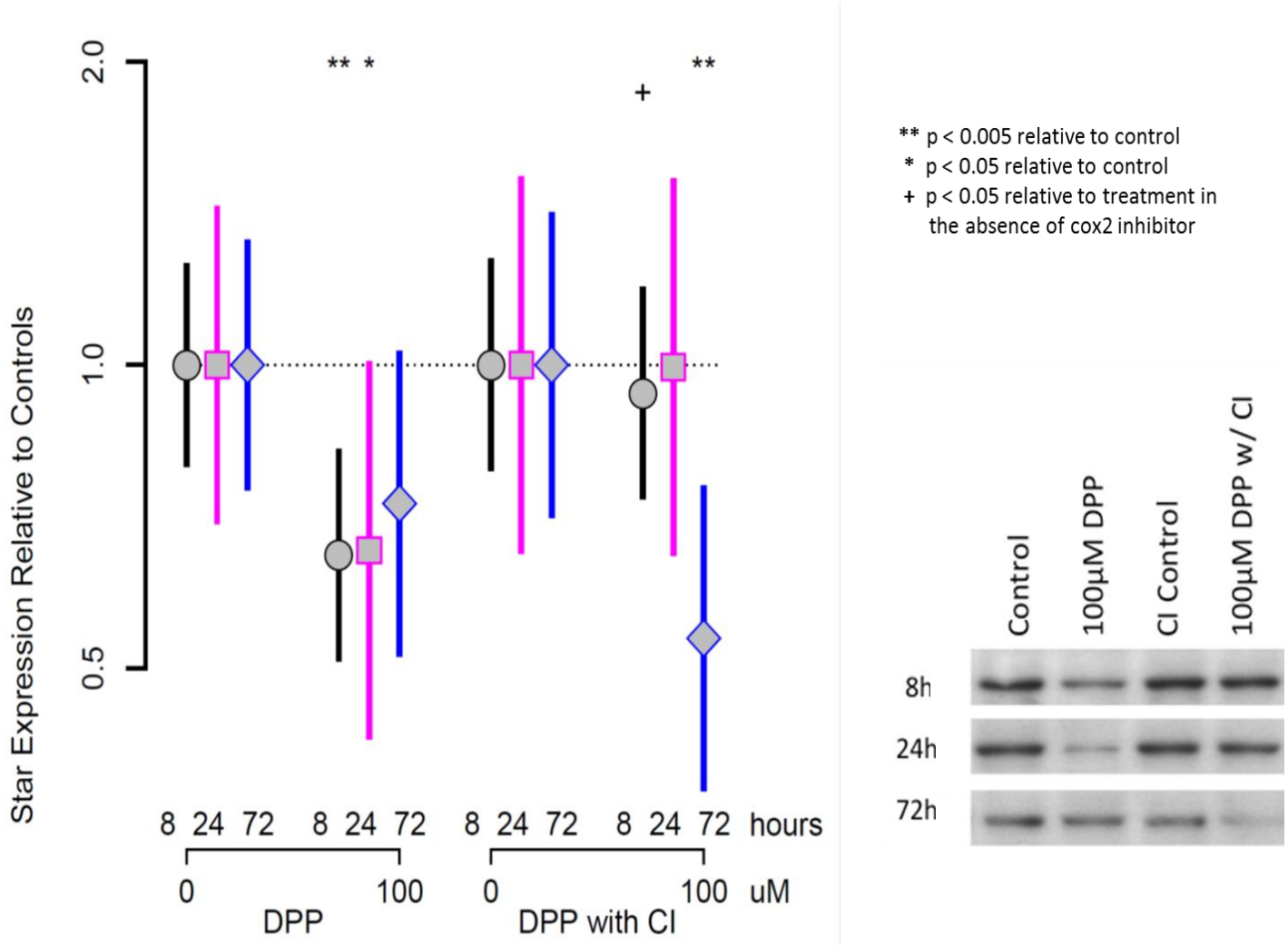

Figure 5. Effect of COX2 inhibition on DPP-induced changes in StAR protein expression. After 8 or 24 h of treatment with 100µM DPP with or without Cox2 inhibitor, protein was harvested, run on western blot, and probed with an antibody to StAR protein. At 8 h, StAR protein expression was significantly decreased in response to DPP phthalate (p=0.0025). Pre-treatment with Cox2 inhibitor (CI) prevented this effect (p=0.019). Each datapoint represents western results from at least 3 independent experiments. Error bars indicate 95% confidence intervals determined by a mixed effects ANOVA.

(Murphy et al., 2014). Furthermore, NHANES data shows a correlation between diethylhexyl phthalate and benzylbutyl phthalate metabolites and markers of inflammation and oxidative stress in humans (Ferguson et al., 2011. Epidemiological evidence suggests that exposure to a range of specific phthalates increases the risk of asthma and allergies (Bornehag and Nanberg, 2010; Kimber and Dearman, 2010). A clearer understanding of the underlying mechanism of the inflammation mediated effects of phthalates will inform risk assessment and facilitate construction of adverse outcome pathways for male reproductive toxicity.

Future studies should address the mechanism of phthalate influence on cox-2 activity. Cox-2 in Sertoli cells has previously been shown to be induced by the cytokine interleukin-1beta (IL1B) via JNK signaling (Ishikawa et al., 2005). This evidence warrants further exploration of these regulatory pathways in response to phthalate exposure. Further research should also explore the cell-type specific mechanisms of phthalate toxicity. These results also need to be replicated with other phthalates to determine whether this inflammatory mechanism is consistent across reproductively toxic phthalates or unique to DPP. Better understanding of the mechanisms of phthalate toxicity will contribute to more informed risk assessment of this ubiquitously used class of chemicals.

Conflict of Interest

Authors have not declared any conflict of interest.

REFERENCES

Alam MS, Ohsako S, Matsuwaki T, Zhu XB, Tsunekawa N, Kanai Y, Sone H, Tohyama C, Kurohmaru M (2010). Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: a possible estrogenic effect of di(n-butyl) phthalate. Reproduction 139 (2):427-37.

Barlow NJ, Phillips SL, Wallace DG, Sar M, Gaido KW, Foster PM (2003). Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate.Toxicol.Sci.73(2):431-41.

Bornehag CG, Nanberg E (2010). Phthalate exposure and asthma in children. Int. J. Androl. 33(2):333-45.

Clark BJ, Cochrum RK (2007). The steroidogenic acute regulatory protein as a target of endocrine disruption in male reproduction. Drug Metab. Rev. 39 (2-3):353-70.

David RM (2006). Proposed mode of action for in utero effects of some phthalate esters on the developing male reproductive tract. Toxicol. Pathol. 34(3):209-19.

Ferguson KK, Loch-Caruso R, Meeker JD (2011). Urinary phthalate metabolites in relation to biomarkers of inflammation and oxidative stress: NHANES 1999-2006. Environ. Res. 111(5):718-26.

Foster PM, Thomas LV, Cook MW, Gangolli SD (1980). Study of the testicular effects and changes in zinc excretion produced by some n-alkyl phthalates in the rat. Toxicol. Appl. Pharmacol. 54(3):392-8. Fromme H, Gruber L, Seckin E, Raab U, Zimmermann S, Kiranoglu M, Schlummer M, Schwegler U, Smolic S, Volkel W (2011). Phthalates and their metabolites in breast milk--results from the Bavarian Monitoring of Breast Milk (BAMBI). Environ. Int. 37(4):715-22.

Gaido KW, Hensley JB, Liu D, Wallace DG, Borghoff S, Johnson KJ, S Hall SJ, Boekelheide K (2007). Fetal mouse phthalate exposure shows that Gonocyte multinucleation is not associated with decreased testicular testosterone. Toxicol. Sci. 97 (2):491-503.

Granholm T, Creasy DM, Pollanen P, Soder O (1992). Di-n-pentyl phthalate-induced inflammatory changes in the rat testis are accompanied by local production of a novel lymphocyte activating factor. J Reprod. Immunol. 21(1):1-14. Gray LE Jr, Ostby J, FurrJ, Price M, Veeramachaneni DN, Parks L (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 58(2):350-65.

Hannas BR, Furr J, Lambright CS, Wilson VS, Foster PM, Gray LE Jr (2011). Dipentyl phthalate dosing during sexual differentiation disrupts fetal testis function and postnatal development of the male Sprague-Dawley rat with greater relative potency than other phthalates. Toxicol. Sci. 120(1):184-93.

Hauser R, Calafat AM (2005). Phthalates and human health. Occup. Environ. Med. 62(11):806-18.

Heger NE, Hall SJ, Sandrof MA, McDonnell EV, Hensley JB, McDowell EN, Martin KA, Gaido KW, Johnson KJ Boekelheide K (2012). Human fetal testis xenografts are resistant to phthalate-induced endocrine disruption. Environ. Health Perspect. 120(8):1137-43.

Howdeshell KL, Rider CV, Wilson VS, Gray LE Jr (2008). Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. Environ. Res. 108(2):168-76.

Ishikawa T, Hwang K, Lazzarino D, Morris PL (2005). Sertoli cell expression of steroidogenic acute regulatory protein-related lipid transfer 1 and 5 domain-containing proteins and sterol regulatory element binding protein-1 are interleukin-1beta regulated by activation of c-Jun N-terminal kinase and cyclooxygenase-2 and cytokine induction. Endocrinology 146(12):5100-11.

Johnson KJ, Heger NE, Boekelheide. K (2012). Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent. Toxicol. Sci. 129(2):235-48.

Jurewicz J, Radwan M, Sobala W, Ligocka D, Radwan P, Bochenek M, Hawula W, Jakubowski L, Hanke W (2013). Human urinary phthalate metabolites level and main semen parameters, sperm chromatin structure, sperm aneuploidy and reproductive hormones. Reprod. Toxicol. 42:232-41.

Kimber I, Dearman RJ (2010). An assessment of the ability of phthalates to influence immune and allergic responses. Toxicology 271(3):73-82.

Kristensen DM, Skalkam ML, Audouze K, Lesne L, Desdoits-Lethimonier C, Frederiksen H, Brunak S, Skakkebaek NE, Jegou B, Hansen JB, Junker S, Leffers H (2011). Many putative endocrine disruptors inhibit prostaglandin synthesis. Environ. Health Perspect. 119(4):534-41.

Lahousse SA, Wallace DG, Liu D, Gaido KW, Johnson KJ (2006). Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl) phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages. Toxicol. Sci. 93(2):369-81.

Lambrot R, Muczynski V, Lecureuil C, Angenard G, Coffigny H, Pairault C, Moison D, Frydman R, Habert R, Rouiller-Fabre V (2009). Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. Environ. Health Perspect. 117 (1):32-7.

Lehmann KP, Phillips S, Sar M, Foster PM, Gaido KW (2004). Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. Toxicol. Sci. 81(1):60-8.

Murphy CJ, Stermer AR, Richburg.JH (2014). Age- and Species-Dependent Infiltration of Macrophages into the Testis of Rats and Mice Exposed to Mono-(2-Ethylhexyl) Phthalate (MEHP). Biol. Reprod. biolreprod-113.

Mylchreest E, Cattley RC, Foster PM (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? Toxicol. Sci. 43(1):47-60.

Onorato TM, Brown PW, Morris PL (2008). Mono-(2-ethylhexyl) phthalate increases spermatocyte mitochondrial peroxiredoxin 3 and cyclooxygenase 2. J. Androl. 29(3):293-303.

Rock KL, Kono H (2008). The inflammatory response to cell death. Annu. Rev. Pathol. 3:99-126.

Silva MJ, Reidy JA, Herbert AR, Preau JL Jr, Needham LL, Calafat AM (2004). Detection of phthalate metabolites in human amniotic fluid. Bull. Environ. Contam. Toxicol. 72(6):1226-31.

Swan SH (2008). Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ. Res. 108(2):177-84.

Swan SH, Liu F, Hines M, Kruse RL, Wang C, Redmon JB, Sparks A, Weiss B (2010). Prenatal phthalate exposure and reduced masculine play in boys. Int. J. Androl. 33(2):259-69.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S. Teague. JL (2005). Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspect. 113(8):1056-61.

Wang X, Dyson MT, Jo Y, Stocco DM (2003). Inhibition of cyclooxygenase-2 activity enhances steroidogenesis and steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells. Endocrinol. 144(8):3368-75.

Wang X, Shen CL, Dyson MT, Eimerl S, J. Orly J, Hutson JC, D. M. Stocco DM (2005). Cyclooxygenase-2 regulation of the age-related decline in testosterone biosynthesis. Endocrinology 146(10):4202-8.

Wegner S, Hong S, Yu X, Faustman EM (2013). Preparation of rodent testis co-cultures. Curr. Protoc. Toxicol. 16-10.

Yu X, Hong S, Moreira EG, Faustman EM (2009). Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: Lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. Toxicol. Appl. Pharmacol. 239(3):325-36.

Yu X, Sidhu JS, Hong S, Faustman. EM (2005). Essential role of extracellular matrix (ECM) overlay in establishing the functional integrity of primary neonatal rat Sertoli cell/gonocyte co-cultures: an improved in vitro model for assessment of male reproductive toxicity. Toxicol. Sci. 84(2):378-93.

Journal of Toxicology and Environmental Health Sciences

Related Journals Published by Academic Journal

- *Journal of Clinical Pathology and Forensic Medicine*
- *Journal of Infectious Diseases and Immunity*
- *Journal of Clinical Virology Research*
- *Clinical Reviews and Opinions*
- *Medical Case Studies*
- *Medical Practice and Reviews*

academicJournals