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Biological markers of harm can be detected in mice exposed for two months to low doses of Third Hand Smoke under conditions that mimic human exposure

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ABSTRACT

Third-hand smoke (THS) is a recently discovered environmental health hazard that results from accumulation and aging of second-hand smoke (SHS) toxins on surfaces of environments where smoking has occurred. Our objective was to determine whether there is a dose-dependent effect of THS exposure on biological markers of harm (BMH) using an *in vivo* exposure system that mimics exposure of humans to THS. THS exposure generated from as low as the 10 cigarettes-smoking regimen, resulted in increased circulating inflammatory cytokines, tumor necrosis factor alpha, interleukin 1 alpha, and granulocyte macrophage colony-stimulating factor. We also found that there was an increase in adrenocorticotropic hormone and superoxide dismutase and a decrease in ATP levels in liver tissue. Many of the altered BMH that are related to oxidative stress and decrease in ATP levels, suggest mitochondrial dysfunction. THS exposure generated from the 20 and 40 cigarettes-smoking regimen resulted in further damage. Our studies are important because virtually nothing is known about the physiological damage caused by different levels of THS exposure. These studies can also serve to educate the public on the dangers of THS and the BMH we identified can potentially be used in the clinic, once verified in exposed humans.

1. Introduction

Cigarette toxins have been shown to affect biological processes as well as organ systems in the body and are detrimental to human health. Recently, however, a new route of exposure to cigarette toxins has been discovered and is known as Third Hand Smoke (THS). THS is Second Hand Smoke (SHS) toxins deposited on surfaces where smoking has occurred as well as in the clothing, skin and hair of people who smoke (Bahl et al., 2014; Hoh et al., 2012; Quintana et al., 2013; Schick et al., 2014). Smoke-free laws are becoming widespread; as of 2016, 24 states in the US had passed laws restricting smoking in work and public spaces (Delgado-Rendon et al., 2017). 347 Public Housing Authorities have adopted anti-smoking ordinances in their units and 19 cities and/or counties (about 4%) in California have complete bans on smoking in multi-unit housing (Delgado-Rendon et al., 2017). THS toxins have been shown to result in health problems ranging from metabolic disorders to hyperactivity and anxiety disorders, with the potential to affect learning in children (Adhami et al., 2016; Ferrante et al., 2013; Goldberger et al., 2012; Hutchinson et al., 2013; Torok et al., 2017). Parental smoking has caused approximately 100,000 infant deaths in this same period due to Sudden Infant Death Syndrome, prematurity, low birth weight, and other conditions (Torok et al., 2017). Children and infants are at most risk for being exposed to THS toxins because they are in closer contact with household surfaces; they frequently put their hands in the mouths and also their surface to volume ratio is large allowing for the absorption of these toxins through the skin in a much more effective way (Ferrante et al., 2013). We have previously determined the minimum amount of time required to cause physiological changes in mice when they were exposed to THS under conditions that mimic human exposure (Adhami et al., 2017). Our findings suggest that specific Biological Markers of Harm (BMH) can potentially be used to determine how long children have been exposed to THS toxins and the extent of cellular and molecular damage that could lead to disease.

In our study to investigate the dose effects to THS exposure, we narrowed the scope of the work to focus on the BMH that were altered by 2-month of exposure. Therefore, the specific BMH that we chose to study are those that are altered at 2 months of THS exposure to the 40 cigarette-smoking regimen (Adhami et al., 2017). We asked the question: What is the minimum dose of exposure required for these toxins to cause harm? Here, we exposed different cohorts of mice to THS

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produced by 10, 20 or 40 cigarette-smoking regimens for 2 months starting at weaning, and then tested the mice to determine the lowest dose that can cause changes for each chosen BMH. The dose of THS exposure was characterized as total particulate matter (TPM) base on the criteria established by Environmental Protection Agency (EPA) and the presence of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in the urine of the mice in comparison with that in the urine of children living in the homes of smokers. We describe this characterization in detail in the Materials and Methods section and in our previously published studies (Martins-Green at all 2014). We found that several of the BMH were increased in the serum and/or in liver and brain tissues. However, it is important to note that ATP production was significantly decreased in both tissues with minimum exposure. Our findings are significant because they show that low levels of THS exposure over 2 months can cause health problems and have the potential to be used as diagnostic tools to detect THS exposure in humans, especially children, once proof-of-concept-studies in humans have been performed.

2. Materials and methods

Animals: Three-weeks-old C57BL/6 mice were divided into control and three experimental groups. The three experimental groups were exposed to THS produced by 10, 20, or 40 cigarettes, respectively. The control group was never exposed to THS toxins. Both control and THS-exposed mice were maintained under controlled environmental conditions – 12-hr light/dark cycle in conventional cages with *ad libitum* access to standard chow (percent calories: 58% carbohydrate, 28.5% protein, and 13.5% fat) and water.

Ethics Statement: All animal experimental protocols were approved by the University of California, Riverside, Institutional Animal Care and Use Committee (UCR-IACUC). Mice were euthanized by carbon dioxide (CO_2) inhalation, which is the most common method of euthanasia used by NIH. The amount and length of CO_2 exposure were approved by UCR-IACUC.

THS exposure method: Common household fabrics such as curtain material, upholstery, and carpet were placed in mouse cages and exposed to SHS generated by a smoke generation system built by Teague Enterprises, Inc., (Woodland, CA) as previously described (Martins-Green et al., 2014). Each cage contained 10 g of curtain material (cotton), 10 g of upholstery (cotton and fiber), and two 103 cm^2 pieces of carpet (fiber) to maintain equal exposure levels across all experimental groups. 10, 20, or 40 3R4F research cigarettes were smoked each day, five days per week for 2 months. The smoke was routed to a mixing compartment to mix with air and the mixture distributed between two exposure chambers, each containing four cages with the materials. The gravimetric method was used to determine the TPM in each smoke chamber using Whatman grade 40 quantitative cellulose filters. The measurements were made halfway through the smoking of the cigarettes; each filter was weighed in an analytical scale and the weight recorded before introducing the filter paper into the filtering device and the device was then allowed to run for 15 min. After 15 min, the filter was weighed again to determine the particulate mass that had accumulated during this time period. This procedure was repeated with two more filters and the average of the three masses gave the TPM for the chamber being measured. The same procedure was used for the other chamber. For the dose-dependence we defined TPM values as follows: “10 cigarettes” as low dose (TPM = 8 ± 5 μg/m^3), “20 cigarettes” as medium dose (TPM = 15 ± 5 μg/m^3), and “40 cigarettes” as high dose (TPM = 30 ± 5 μg/m^3). 30 ± 5 μg/m^3 is the value that the EPA (U.S. Environmental Protection Agency, 2002) found in the homes of smokers by the EPA. At the end of each week, cages were removed from the exposure chamber, bagged, and transported to the vivarium where mice were transferred from the previous cages into the new cages with the THS exposed material. The used materials from the previous cages were put in new cages which were brought to the laboratory for exposure to THS for the following week. At the end of the week the cages with these materials were taken to the vivarium and the animals transferred to them. This cycle was followed for 4–6 weeks or until the material became soiled. By using two sets of cages and materials, each of which was exposed on alternating weeks, we ensured that mice inhabited cages containing fabric that had been exposed to fresh THS in the beginning of each week and aged smoke towards the end of the week. This is what happens in houses of smokers; exposure to fresh THS toxins and then to aged THS toxins. Because we did not want breeding to occur during these experiments, the males and females were kept in separate cages. Age-matched control mice were kept in clean air in separate vivarium room.

Blood collection: Blood was extracted directly from the heart and allowed to coagulate at room temperature for 1 h. The samples were then centrifuged at 10,000 rpm for phase separation. The serum was used immediately for assays or frozen and stored at −80 °C for later assays.

Tissue collection: After each dose of THS exposure, brain and liver tissue were collected from THS-exposed or control mice, and immediately frozen and stored at −80 °C. The liver tissue was collected from the periphery of the lobes to avoid major vessels and ducts. The brain tissue was cut into two halves and collected from the region of hypothalamus and pituitary. Liver and brain pieces (10 mg) were placed in ice-cold RIPA buffer containing 50 mM Tris (50 mM pH 7), NaCl (150 mM), SDS (0.1%), sodium deoxycholate (0.5%) and Triton X (1%) and homogenize using a Bullet Blender Homogenizer to extract total protein. The samples were centrifuged at 10,000 rpm for 15 min at 4 °C, and the resulting supernatants were stored at −80 °C for later to analyze by the various assays we used for this study. Protein quantification was done using the Bradford assay (Bio-Rad).

Measurement of aspartate aminotransferase (AST) activity: A MybioSource ELISA Kit (Cat # MBS043425) was used to measure AST levels in the serum, which were quantified by incubation of the 25 μl of serum samples in anti-AST antibody coated plates, followed by HRP–conjugated secondary antibody. A standard curve was used to quantify the activity of AST in the serum. The plate was read at 580–630 nm wavelength.

Measurement of urea level: Urea quantization in the serum was determined using the OxiSelect Urea Assay Kit from Cell Biolabs (Cat#STA-382). 10 μl of serum samples or standards were incubated for 10 min with the enzyme urease. Urea contained in samples and standards was first degraded into ammonia and carbon dioxide, which further reacted with an alkaline developer to produce a blue-green colored product that was measured in a spectrophotometric plate reader at an optical density between 580 and 630 nm wavelength. A standard curve was used to quantitate urea in the serum.

Measurement of adrenocorticotropic hormone (ACTH) level: A MybioSource ELISA Kit (Cat # MBS720035) was used to measure ACTH level in the serum, which was quantified by incubation of 50 μl of serum in an anti-ACTH antibody-coated plate, followed by HRP–conjugated secondary antibody. A standard curve was generated and used to quantitate ACTH level at 450 nm wavelength.

Measurement of epinephrine level: A MybioSource ELISA Kit (Cat # MBS011202) was used to measure epinephrine levels in the serum. Epinephrine levels were quantified by incubation of 25 μl of serum in an anti-epinephrine antibody-coated plate, followed by HRP–conjugated secondary antibody. A standard curve was generated and used to quantitate epinephrine level at 450 nm wavelength.

Measurement of cytokines level: The levels of cytokines in the serum were quantified using a Multi-Analyte ELISArray Kits from QIAGEN (Cat # MEM-004A). This ELISArray kit allowed for the analysis of multiple cytokines and chemokines simultaneously, IL-1α, MCP-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-α, TNF-α, G-CSF and GM-CSF. 50 μl of diluted serum was incubated in corresponding ELISA wells of each cytokine tested, incubated with the corresponding antibody, followed by HRP–conjugated secondary antibody. A standard curve was generated and used to quantitate the levels of each cytokine tested.

Measurements of hydrogen peroxide (H_2O_2) level: A Cayman Hydrogen Peroxide Assay Kit (Cat # 600,050) was used to measure H_2O_2 level in the liver and brain, respectively. H_2O_2 was detected using 10-acetyl-3,7-dihydroxyphenoxazine (ADHP), a highly sensitive and stable probe for H_2O_2 by using homogenate supernatant of liver and brain. In the presence of horseradish peroxidase, ADHP reacts with H_2O_2 in a 1:1 stoichiometry ratio to produce a highly fluorescent resorufin (excitation = 530–560 nm; emission = 590 nm). The standard curve generated was used to quantify the activity of hydrogen peroxide in the liver and brain.

Measurement of superoxide dismutase (SOD) activity: Cayman Superoxide Dismutase Assay Kit (Cat # 706,002) was used to measure SOD levels in the liver and brain. SOD activity was quantified by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine by using homogenate supernatant of liver and brain. A standard curve was generated and used to quantify the activity of SOD in the liver and brain samples. The plate was read at 450 nm wavelength.

Measurement of nitrosylation of proteins: Protein damage was quantified by measuring the nitrosylation of tyrosine residues in proteins. The amount of 3-nitrotyrosine in the liver and brain proteins was determined using the OxiSelect Nitrotyrosine ELISA Kit from Cell Biolabs (Cat#STA-305). The liver samples or nitrated BSA standards were first added to a nitrated BSA pre-absorbed enzyme immunoassay (EIA) plate. After a brief incubation, an anti-nitrotyrosine antibody was added, followed by an HRP conjugated secondary antibody at 450 nm wavelength. The protein nitrotyrosine content in the liver samples were determined by comparing with a standard curve that was prepared from predetermined nitrated BSA standards provide in the kit.

Measurement of ATP level: The ATP level in liver and brain was quantified using BioVision's ATP Colorimetric and Fluorometric Assay kit (Cat# K354-100), which is designed to detect low quantities of ATP (~50 pmol) by measuring the phosphorylation of glycerol. The assay was performed according to the protocol included in the kit. 50 µl of sample supernatant of liver or brain tissue, containing the same amount of protein, were added to a 96-well plate in duplicates. 50 µl of the Reaction Mix (44 µl ATP Assay buffer; 2 µl ATP probe; 2 µl ATP converter; and 2 µl developer) were added to each well containing the ATP standards and samples to initiate the phosphorylation of glycerol reaction. The plate was mixed well and incubated for 30 min at room temperature away from light. During this incubation period, glycerol was phosphorylated. The product of this phosphorylation reaction was read at λ = 570 nm and the absorbance readings of the standards were used to generate a standard curve. The unknown amount of ATP in the samples was then calculated using the standard curve and the observances reading of the samples.

MitoTracker™ Red Staining of Liver Sections: For each control and 40 cigarettes group mice, a piece of liver tissue was fixed in 10% paraformaldehyde overnight, incubated with sucrose to decrease ice crystal damage during freezing and then embedded in Optimal Cutting Temperature compound (OCT compound). 7 µm-thick sections were made using a cryostat. The OCT was removed from the sections and then they were stained with 200 µL of 1 mM rosamine MitoTracker® probes (M7511, M7513, Invitrogen, USA) for 15min and protected from light. The excess dye was washed, Vecta-shield applied and coverslips sealed with nail. The stain was visualized within 48 h using Nikon Microphot-FXA fluorescence microscope with a Nikon DS-Fi1 digital camera and Nikon NIS-Elements software.

Power analysis: Our power analysis, takes into account the differences we have previously observed in similar studies, predicts that for a standard deviation of 0.70, we would need 6–7 mice in each condition to reach 90% confidence. Because our data is so tight we have used 6 animals per group. The reason for that is two-fold: (1) these mice are genetically practically identical and (2) the conditions of exposure are very controlled and standardized.

Statistical Analysis: For statistical analysis in this study, we used Kolmogorov-Smirnov test to conduct normality tests for the variables and IBM SPSS 20.0 (IBM, New York, USA) to conduct one-way analysis of variance (ANOVA) followed by LSD test for multiple comparisons. All data are presented by mean ± SD. Means were considered significantly different when $p < 0.05$.

Table 1
Biomarkers examined to test the damage caused by THS exposure in a dose-dependent manner.

Biological Markers of Harm	Detected In
Aspartate aminotransferase (AST)	Serum
Urea	Serum
Adrenocorticotropic hormone (ACTH)	Serum
Epinephrine	Serum
Interleukin-1α (IL-1α)	Serum
Interleukin-4 (IL-4)	Serum
Interleukin-10 (IL-10)	Serum
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Serum
Tumor necrosis factor alpha (TNF-α)	Serum
H_2O_2	Liver; Brain
Superoxide dismutase (SOD)	Liver; Brain
Nitrotyrosine	Liver; Brain
ATP	Liver; Brain

3. Results

For all of the studies shown here, we investigated levels of molecules that had shown to be altered in our previous studies of the time-dependent effect of THS exposure (Adhami et al., 2017). We chose to determine whether those same molecules were altered in a dose-dependent manner in the serum, and liver and brain tissues of different cohorts of mice exposed for 2 month to doses of THS generated by the 10, 20 and 40 cigarette-smoking regimen and tested these mice against 13 BMH (Table 1). Nine biomarkers were tested in serum and four in liver and brain tissue.

The data on the effects of THS exposure are presented in four sections: (1) serum circulating BMH that result in tissue stress (AST, Urea, ACTH and Epinephrine); (2) serum markers of inflammation (GM-CSF, IL-1α, TNF-α, IL-4, and IL-10); (3) THS-induced oxidative stress in liver and brain tissues (H_2O_2, SOD, nitrotyrosine); and (4) decreased energy production by the mitochondrial (ATP). In the text, when we refer to 10, 20, 40 cigarettes it means that the mice were exposed to THS generated by the regimen of smoking these numbers of cigarettes 5 h s/day, 5 day/week with the TPM adjusted to 8, 15, 30 ± 5 µg/m^3, respectively.

3.1. Dose required for THS exposure to cause changes to circulating stress BMH

We found that serum AST levels were increased significantly ($p < 0.01$) in a dose-dependent manner as the number of cigarettes increased starting at a dose of the 20 cigarette-smoking regimen suggesting that AST synthesis is induced by THS exposure. With the 40 cigarette-smoking regimen AST levels doubled when compared to those of the control (Fig. 1A). We also found that urea levels, were significantly increased ($p < 0.05$) after THS-exposure generated by the 40 cigarettes-smoking regimen, but showed already elevation after smoking of 20 cigarettes (Fig. 1B) confirming alteration in liver function. Furthermore, we found significant increases ($p < 0.05$) in the circulating stress hormone, ACTH, which is produced in the brain starting at a dose of the 10 cigarettes-smoking regimen and significant increases were found in ACTH at a dose of the 20 ($p < 0.01$) and 40 ($p < 0.001$) cigarette-smoking regimen (Fig. 1C). ACTH in turn leads to the production of the stress hormone epinephrine by the adrenal glands. Indeed, we found a dose-dependent increase in circulating epinephrine after exposure to THS generated by the 40 cigarette-

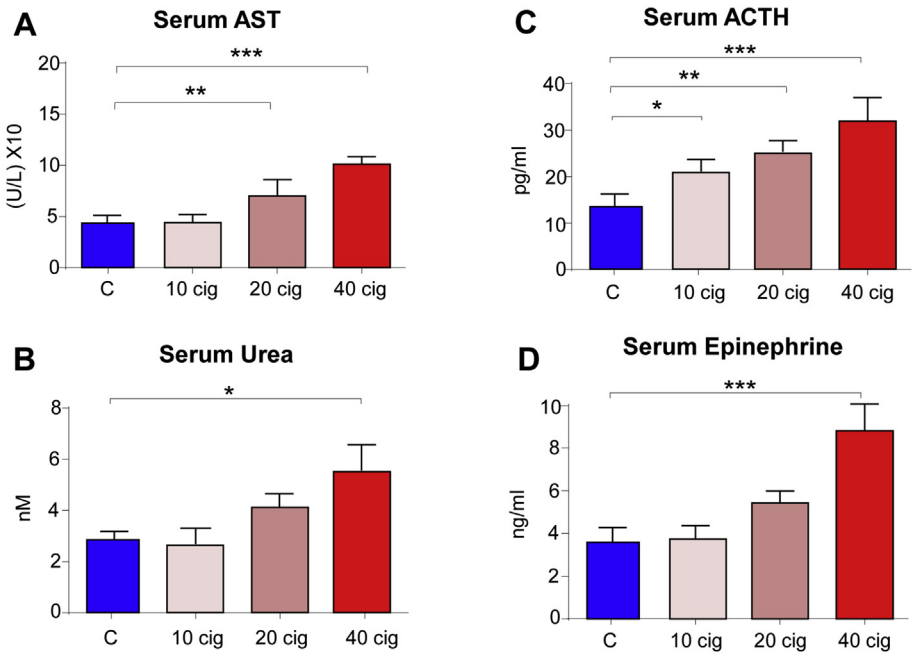

Fig. 1. THS effects on serum circulating BMH that result in tissue stress. Three-weeks-old 57BL/6 mice were divided into control and three experimental groups (6 mice in each group). The three experimental groups were exposed to THS produced by the 10, 20, or 40 cigarette smoking regimen (see M&M). The control group was never exposed to THS toxins. Blood was extracted directly from the heart at the end of experiment, and serum collected from the coagulated blood by centrifugation. THS exposed mice showed progressively increased levels of AST(**A**), Urea (**B**), ACTH(**C**), and epinephrine (**D**). All data are mean ± SD * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n = 6 per group. P values were adjusted for the number of times each test was run.

smoking regimen, which induced significantly increase in epinephrine ($p < 0.001$) when compare to the control **(**Fig. 1D**).** Thus, starting at the dose of 10, and 20 cigarettes-smoking regimen we see a general trend of increased changes in liver function and circulating stress hormones.

3.2. Dose required for THS exposure to increase markers of inflammation

In order to determine whether exposure to THS toxins results in an increase in inflammation in a dose-dependent manner, we examined the levels of cytokines in the serum. We found that several pro-inflammatory cytokines were elevated. Mice exposed to THS generated by the 10 cigarette-smoking regimen had significant increases in the pro-inflammatory cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin 1 α (IL-1α), and tumor necrosis factor α (TNF-α) compared with those of the controls (Fig. 2A–C**)**. From Fig. 2A, we saw that GM-CSF is a very sensitive indicator of THS exposure. Indeed, even at the 10 cigarette-smoking regimen, the level of GM-CSF were significantly increased ($p < 0.001$) when compared to the control mice. This was also true for the 20 and 40 cigarette-smoking regimen which showed highly significant ($p < 0.001$) increased levels of GM-CSF when compared to the control mice. We found that mice exposed to the 10, 20 and 40 cigarette-smoking regimen have significantly ($p < 0.001$) higher IL-1α levels than control. TNF-α is also a very sensitive indicator of inflammation caused by THS exposure. We found that its levels increased significantly ($p < 0.01$or $p < 0.001$) in all cigarette-exposed groups compared to the control group **(**Fig. 2C**).** Collectively these cytokines indicate that THS-exposure results in a pro-inflammatory condition. THS exposed mice also have a significant increase of the anti-inflammatory cytokines IL-10 and IL-4 at the dose of the 20 and 40 cigarettes-smoking regimen, compared to the controls **(**Fig. 2 **D, E).** IL-10 level showed significant increase ($p < 0.05$ or $p < 0.001$) compared to control group when the mice were exposed to the 20 and 40 cigarette-smoking regimen, respectively **(**Fig. 2D**).** In the same way, the IL-4 levels in the THS exposed mice were significantly different ($p < 0.001$) from the control group when the mice were exposed to the 40 cigarette-smoking regimen. However, mice exposed to the 10 and 20 cigarettes-smoking regimen have no significantly higher IL-4 levels than control **(**Fig. 2E**).** These data show that as low as a dose of THS produced by the 10 cigarettes-smoking regimen, causes a state of pro-inflammation that cannot be reversed by the anti-inflammatory cytokines.

3.3. Dose required for THS exposure to cause oxidative stress in liver and brain

To determine whether mice exposed to THS have oxidative stress in the liver, we measured the levels of H_2O_2 and found that a dose of THS generated by the 20 cigarette-smoking regimen caused a significant increase of H_2O_2 levels ($p < 0.01$) in the liver tissue of exposed mice which increased as the dose increased. The H_2O_2 concentrations steeply increased ($p < 0.001$), more than two fold, when the mice were exposed to THS at the 40 cigarette-smoking for 2 months **(**Fig. 3A**).** Because H_2O_2 is produced by superoxide dismutase (SOD) we measured the activity of this enzyme to determine whether it was also elevated. Indeed, we found that SOD activity was significantly increased ($p < 0.01$) by exposure to THS generated by as low as the 10 cigarette-smoking regimen and the activity increased in a dose-dependent manner ($p < 0.001$) **(**Fig. 3B**).** We found that exposure to THS generated by the 20 cigarette-smoking regimen was sufficient to cause nitrotyrosine modification in proteins at the statistically significant difference of $p < 0.05$ and that nitrotyrosynilation increased further at a dose of the 40 cigarette-smoking regimen with a significance of $p < 0.001$ (Fig. 3C).

We have previously shown that THS exposure affects the brain with consequences that changes the behavior of exposed mice (Martins-Green et al., 2014) and thus we determined whether there was a dose-dependent increase in oxidative stress in the brain as well. We measured the levels of H_2O_2 and those of SOD and nitrotyrosine modifications in the brain and found that neither H_2O_2 levels nor SOD activity were increased with any of the three doses of THS (Fig. 4A and B). However, we observed oxidative stress mediated molecular damage in the brain of THS exposed mice with significantly elevated ($p < 0.001$) levels of nitrotyrosynilation that occurred after THS-exposure with the 20 cigarette-smoking regimen (Fig. 4C**)**. We found that the 40 cigarette-smoking exposed-mice showed 3 times higher nitrotyrosine concentration comparing to the control group ($p < 0.001$).

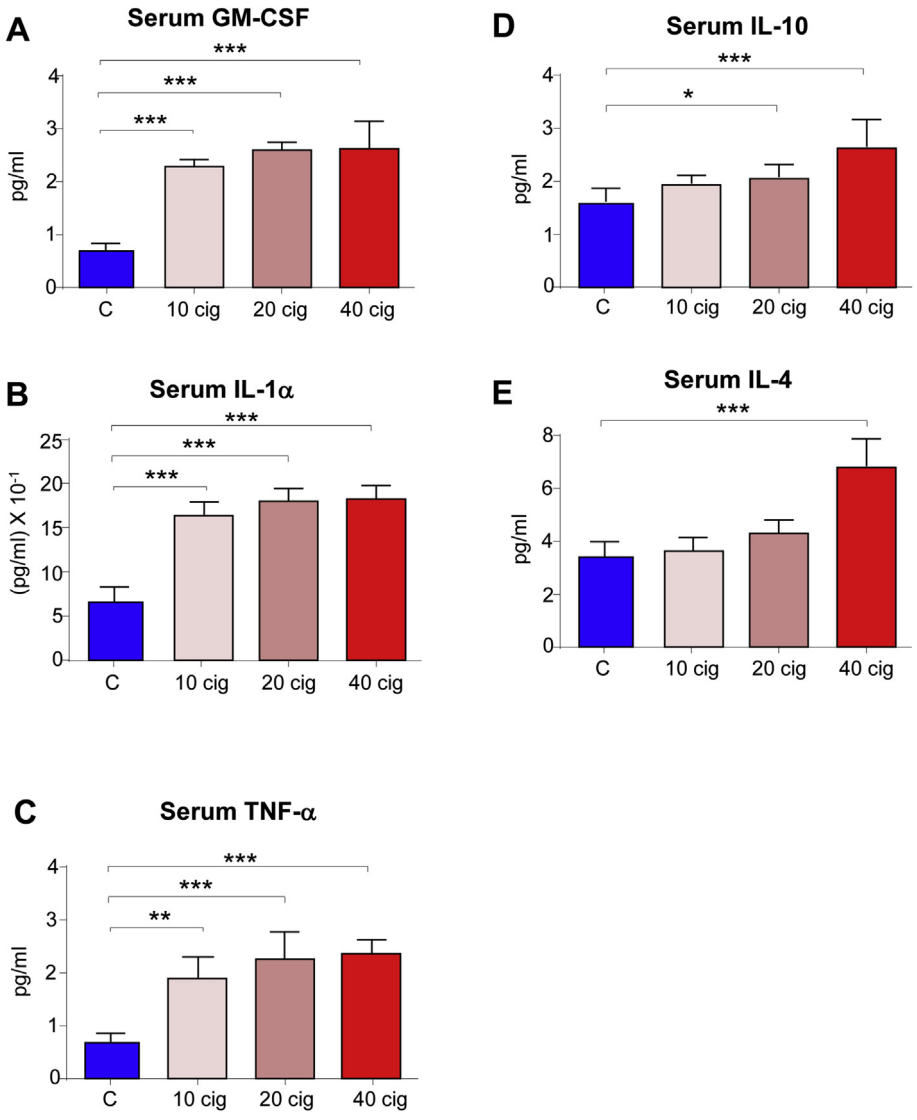

Fig. 2. THS effects on inflammation-related BMH present in serum. Exposure and serum collection was done as described in Fig.1. Increasing concentration of THS exposure resulted in progressively increased levels of GM-CSF (**A**), IL-1α (**B**), and TNF-α (**C**). Increased levels of IL-10 (**D**) and (**E**) were observed with the 40 cigarettes-smoking regimen. All data are mean ± SD * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n = 6 per group. P values were adjusted for the number of times each test was run.

3.4. Dose required for THS exposure to cause mitochondrial dysfunction in liver and brain

A recurring theme we see in our studies of THS exposure is mitochondrial dysfunction. We found that exposure to THS generated by the 40 cigarette-smoking regimen shows mitochondrial fragmentation (Fig. 5 **A, B**). In Fig. 5 **A**, we can see that the fluorescent dots are concentrated and bright whereas in Fig. 5 **B**, we see that the fluorescent dots are scattered and dim, suggesting fragmented mitochondria. Hara et al. (2013) have reported similar results by using MitoTracker Red staining. We then measured the levels of ATP in the liver and found a dose-dependent decrease in ATP levels and the significant effect ($p < 0.01$) was already seen at THS exposure generated by the 10 cigarette-smoking regimen. In terms of numerical values, the ATP level is reduced to one-third of the control group **(**Fig. 5C**).** In the brain, this effect was less dramatic and began to be ostensively seen at the 20 cigarette-smoking regimen ($p < 0.05$). ATP levels were one-half of the control group after the 40 cigarette-smoking regimen exposure **(**Fig. 6**).**

4. Discussion

The goal of this study was to determine the minimum dose required to cause physiological changes in mice when they are exposed to THS generated by different number of cigarettes in an exposure system that mimics human exposure. We tested mice exposed for two months to three different doses of THS generated by the 10, 20 and 40 cigarette-smoking regimens as described in the methodology. With the 10 cigarette-smoking regimen, we found increases in ACTH, GM-CSF, IL-1α TNF-α in the serum, decreased levels of ATP in liver and brain tissue and increased levels of in SOD activity in liver. At the 20 cigarette-smoking regimen, we found in addition that AST and IL-10 increased in the serum, that increased H_2O_2 and nitrotyrosine levels in liver, and that nitrotyrosine levels increased whereas ATP levels decreased in brain. At 40 cigarettes we found that in addition urea, epinephrine, and IL-4 were increased in the serum (Fig. 7). Once these BMH are identified in children who live in homes of smokers, it is possible that they will be used as diagnostic tools to detect THS exposure levels.

Changes in the external environment, such as THS exposure, can cause tissue stress. We found a dose-dependent increase in serum AST,

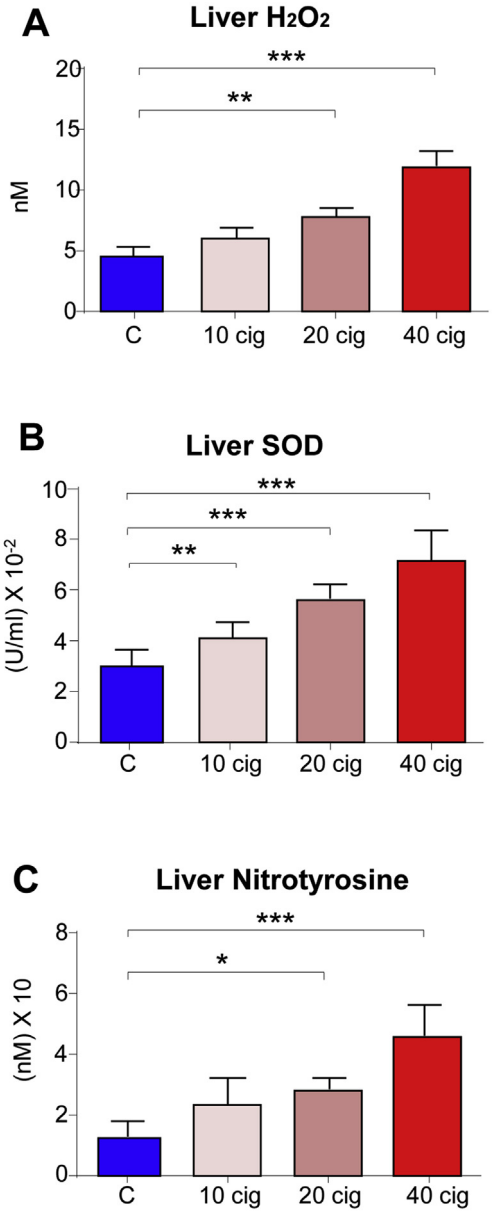

Fig. 3. THS induces oxidative stress and oxidative stress-mediated damage in the liver. Exposure as described in Fig.1. Liver tissue was collected from THS-exposed or control mice at the end of experiment. 10 mg liver tissue were placed in ice-cold RIPA buffer and homogenize using a Bullet Blender Homogenizer to extract total protein. The resulting supernatants were used for this study. Increasing concentrations of THS exposure resulted in progressively increased levels of H_2O_2 (**A**), SOD (**B**), and nitrotyrosine (**C**). All data are mean ± SD * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n = 6 per group. P values were adjusted for the number of times each test was run.

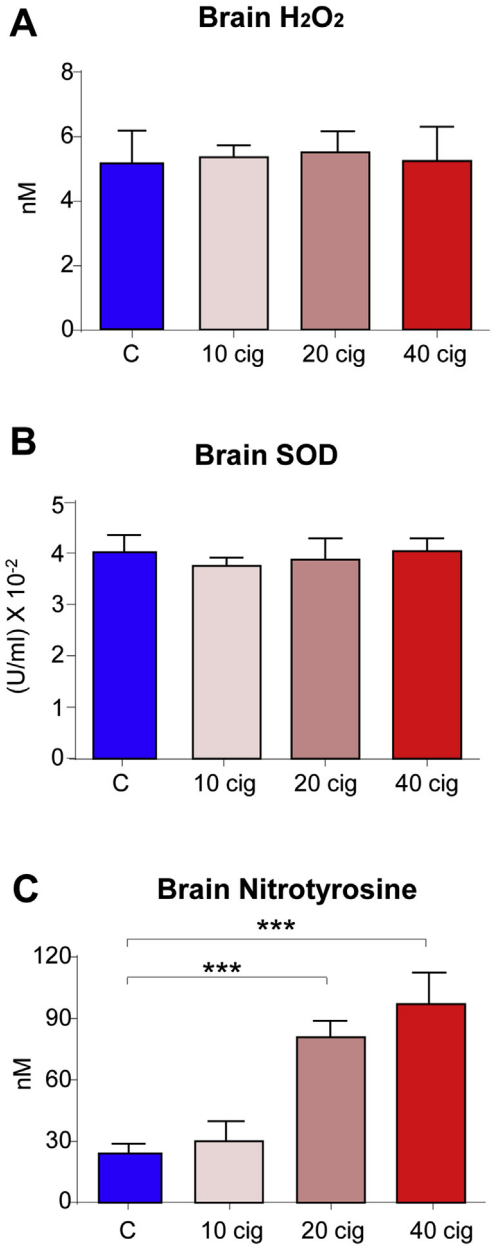

Fig. 4. THS exposure induces oxidative stress and oxidative stress-mediated damage in the brain. Exposure was done as described in Fig.1. Brain tissue was collected from THS-exposed or control mice at the end of experiment. 10 mg brain taken from the region of the hypothalamus and pituitary were placed in ice-cold RIPA buffer and homogenize using a Bullet Blender Homogenizer to extract total protein. The resulting supernatants were used for this study. After different doses of THS exposure, unchanged levels of H_2O_2 (**A**) and of SOD activities (**B**) were seen but progressively increased levels of nitrotyorsine (**C**) were observed. All data are mean ± SD *** = $p < 0.001$. n = 6 per group. P values were adjusted for the number of times each test was run.

starting after 20 cigarettes. The increase of AST levels in the serum indicate alterations in the enzymatic activity of the liver. In support of our results, it has been shown that AST levels can be significantly increased after mice are exposed to THS for 6 months (Flores et al., 2016) and rats that are exposed to SHS for 4 weeks also show elevated AST (Ogenyi et al., 2015).

In major depressive disorder, higher CRH, ACTH, and cortisol levels have been reported (Stetler and Miller, 2011; Vrshek-Schallhorn et al., 2013). The paraventricular nucleus of the hypothalamus produces corticotrophin-releasing hormone (CRH), that is released into the anterior pituitary gland, stimulating the release of ACTH. We find that ACTH is increased in a dose-dependent manner with significant changes after 10 cigarettes. Epinephrine, which is involved in the emotional modulation of memory (Packard and Wingard, 2004), is another hormone significantly changed BMH. We found that epinephrine levels increased in a dose dependent manner with significant changes after the 40 cigarette-smoking regimen, but the levels were obviously increased after 20 cigarettes exposure. The changes in these important biomarkers of disease in the serum, show that the body is under significant stress when exposed to THS and this effect is dependent on the dose of exposure.

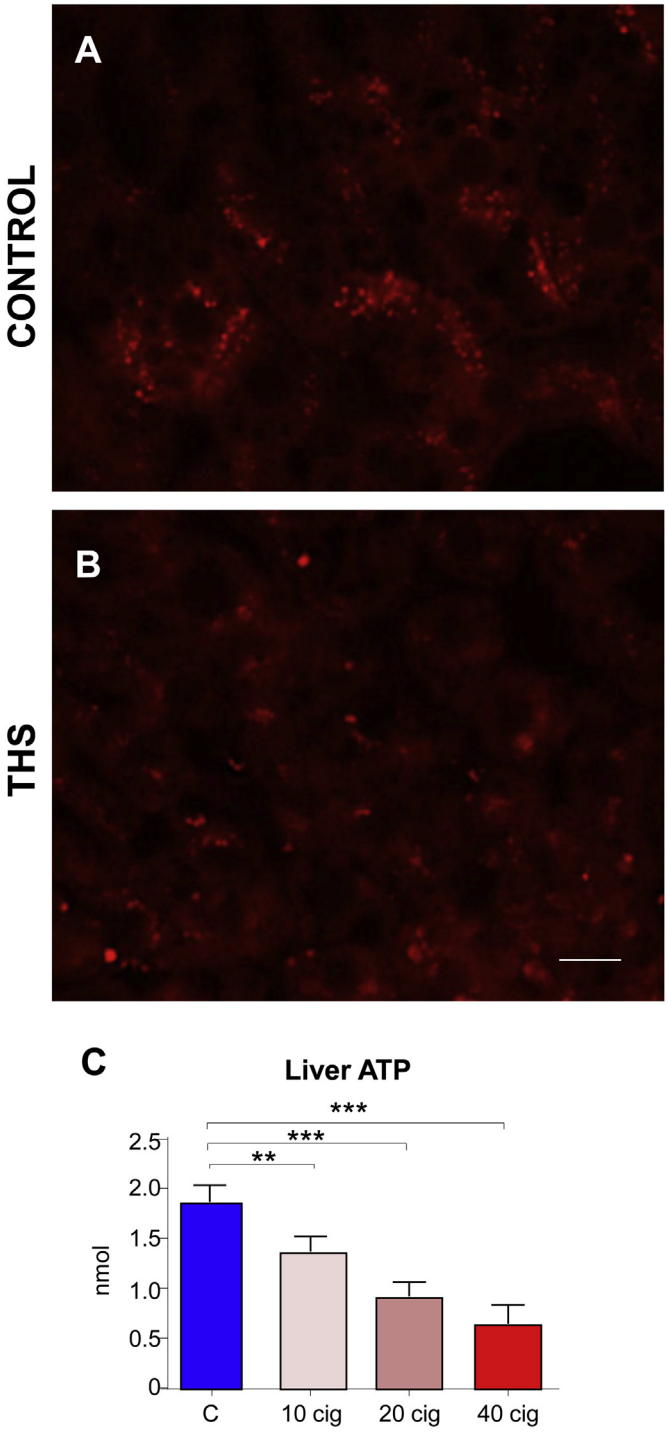

Fig. 5. THS exposure results in mitochondrial dysfunction in the liver. Liver tissue from control mice and those exposed to THS generated by the 40 cigarettes-smoking regimen, were prepared as described in the M&M and 7 μm-thick sections were made using a cryostat. The sections were stained with 1 mM rosamine MitoTracker® probes for 15min in the dark. Pictures were taken using a Nikon Microphot-FXA fluorescence microscope with a Nikon DS-Fi1 digital camera and Nikon NIS-Elements software. Control mice showed numerous mitochondria in the hepatocytes (**A**), whereas THS exposed mice showed significant reduction in the number of mitochondria per cell (**B**), and progressively decreased levels of ATP (**C**). Scale bar = 10 μm. All data are mean ± SD ** = $p < 0.01$, *** = $p < 0.001$. n = 6 was used for (C). P values were adjusted for the number of times each test was run.

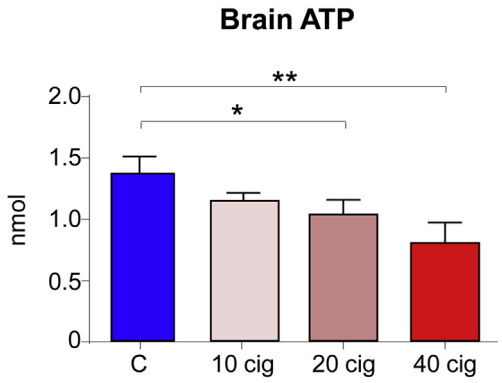

Fig. 6. THS exposure results in mitochondrial dysfunction in the brain. Exposure was done as described in Fig.1. Brain tissue was collected from THS-exposed or control mice at the end of experiment and 10 mg brain taken from the region of the hypothalamus and pituitary and placed in ice-cold RIPA buffer and homogenize using a Bullet Blender Homogenizer to extract total protein. The resulting supernatants were used to detect the levels of ATP. Increasing THS exposure led to progressively decreased levels of ATP. Starting at 20 cigarettes exposure, the ATP levels changed significantly comparing to the control. All data are mean ± SD * = $p < 0.05$, ** = $p < 0.01$. n = 6 per group. P values were adjusted for the number of times each test was run.

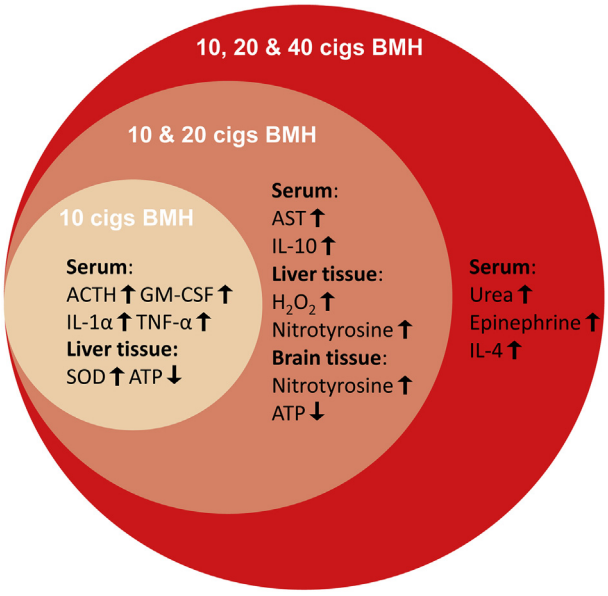

Fig. 7. Summary of the Biological Markers of Harm (BMH) we found altered in response to dose of THS exposure. "↑" indicates biological marker is upregulated by THS exposure; "↓" indicates biological marker is downregulated by THS exposure.

The results presented here indicate that THS causes damage to the liver and brain and stimulates a state of pro-inflammation. Some of these effects are dose-dependent and can be seen at 10 cigarttes/day/5 day/week smoking regimen for 2 months but others require a dose of 20 or 40 cigarettes/day/5 days/week for 2 months. The increase in pro-inflammatory cytokines could have serious implications for the ability of the immune system to fight infections and other diseases (Franceschi et al., 2000; Glaser and Kiecolt-Glaser, 2005; Kronfol and Remick, 2000). Indeed, if the immune cells are being stressed constantly by exposure to the THS toxins they will not be able to play a role as the major defense mechanisms against any kind of inflicted injury. IL-1α for example is a cytokine that is increased during states of inflammation and plays roles in furthering the inflammatory response by increasing production of other cytokines such as TNF-α (Descampslatscha et al.,

1995) TNF-α can induce cell death, mitochondrial fission and/or breakdown, as well as activating inflammatory pathways (Woolf et al., 1997). We found that THS exposure generated by the 10 cigarette-smoking regimen resulted in increase of TNF-α levels, not only indicating inflammation, cell damage and death, but also potentially explaining mitochondrial dysfunction that we observe with THS exposure. GM-CSF is another cytokine that is increased during states of inflammation and plays roles in monocyte recruitment and stimulation of additional inflammatory cytokines (Shaw et al., 2014). GM-CSF also plays roles in fighting infection by being a signal transducer and transcription activator of macrophages, which leads to their production of reactive oxygen species levels needed for proper function during the response to injury (Kraaij et al., 2010). GM-CSF levels are increased with THS exposure generated by the 10 cigarette-smoking regimen and the levels increase as the dose increases. In addition to the increase in the levels of these inflammatory cytokines, we also see anti-inflammatory cytokines such as IL-10 and IL-4 increased in THS-exposed mice in the 20 and 40 cigarette-smoking regimens, respectively, as the body attempts, unsuccessfully, to resist the inflammation caused by THS exposure. These data add to the evidence that exposure to THS result in a pro-inflammatory condition in the exposed mice and further explain the high levels of reactive oxygen species and oxidative stress in the tissues (Chen et al., 2017; Hussain et al., 2009).

In the liver, the data we obtained on the oxidative stress levels after THS exposure shows increases in H_2O_2 levels and SOD activity with the 20 and 10 cigarette-smoking regimen, respectively. Cigarette smoke toxins, are potent sources of reactive oxygen species (ROS) (Reilly et al., 2017). SOD in the liver converts O_2·- into H_2O_2 which is a substrate for catalase and GPx (Weydert and Cullen, 2010), two critical anti-oxidant enzymes. We did not detect changes in catalase and GPx enzymatic activity in the THS-exposed mice at different doses for 2 months, even at the highest exposure dose (40 cigarettes-smoking regimen). This shows, that the activity of these antioxidant enzymes is not sufficient to combat the oxidative stress induced by THS-exposure. Indeed, catalase and GPx enzymatic activity in the cell breaks down to $H_2O + O_2$ or H_2O_2, respectively. If the activity of these enzymes does not increase, the excess H_2O_2 will participate in the Fenton reaction by reacting with Fe^{2+} ions in the tissue leading to the production of hydroxyl radicals (Pignatello et al., 2006), which are damaging to proteins, lipids and DNA. This damage in the liver is significant because it can affect the capability of this organ to detoxify the body (Imlay et al., 1988). We observe that at the 20 cigarette-smoking regimen we can already detect the presence of nitrotyrosynilation in the liver.

In our previous time-dependent study, the brain takes much longer (4 months) for THS exposure with the 40 cigarette-smoking regimen to result in the accumulation of H_2O_2 (Adhami et al., 2017). We speculate that the blood-brain barrier is most likely the reason why the brain is more “protected” from THS exposure and molecular damage takes longer to occur. These finding are supported by previous work (Fuller et al., 2010). However, nitrotyrosine levels were elevated in the brain. Nitrotyrosynilation is widely used as a marker of post-translational modification by the nitric oxide (·NO, nitrogen monoxide)-derived oxidant peroxynitrite (ONOO·-), which forms when superoxide anions are not effectively cleared by the natural antioxidant defense systems (Brennan et al., 2002). Nitrotyrosinylation of proteins can potentially changes their function compromising tissue function (Kaminski and Andrade, 2001). We observe that the 20 cigarette-smoking regimen caused nitrotyrosynilation in the brain, suggesting that ONOO·- can readily penetrate the blood brain barrier damaging many molecular structures in brain tissue.

We have shown in our previous studies, that THS exposure results in a wide array of mitochondrial damage and dysfunction. We first observed these effects when we found decreased levels of ATP in both the liver and the brain of mice exposure to THS for 2 months (Adhami et al., 2017). In this study, we see that with a dose as low as the 10 cigarette-smoking regimen, mice exposed to THS have decreased ATP levels in the brain and liver, which occurs in a dose-dependent manner. The lack of ATP can be very damaging to the function of liver and brain given that the cells will be in a deficit for energy production.

5. Conclusion

We show that THS toxins have major effects on liver and brain, and on circulating hormones and cytokines, which show a general trend of increased susceptibility of the body to metabolic diseases and inflammation as the dose of THS exposure increases. We identified testable serum BMH altered as a result of THS exposure at different doses of exposure. These BMH can potentially be used to determine levels of THS toxin exposure and the extent of cellular and molecular damage that could lead to disease. Once the translatability from mouse to human is confirmed, our results may be important to inform the public, in particular the parents of children living in the homes of smokers, about the danger of exposure to THS.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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