In utero thirdhand smoke exposure modulates platelet function in a sex-dependent manner

Thirdhand smoke (THS), the persistent residue of tobacco smoke that remains after a cigarette is extinguished, materialized as a threat for human health over the last decade. These toxic residues end up depositing on surfaces and objects where tobacco has been used (e.g., homes) and persist for weeks/months after the last smoking.[1] THS toxicants undergo chemical reactions and changes over time potentially making them more toxic.[2] Given that the routes of exposure to THS involve skin absorption, inhalation and ingestion,[3] it is thought to be more toxic by producing more toxicants in the blood of the exposed person.[4] Indeed, there is a growing body of evidence documenting THS-induced health risks,[5] including cardiovascular disease (CVD). For example, we previously showed that THS exposure modulates platelet function and enhances thrombogenesis in adult exposed mice.[6] However, it has not yet been established whether prenatal/*in utero* THS exposure impacts platelet function and related disorders, which is paramount since the developing embryo is especially sensitive to environmental toxicants, including cigarette smoke.[7] Therefore, this study was designed to address this issue, utilizing the offspring of exposed females. In addition, we also examined whether sex differences exist in THS-induced effects.

We employed our innovative THS exposure approach which has been peer-reviewed[4,6] and accepted as one that provides exposure conditions that mimic those in multiple real-life human situations.[4] According to our experimental design, the female breeders were exposed to THS smoke or clean air starting 1 week before mating and throughout the whole pregnancy period by placing them in cages that are furnished with either THS or clean-air exposed materials. After delivery (post-natal day 4), the offspring were moved to clean air cages and housed until 8-10 weeks of age, before experimentation. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee.

We first sought to investigate the *in vivo* effect of *in utero* THS exposure on hemostasis and thrombosis. Thus, the tail bleeding time assay revealed that the THS-exposed males and females exhibit substantially shortened bleeding time compared to clean air exposed controls (Figure 1A). In fact, the average bleeding time in males was 395 ± 65.14 seconds (sec) in clean air group *versus* 68.80 ± 14.87 sec in the THS group; whereas in females it was 449.40 ± 45.07 seconds and 44.50 ± 12.56 sec for clean air and THS groups, respectively. As the time needed for cessation of bleeding was significantly reduced in the *in utero* THS–exposed mice, we therefore hypothesized that these mice are more vulnerable to thrombosis. This was tested by employing the $FeCl_3$ carotid artery injury–induced thrombosis model. As depicted in Figure 1B, *in utero* THS mice of both sexes displayed a significant reduction in the occlusion time, with the average in males being 1,080 ± 60.00 sec in clean air group *versus* 210.80 ± 79.37 sec in THS group; whereas the females recorded 1,150 ± 114.30 sec and 281 ± 116.50 sec for the control and experimental groups, respectively. Taken together, these results show that *in utero* THS exposure enhances hemostasis and renders mice at higher risk of developing thrombosis. However, when males are compared with females, the results did not show any sex-based differences for either hemostasis or thrombus formation.

Notably, the platelet and other blood cells count was measured in both *in utero* THS- and clean air-exposed mice, as changes in platelet number may contribute to the hemostasis and thrombosis phenotype observed. The *in utero* THS exposure did not affect the platelet count or other hematological parameters (Table 1).

In light of the bleeding time and thrombosis data, another set of experiments evaluated the manifestation of the potential prothrombotic phonotype at the level of platelet physiology by studying platelet functional parameters *in vitro*. Hence, we first determined the effect of *in utero* THS exposure on agonist-induced platelet aggregation, which was found (Figure 2A) to be substantially increased, in response to either thrombin (0.1 U/mL) or ADP (1 μmol/L) in male and female mice exposed to THS *in utero*. However, when comparing platelet aggregation of both sexes, our analysis did not

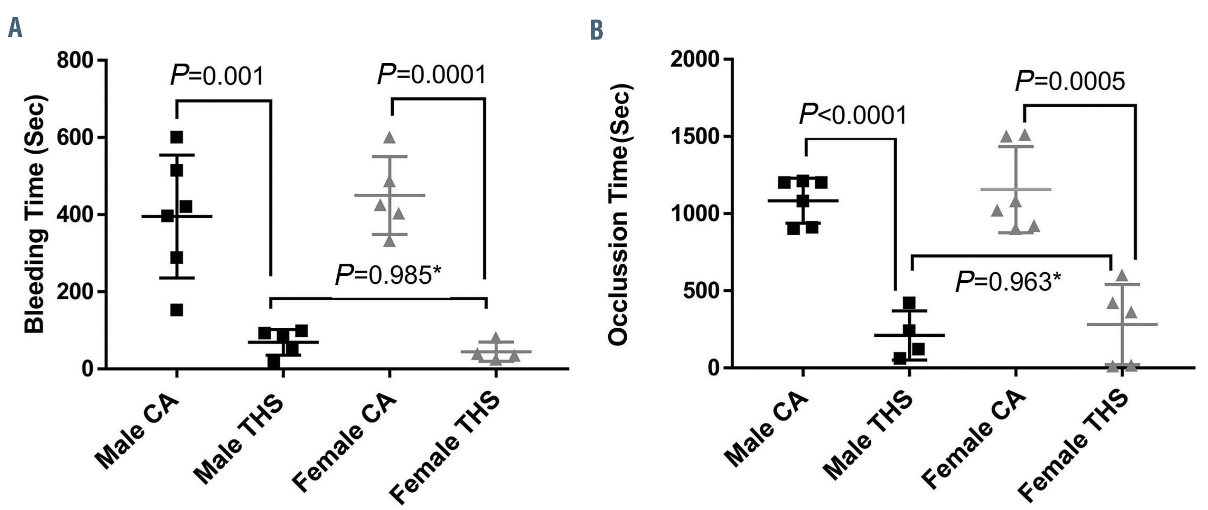

Figure 1. *In utero* thirdhand smoke exposure shortens the bleeding time in the tail bleeding time assay, and the time to occlusion in the ferric chloride *in vivo* thrombosis model both in males and females. (A) Tail bleeding time assay in the *in utero* thirdhand smoke (THS)- and clean air (CA)–exposed mice compared in males and females. Each point represents the tail bleeding time of a single animal. (B) Ferric chloride–induced thrombosis model (time to occlusion) in the *in utero* THS- and clean air–exposed compared in males and female mice. Each point represents the occlusion time of a single animal. *Male and female data compared using two-way ANOVA while THS *vs.* CA comparison within the same sex was done using Student's *t*-test.

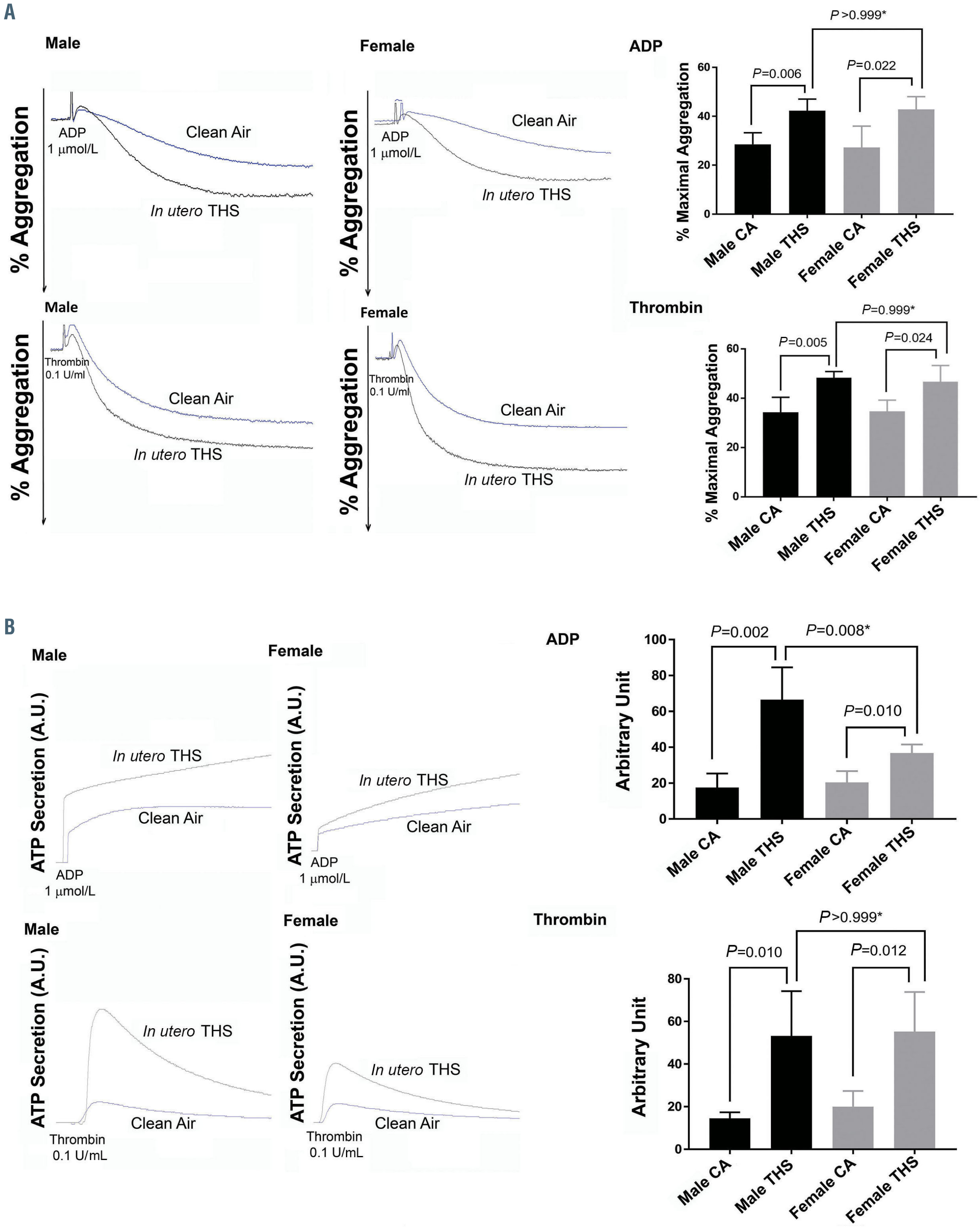

Figure 2. Effect of *in utero* thirdhand smoke exposure on platelet physiology in the exposed male and female mice compared to clean air-exposed controls. (A) Thirdhand smoke (THS) *in utero* exposure enhanced platelet aggregation in the exposed male and female mice compared to clean air (CA)-exposed controls. Platelets from THS- *in utero* exposed and CA–exposed mice were stimulated with 1 μmol/L ADP or 0.1 U/mL thrombin before their aggregation response was measured. The experiment was repeated 3 times, with blood pooled from at least 6-8 mice each time. (B) *In utero* THS exposure enhanced ATP secretion in the *in utero* exposed male and female mice, compared to CA-exposed controls. Platelets from THS *in utero* exposed and CA–exposed mice were stimulated with 1 μmol/L ADP or 0.1 U/mL thrombin before their dense granule secretion (ATP secretion) was determined. Dense granules secretion responses were measured in a lumi-aggregometer. Platelets were incubated with luciferase/luciferin (12.5 μL) for the dense granule measurements. The experiment was repeated 3 times, with blood pooled from at least 6-8 mice each time. *Female and male data compared and *P* value calculated by two-way ANOVA while comparison within the same sex was done using Student's *t*-test. Error bars represent standard deviation.

Table 1. Peripheral blood cell counts in *in utero* thirdhand smoke- and clean air–exposed male and female mice.

Cell type	Male			Female		
	Clean air	*in utero* THS	*P* value	Clean air	*in utero* THS	*P* value
Platelets	627.40 ± 88.57	649.20 ± 42.13	0.63	504.80 ± 46.98	517.30 ± 7 7.80	0.77
MPV	4.84 ± 0.27	4.82 ± 0.08	0.89	4.82 ± 0.13	4.86 ± 0.11	0.62
Red blood cells	6.92 ± 0.50	7.45 ± 0.27	0.11	6.71 ± 0.83	6.69 ± 0.57	0.96
Lymphocytes	1.40 ± 0.40	1.93 ± 0.70	0.18	1.60 ± 0.49	2.17 ± 0.59	0.13
Monocytes	0.13 ± 0.06	0.09 ± 0.02	0.12	0.13 ± 0.06	0.09 ± 0.02	0.12
Granulocytes	1.86 ± 0.52	2.39 ± 0.80	0.24	1.86 ± 0.50	2.51 ± 0.67	0.14
HCT	32.00 ± 2.25	34.13 ± 1.41	0.16	31.98 ± 4.05	31.06 ± 3.17	0.69

All counts are expressed as thousands per microliter, except for red blood cells, which are expressed as millions per microliter. Data are presented as mean ± standard deviation. HCT: hematocrit; MPV: mean platelet volume; THS: thirdhand smoke.

reveal a statistical significance, with either of the agonists.

Given that platelet granule secretion is known to contribute significantly to platelet activity,[8] we investigated agonist-induced ATP release and P-selectin surface expression as markers for dense and α-granules release, respectively. Dense granules as well as α-granules secretion were increased in platelets obtained from *in utero* THS exposed mice, in response to either ADP or thrombin (Figure 2B; *Online Supplementary Figure S1i and ii*). These data revealed that platelet secretion contributes to the THS prothrombotic phenotype. In terms of sex-dependent differences, the *in utero* THS-exposed males showed much higher ADP-induced dense granule secretion compared to females, but no statistical difference was observed in the clean air-exposed mice (Figure 2B). Moreover, no differences between the two sexes were observed with thrombin regardless of exposure type (Figure 2B). In contrast, α-granules secretion was significantly elevated in THS exposed females compared to males following stimulation by thrombin; but this was not the case with ADP (*Online Supplementary Figure S1i and ii*).

Next, we investigated the impact of *in utero* THS on αIIbβ3 activation; which was more pronounced in the THS exposed mice, in response to 5 μmol/L ADP and 0.1 U/mL thrombin (*Online Supplementary Figure S1iii and iv*); which is in accordance with the enhanced aggregation response and was demonstrated in both sexes. Interestingly, our analysis revealed a significant sex-based difference (higher in males) with both agonists.

As platelets are activated, phosphatidylserine (PS) becomes exposed at their outer surface, for the assembly of coagulation factor complexes.[9] Subsequently, we determined the impact of *in utero* THS exposure on PS expression. We found PS expression to be markedly enhanced upon stimulation with thrombin or ADP following THS *in utero* exposure (*Online Supplementary Figure S1v and vi*), which was documented in males and females. However, when both sexes were compared, it was found that the ADP effects were more pronounced in THS-exposed females compared to males. In contrast, when thrombin was analyzed, the effects in males were found to be higher than females. This discrepancy between the release of dense granules *versus* α-granules in males and females might be attributed to the fact that the former was performed using platelet-rich plasma whereas the latter using washed platelets; given that the presence of other plasma factors makes it difficult to assess whether the sex difference is inherent to platelets or related to plasma.[10] As for PS exposure, female platelets showed more significant elevation in comparison to those from males with ADP. However, this trend is completely reversed when thrombin-stimulated platelets were utilized with a significant elevation in PS in males compared to females. This could be explained by the variation in dose-response between males and female platelets.[11] It also should be noted that these are different platelet functional responses. In addition, analogy could be inferred from the race disparity of thrombin PAR4 receptor that triggers enhanced platelet aggregation as well as calcium mobilization when activated in African lineage compared to Caucasian.[12] Similarly, a sex disparity in the receptors of different agonists or their downstream signaling pathways could explain the aforementioned discrepancies.

Collectively, our functional assays provide evidence that *in utero* exposure to THS triggers a state of platelet hyperactivity and contributes to the prothrombotic phenotype in the offspring mice.

In summary, these data provide evidence that the negative health effects of maternal THS exposure extend to the “non-exposed” offspring. Thus, our findings document for the first time that *in utero* THS exposure drives platelets into a state of hyperactivity, that manifests in a host of enhanced functional responses (e.g., aggregation). Together, these effects ultimately lead to a prothrombotic phenotype. It is noteworthy that this danger, according to our current and published data, does not only affect “directly” THS-exposed mice as we have shown before[6] but expands to the offspring of the exposed pregnant mice as well. Interestingly and importantly, this prothrombotic phenotype endured despite the fact that offspring mice were not exposed to THS as they were moved to clean-air exposed cages until they reached 8-10 weeks of age. These data also highlight the underestimated risk of exposure to THS toxicants that persist up to several months after the last smoking has taken place.[1,13] It is also important to note that this phenotype is consistent with the state of hyperactive platelets we reported previously as a result of exposure to other forms of tobacco that are perceived as safe, namely e-cigarettes[14] and hookah/waterpipe.[15]

As for the comparisons between males and females, although no sex differences could be demonstrated in bleeding time, thrombosis or platelet aggregation, we did observe significant differences in dense and α-granule secretion, αIIbβ3 activation as well as PS exposure when compared sex-wise.

In conclusion, our data clearly demonstrates for the first time that *in utero* THS exposure modulates the platelet biology in the non-exposed offspring, making them more susceptible to cardiovascular diseases.

Hamdy E. A. Ali,[1] Ahmed B. Alarabi,[2] Zubair A. Karim,[3] Victor Rodriguez,[3] Keziah R. Hernandez,[3] Patricia A. Lozano,[2] Medhat S. El-Halawany,[1] Fatima Z. Alshbool[2] and Fadi T. Khasawneh[1].

[1]Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, Texas A&M University, Kingsville; [2]Department of Pharmacy Practice, Irma Lerma Rangel College of Pharmacy, Texas A&M University, Kingsville and [3]Department of Pharmaceutical Sciences, School of Pharmacy, The University of Texas at El- Paso, El Paso, TX, USA.

Correspondence:

FADI T. KHASAWNEH - fkhasawneh@tamu.edu.

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