



Effects of electronic cigarette flavorants on human platelet aggregation *ex vivo*

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ABSTRACT

Because little is known about the effects of individual flavorants in electronic cigarette (e-cig) fluids on human platelet aggregation, we tested for the direct effects of 15 common e-cig flavorants on adenosine diphosphate (ADP)-induced human platelet aggregation *ex vivo*. To better understand a potential mechanism of action of flavorants, we quantified 2 phases of aggregation. Human platelet-rich plasma (PRP) was obtained from whole blood of healthy volunteers and used in a platelet aggregometry assay. PRP was incubated with 1 of 15 different flavorant compounds (e.g., benzyl alcohol, eugenol, citronellol, menthol, menthone, diacetyl, maltol, limonene, methylbutyric acid, isoamyl acetate, acetylpyridine, eucalyptol, 2,5-dimethylpyrazine, cinnamaldehyde, and vanillin) at 100 μ M for 5 min at 37 °C prior to addition of ADP (10 μ M). Subsequent ADP-induced platelet aggregation was tracked for 5 min using an aggregometer. Aggregation curves were analyzed for flavorant-induced effects on total (%) aggregation, Phase 1 and Phase 2 components, and compared with their ADP-only control via One-Way ANOVA. Notably, eugenol significantly inhibited total aggregation; an effect due solely to inhibition of Phase 2. No other flavor tested had any effect on total or phase-specific ADP-induced platelet aggregation. These results indicate that parent flavorant compounds commonly found in e-cig liquids neither activate nor inhibit ADP-induced human platelet aggregation. However, as flavorants are chemically altered during heating of e-cig, thermally-derived products of flavorants (e.g., flavor acetals) also will need to be tested for effects on platelet activation.

1. Introduction

Electronic cigarettes (e-cigs) or electronic nicotine delivery systems (ENDS) are marketed as “healthier alternatives” to conventional tobacco cigarettes, yet the cardiovascular disease (CVD) risk associated with chronic e-cig use is unknown. Moreover, given that e-cigs use has increased dramatically among youth and young adults, there are

concerns about renormalization of tobacco use and the potential increased CVD risks associated with acute and chronic use of e-cigs. From 2017–2018, the use of e-cigs among high school and middle school students increased from 11.7% to 20.8% and 3.3–4.9%, respectively [1]. In 2021, the National Youth Tobacco Survey in the United States reported that e-cigs are the most commonly used form of tobacco products among youth, with an estimated 2.06 million students documented as

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; CVD, cardiovascular disease; E-cig, electronic cigarette; ENDS, electronic nicotine delivery systems; GRAS, generally regarded as safe; NO, nitric oxide; P1, phase 1 aggregation; P2, phase 2 aggregation; PAF, platelet activating factor; PG, propylene glycol; PPP, platelet-poor plasma; PRP, platelet-rich plasma; ROS, reactive oxygen species; TxA₂, thromboxane A₂; VG, vegetable glycerol.

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current e-cig users [2]. Additionally, 84.7% of those students use flavored e-cigs, primarily fruit-flavored, and thus, the increase in youth use of e-cigs appears to be due, in large part, to the many flavors offered, including e-cigs with appealing names such as “Cotton Candy,” “Gummi Bear,” and “Bubble Gum” [2,3]. Flavor profiles (>7700) are a product of mixing a variety of chemical compound additives (flavorants) including pure chemicals such as menthol, benzaldehyde, eugenol, cinnamaldehyde, or ethylvanillin [4]. These compounds may be at relatively high concentrations (0.1–1% v/v) with cinnamaldehyde being present at up to 4% [5,6]. Although many of these flavoring compounds are generally regarded as safe (GRAS) for ingestion, there is little known about potential toxicity of heated flavorants as inhaled during typical e-cig use. Notably, commonly used e-cig-derived flavoring compounds, without nicotine, induce concentration-dependent increases in reactive oxygen species (ROS) and cytotoxic effects in human monocytic cells [7]. Specifically, Muthumalage et al. [7] report that cinnamaldehyde and vanillin are more toxic other flavorants (e.g., diacetyl, acetoin, maltol, pentanedione, *o*-vanillin, and coumarin) tested in human monocytic cells for induction of oxidative and inflammatory responses [7].

It is critical to assess overall CVD risk of e-cig use, and thus, there is a need to assess the contribution of individual constituents in e-cigs such as flavorants to the overall CVD risk [8]. For example, exposure of human endothelial cells to each of nine different flavoring compounds led to concentration-dependent reduction in nitric oxide (NO) production and concurrent increases in markers of oxidative stress, inflammation, and apoptosis [9]. As NO inhibits platelet activation, [10] flavorants that diminish endothelial cell production of NO may increase

the risk of platelet activation [11]. In fact, acute exposure of mice to e-cig aerosols induces prothrombotic effects [12]. Inhalation exposures *in vivo* are complex, though, and thus, use of a more direct, higher throughput *in vitro* assay may allow for identification of individual e-cig flavorants that exert platelet toxicity.

Despite this urgent need to evaluate e-cigs and their constituents for prothrombotic effects, there has been little investigation into whether flavorants directly induce platelet activation. The present study investigated how unaltered parent flavorants that are commonly found in e-cig liquids may influence prothrombotic events, specifically platelet aggregation. We hypothesized that direct exposure of platelets to the parent flavorings induces significant changes in biphasic platelet aggregation *ex vivo*. Thus, the goal of this study was to quantify the effects of common e-cig flavorants on adenosine diphosphate (ADP)-induced human platelet aggregation. Furthermore, we investigated whether flavorants would affect either platelet initiation (Phase 1) or secondary aggregation and amplification (Phase 2) to better understand direct actions of flavorants.

2. Materials and methods

2.1. Materials

2.1.1. Flavorants

The following 15 e-cig flavorants were used in this study (Fig. 1): benzyl alcohol, citronellol, eugenol, menthol, cinnamaldehyde, vanillin, isoamyl acetate, eucalyptol, limonene, diacetyl, menthone,

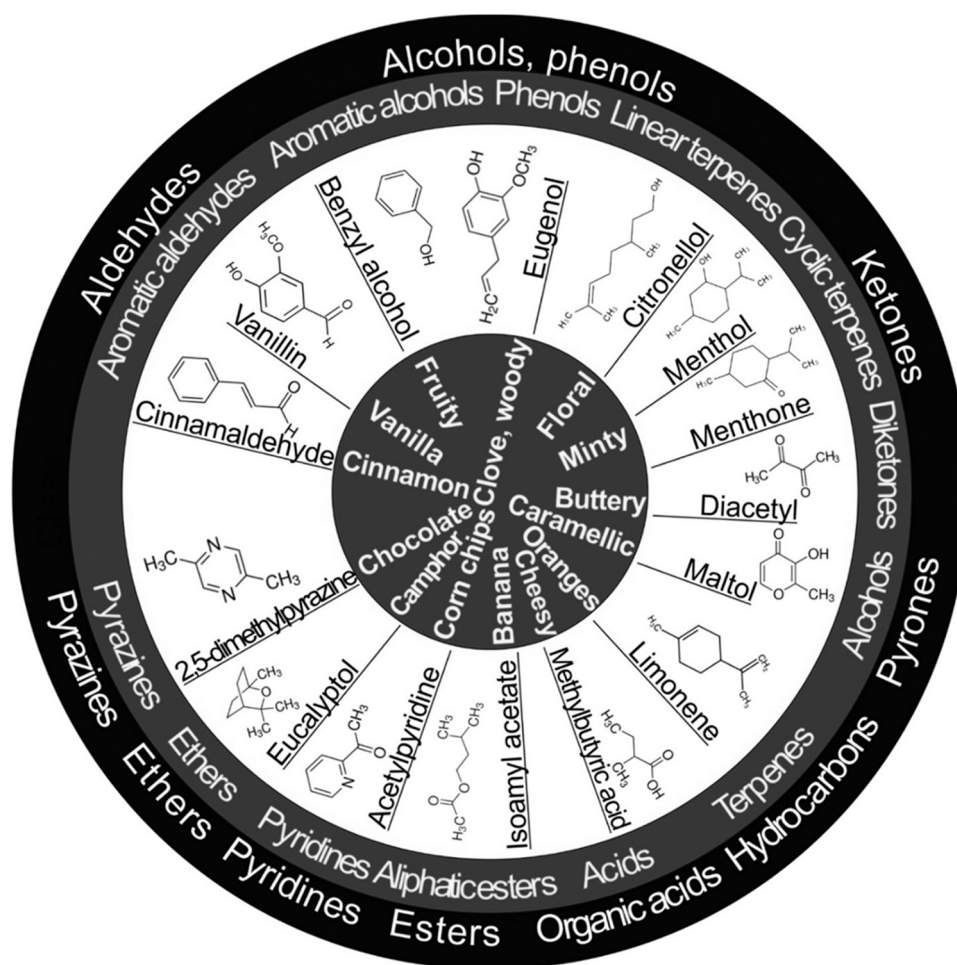


Fig. 1. Electronic cigarette flavorant additives used in this investigation, organized by flavor profile (innermost circle), compound nomenclature, compound structure, structural group, and chemical class (outermost circle).

methylbutyric acid, 2,5-demethylpyrazine, acetylpyridine, and maltol. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. ADP was purchased from Chrono-log Corp. (Haverstown, PA). A 1 mM stock solution was made by reconstituting 2.5 mg of lyophilized ADP in sterile saline and stored at -80°C until used.

2.1.2. Preparation of flavorants

Each parent flavorant was prepared fresh before testing and dissolved in ethanol to obtain stock solution. Ethanol was used as vehicle control.

2.2. Humans

Blood (~15 mL per donor) was collected into tubes containing 3.8% trisodium citrate from self-reported healthy male and female human volunteers at the University of Louisville Health Sciences Center campus. This exempt protocol was approved by the University of Louisville Institutional Research Board, and no participant information was disclosed to investigators.

3. Methods

3.1. Preparation of human platelets

Samples were analyzed within 3 h of blood draw. Samples were gently mixed for 5 min on a tube rocker at RT. To obtain PRP, citrated blood samples were centrifuged (180xg; 15 min; RT). Remaining blood was centrifuged again (1,300xg; 20 min; RT) to obtain platelet-poor plasma (PPP). PRP was adjusted to 3×10^8 PLT/mL with PPP used as the reference sample.

3.2. Platelet aggregometry assay

The Born method of turbidimetric aggregation was used [13]. A four-channel Chrono-log Lumi-aggregometer 480VS was used to measure aggregation photometrically over 5 min post ADP-induction (10 μM ; Fig. 2). PRP or PPP reference samples (350 μL) were incubated at 37°C in cuvettes (Chrono-log, P/N 312) with a stir bar (Chrono-log, P/N 311; 1000 rpm). Flavorants at 100 μM (or eugenol at 10–100 μM) or ethanol control were added in PRP and pre-incubated (37°C) for 5 min. After pre-incubation, ADP was added to stimulate

biphasic aggregation over 5 min. ADP initiated a rapid shape change followed by onset of rapid aggregation (Phase 1, P1) and then greater aggregation and amplification (Phase 2, P2) responses.

3.3. Calculations

‘Total aggregation’ was defined as the final aggregation (%) at 5 min after ADP (Fig. 2). P1 (%) was defined from onset of aggregation until an inflection point or ‘leveling off’ of the initial P1 slope. P2 (%) was calculated as the difference between Total aggregation and P1 (Total aggregation - P1 = P2). For example, if a compound had anti-aggregation activity, then the P2 value could be negative. To account for variation in participants’ baseline aggregation responses to ADP, both P1 and P2 values also were normalized as a percentage of the total aggregation to 100%.

3.4. Statistical analyses

Total, P1, and P2 aggregation values are means \pm SE. Percentage responses for Total aggregation, P1, and P2 were compared between control (ADP only) and each flavorant compound using a One-Way ANOVA (or One-Way ANOVA with repeated measures for eugenol) (SigmaPlot ver. 12). Statistical significance was accepted at $p < 0.05$.

4. Results

4.1. Direct effect of flavoring additives on ADP-induced platelet aggregation in human PRP

We tested 15 flavorants at 100 μM for their direct effects on platelet aggregation induced by ADP (Table 1). Except for eugenol, none of the other 14 flavorants had any effect on total percentage of ADP-induced aggregation. Eugenol, in contrast to all other flavorants tested, had a strong inhibitory effect on total platelet aggregation (Table 2).

4.2. Total aggregation as function of Phase 1 and Phase 2 responses

We recorded the responses of platelets due to ADP stimulation as a collective in real-time. The total aggregation through 5 min has 3 distinct elements: 1) initial shape change (addition of agonist); 2) initiation of platelet activation (P1); and 3) feed forward aggregation due to platelet secretion of granule contents including ADP, serotonin, and thromboxane that further stimulate aggregation (P2). In quantifying these phases, ADP alone stimulated $57 \pm 2\%$ of total aggregation in P1 (Table 1). The remaining fraction was P2 ($43 \pm 2\%$). Because most flavorants tested had no effect on total aggregation, we did not expect, nor did we find, any effect of most flavorants on either P1 or P2% aggregation (Table 1).

In contrast, as eugenol significantly decreased total aggregation, we observed that all of this inhibitory effect resulted from inhibition of P2 response (Table 2). The inhibitory effect on P2 was concentration-dependent and was significant even at the lowest concentration tested of 10 μM . ADP alone control stimulated $62 \pm 3\%$ of total aggregation in P1 (Table 2). The remaining fraction was P2 ($38 \pm 3\%$). In the presence of eugenol, ADP stimulated a normal P1 aggregation yet P2 was blunted in a concentration-dependent manner. These results indicated that P2 was inhibited by eugenol. At the highest eugenol concentration tested (100 μM), we observed disaggregation in which our traces reversed post-P1 activity resulting in negative P2 values (Table 2). Together, these data indicate eugenol is an inhibitor of ADP-induced platelet aggregation.

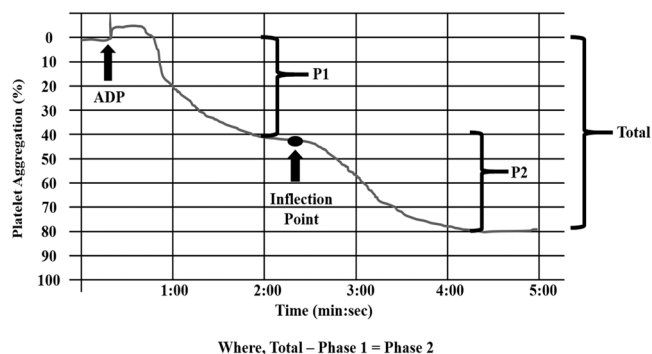


Fig. 2. Schematic of adenosine diphosphate (ADP)-induced phase 1 and phase 2 human platelet aggregation *ex vivo*. A flavorant is added to a sample of PRP and is then placed into the aggregometer (5 min, 37°C). Addition of ADP stimulates rapid change of platelets from a quiescent state to an activated state, causing phase 1 aggregation in which platelet alpha and dense granules to secrete. This initial release of granules subsides (inflection point), and the secreted granules activate neighboring platelets, thus amplifying a secondary aggregation response (phase 2). These aggregating responses occur within 5 min before stabilizing. Total aggregation (%) is recorded after 5 min. The inflection point is recorded as the sole response of phase 1 (%). Phase 2 (%) response is calculated as Total minus Phase 1.

Table 1
Effect of flavorants (100 μ M) on ADP-induced (10 μ M) human platelet total and biphasic aggregation *ex vivo*.

Flavorant	N	100 μ M			Normalized to 100% aggregation	
		Total (%)	Phase 1 (%)	Phase 2 (%)	Phase 1 (%)	Phase 2 (%)
Control	24	65 \pm 3	37 \pm 2	28 \pm 2	57 \pm 2	43 \pm 2
Cinnamaldehyde	16	63 \pm 3	38 \pm 2	25 \pm 3	61 \pm 3	39 \pm 3
Menthol	21	61 \pm 6	36 \pm 2	26 \pm 2	58 \pm 2	42 \pm 2
Benzyl Alcohol	11	64 \pm 3	38 \pm 2	27 \pm 2	59 \pm 2	41 \pm 2
Vanillin	21	61 \pm 2	35 \pm 1	26 \pm 2	58 \pm 2	42 \pm 2
Maltol	6	61 \pm 4	34 \pm 1	27 \pm 3	56 \pm 2	44 \pm 2
Limonene	7	61 \pm 3	37 \pm 2	24 \pm 1	61 \pm 2	39 \pm 2
Menthone	13	65 \pm 3	35 \pm 2	30 \pm 2	54 \pm 2	46 \pm 2
Methylbutyric acid	14	67 \pm 3	36 \pm 2	31 \pm 2	54 \pm 2	46 \pm 2
Diacetyl	16	63 \pm 2	37 \pm 2	26 \pm 2	59 \pm 3	41 \pm 3
Acetylpyridine	14	61 \pm 3	33 \pm 1	28 \pm 3	56 \pm 2	44 \pm 2
Citronellol	8	63 \pm 3	38 \pm 3	25 \pm 2	61 \pm 3	39 \pm 3
Eucalyptol	18	62 \pm 2	33 \pm 1	29 \pm 2	54 \pm 2	46 \pm 2
Isoamyl acetate	14	60 \pm 3	34 \pm 2	26 \pm 2	57 \pm 2	43 \pm 2
2,5-dimethylpyrazine	12	63 \pm 2	35 \pm 1	29 \pm 2	55 \pm 2	45 \pm 2

Data are mean \pm SEM. Abbreviations: ADP, adenosine diphosphate; N, number of human subjects. Total aggregation (%) - Phase 1 (%) = Phase 2 (%).

Table 2
Concentration-dependent effects of eugenol on ADP-induced (10 μ M) human platelet total and biphasic aggregation *ex vivo*.

Eugenol [μ M]	N	Eugenol+ADP			Normalized to 100% aggregation	
		Total (%)	Phase 1 (%)	Phase 2 (%)	Phase 1 (%)	Phase 2 (%)
0; Control	24	66 \pm 4	41 \pm 3	25 \pm 3	62 \pm 3	38 \pm 3
10	9	49 \pm 2 *	38 \pm 3	13 \pm 4 *	79 \pm 4 *	21 \pm 4 *
25	12	42 \pm 3 *	43 \pm 2	-1 \pm 1 *	99 \pm 3 *	1 \pm 3 *
100	17	41 \pm 2 *	43 \pm 2	-2 \pm 1 *	106 \pm 3 *	-6 \pm 3 *

Data are mean \pm SEM. Abbreviations: ADP, adenosine diphosphate; N, number of human subjects. Total aggregation = Phase 1 (%) + Phase 2 (%). *P < 0.05, One-Way ANOVA with repeated measures.

5. Discussion

5.1. Purpose of study

To our knowledge, no one has investigated the direct effects of these 15 common flavorants on ADP-induced human platelet aggregation. We hypothesized that direct exposure of platelets to flavorants may enhance ADP-induced platelet aggregation *ex vivo* by altering either or both of the aggregation phases. Under the conditions used, our data indicate nearly all of the flavoring compounds tested do not influence direct platelet aggregation induced by ADP. Importantly, eugenol, a known anticoagulant and a banned tobacco flavorant, acts as a strong positive control. Our results demonstrate that the conditions used herein are appropriate to detect either activation or inhibition of ADP-induced platelet aggregation by common e-cig flavorants.

5.2. Strengths of study

This study has several strengths: 1) human platelets; 2) wide range of common flavorants tested; 3) appropriate test conditions and concentrations; and, 4) a strong positive control flavorant.

5.2.1. Human platelets

Both animal and *in vitro* research models are on the rise to reduce the need of human-based studies. However, research using human samples is most effective in modeling the true impacts of toxicant exposure on human health. Agonist-induced platelet activation and aggregation may vary from species to species, in part, by their mechanism of action or potency. Studies involving human platelets may differ from animal-based platelet studies because: 1) human-based studies reflect variation due to a person's way of life and diet as well as use of certain medications, e.g., aspirin, that influence platelet behavior; 2) genotypic differences between species; 3) animal-based studies are controlled: genotype, housing, route and dose of exposure; and, 4) the potency of

agonists vary between animals and humans [14,15]. Thus, a strength of our study is the direct testing of flavorants on human platelets avoiding need of translational inference.

5.2.2. Wide range of common flavorants tested

Our study uses common flavorants found in thousands of e-cig flavoring profiles currently on the tobacco market [16–18]. The diversity of flavor profiles continues to increase over time to attract new e-cig users [4]. Our study investigates the effect of pure flavorants on platelet aggregation rather than the effects of flavor profiles that are more chemically diverse, and thus, may exert more complex effects on platelets than the pure compounds used herein.

5.2.3. Appropriate test concentration

Our series of experiments involved testing effects of flavorants (100 μ M) on ADP-induced biphasic platelet aggregation. Blood levels of flavorant compounds are unreported. To justify levels of flavorants used in this study, we estimated potential blood levels based on reported concentrations of common flavorants in e-cig liquids (Table 3).

Using the highest reported e-liquid concentrations in Table 3, we estimated the blood levels of flavorants after vaping using convenient assumptions, e.g., 100% efficiency in both transfer from e-liquid to aerosol and from aerosol to the blood (Table 3). During e-cig use, e-liquid consumption is minimally dependent on user topography and e-cig power settings, and so e-liquid consumption will vary in different studies. Although the volume of e-liquid aerosolized in any puff depends on several factors (e.g., power, puff volume, puff duration, etc.), we used 2 μ L as a conservative estimate of e-liquid consumed during a 100 mL puff [19,20]. This is based on: 1) the literature [20]; 2) our experience in an experimental setting wherein a 91.1 mL puff consumed 4.0–6.9 mg of e-liquid, which is more volume than we use in the calculation [21]; and, 3) because we previously used this same volume in estimates of cinnamaldehyde uptake [19]. Moreover, as pulmonary blood is first to receive the compounds in inhaled e-cig aerosols, we chose a modest blood

Table 3

Levels of common flavorants in electronic cigarette liquids, and the estimated blood levels of flavorants of e-cigarette users.

Flavorant (M.W.)	Flavorant [mg/mL]	~µg per puff ^a	~blood [µg/dL] ^b	~blood [µM] ^c	Ref.
Cinnamaldehyde (132.16)	0.8 – 155	310	310	23.46	[3,36]
Menthol (156.27)	0.1 – 84	168	168	10.75	[3,5,16]
Benzyl Alcohol (108.14)	0.1 – 39	78	78	7.21	[3,36]
Vanillin (152.15)	0.1 – 33	66	66	4.33	[3,16]
Eugenol (164.20)	1.9 – 12	24	24	1.46	[3,36]
Maltol (126.11)	0.8 – 4.9	9.8	9.8	0.77	[3,36]
Limonene (136.24)	0.7 – 2.9	5.8	5.8	0.43	[3]
Menthone (154.25)	0.6 – 1.4	2.8	2.8	0.18	[3,36]
Methylbutyric acid (102.13)	0.6	1.2	1.2	0.12	[3]
Diacetyl (86.09)	0.0004 – 0.24	0.48	0.48	0.006	[16]

^a Estimated µg of flavorant aerosolized in a puff. Estimated µg was based on volume of e-liquid consumed per puff [2 µL] * [flavorant]. [21] ^b For simplicity, we assumed 100% of aerosolized flavorant was absorbed into 100 mL blood. ^c To calculate flavorant [molar], blood concentration was divided by flavorant M.W.

volume (100 mL; 2 heart beats worth) for dilution of pure flavorants. Using cinnamaldehyde as our example flavorant with a reported concentration range of 0.8 – 155 mg/mL (Table 3), 2 µL of aerosolized e-liquid [155 mg/mL] contains 0.31 mg cinnamaldehyde. Per our assumptions, 0.31 mg cinnamaldehyde is absorbed into blood, i.e., 100 mL blood = 0.31 mg/dL. Thus, under these conditions, blood cinnamaldehyde concentration would be approximately 23.46 µM. Of course, we appreciate that whole-body dilution will occur, yet not instantaneously. Moreover, platelet aggregation is a rapid event, and thus, a thrombus could form rather quickly upon activation. We chose a relatively high level of pure flavorant (100 µM) to test this idea in vitro, and more critically to not miss a toxicological threshold of these common flavorants. Additionally, during active e-cigarette use, flavorants may accumulate in blood for many reasons. Notably, most of these common flavorant compounds are GRAS, yet this designation is for their ingestion, and toxicity data regarding their inhalation is not available. Finally, the thermally-derived products of flavorants after heating need to be better characterized and tested for their effects on platelets.

5.2.4. Strong positive control flavorant

Clove oil has been demonstrated as a potent antithrombotic agent of platelet aggregation [22,23]. Eugenol as a major constituent of clove oil is also well-documented for having potent anti-platelet aggregation effects [24]. In human PRP, eugenol significantly inhibits arachidonic acid- (AA; 0.8 mM), collagen- (20 µg/mL), and platelet activating factor- (PAF; 0.8 µM) induced aggregation [25]. In our current study, we tested the direct effects of eugenol on ADP-induced human platelet aggregation. We found that eugenol at a concentration as low as 10 µM inhibits ADP-induced aggregation and does so solely via inhibition of phase 2 – a step that is dependent on platelet secretion of granules and platelet recruitment. This finding is consistent with the known mechanism of clove and eugenol suppression of platelet action that is similar to aspirin and other inhibitors of prostaglandin biosynthesis, e.g., thromboxane A₂ (TxA₂) [24–26]. In this study, eugenol proved useful as a strong positive control flavorant that directly inhibits ADP-induced platelet aggregation, and these data confirm that proper testing conditions were used for isolated human platelets.

5.3. Limitations of study

This study also has a few limitations: 1) only one agonist (ADP) was tested; 2) contrasting results with literature; and, 3) parent flavorants vs. flavorant thermal degradation products.

5.3.1. Use of ADP agonist

We did not test for effects of flavorants on other agonists of platelet aggregation (e.g., thrombin, TxA₂, serotonin, collagen, AA, or epinephrine) because these agonists, with the exception of epinephrine, do not induce a biphasic platelet response [27]. The biphasic response of ADP provides an opportunity to reveal mechanisms by which flavorants, in combination with ADP, act on platelets. Thus, we chose ADP as the ‘best option’ of all agonists for this study because it stimulates a biphasic response [28]. We recognize that other agonists reveal other pathways of platelet aggregation that may become activated (or inhibited) upon exposure to a flavorant, which are also important to study.

5.3.2. Comparisons with literature

Our study found no effects of common e-liquid flavorants (excepting for our positive control eugenol) on ADP-induced platelet aggregation, although this general finding is contradicted by a few published studies. For example, several flavorants that we tested, cinnamaldehyde, vanillin, menthol, and menthone, are reported to have anti-platelet activity. For example, Takenaga et al. finds that cinnamaldehyde (300–1000 µM) inhibits ADP (2 µM)- and collagen (0.5 µg/mL)-induced human platelet aggregation via suppressing release of AA and TxA₂ and B₂ in platelet granules [29]. In rat PRP, cinnamaldehyde (150–600 µM) also decreases collagen- (100 µg/mL)- and thrombin- (3 U/mL) induced platelet aggregation [30]. Vanillin (100 ng/mL, 0.657 µM) suppresses AA- (100 µM) induced rabbit platelet aggregation [31]. To our knowledge, no other study using vanillin was conducted in human PRP with ADP as the agonist. Both menthol (5 mM) and menthone (5 mM) inhibit collagen- (5 µg/mL) and ADP- (5 µM) induced human platelet aggregation *ex vivo*, [32] however, again the level used (5 mM) is an order of magnitude higher than both our estimated blood level and our test concentration of 100 µM. Last, limonene (500 µM; 5x our test [100 µM]) inhibits thrombin-induced platelet activation via activation of the adenosine A_{2A} receptor [33]. Despite apparent contradictions between our findings and published studies, differences in effects of parent

flavorants appear attributable to use of higher concentrations and different agonists of platelet aggregation.

5.3.3. Flavorant thermal degradation products

E-cigs heat their liquids that can result in thermal degradation of parent flavorants. Although we tested 15 common flavorants in e-liquids representing seven distinct chemical classes, we did not test the variety of compounds formed within and during the heating of e-liquids. For example, flavor acetals or thermally-derived flavor products that result from chemically altering the parent flavorants and forming chemical adducts during heating of e-cig solvents or that appear as stable adducts with propylene glycol (PG) and vegetable glycerol (VG) at room temperature, were not tested [34]. Interestingly, some acetal adducts are more toxic than their parent flavorants in lung epithelial cells [35]. The effect of flavor acetals on platelet function have not been studied. As such, we did not address the effects of flavorant thermal degradation products or PG:VG acetals on platelet aggregation because establishing a baseline of the effects of pure flavorant compounds, which can likely reach [μ M] levels in the pulmonary blood (see Table 3), on human platelets is needed.

6. Summary and conclusion

Under the conditions tested, our range of common e-cig fluid flavorants had limited direct effect on isolated human platelets aggregation stimulated by ADP. As discussed, flavorants may have agonist-dependent effects, e.g., augment platelet activation of specific agonists such as collagen, thrombin, etc., however, past reports used incredibly high levels of flavorants that are not justified relative to e-cigarette use. Additionally, it is important to note that e-cig products are heated instruments. The chemical structure of flavorants can be altered when heated, and thus, these new compounds (e.g., flavor acetals) may impact platelet behavior. In fact, some flavorant acetals are more potent biologically than their parent compounds [35]. To address this gap in knowledge, future studies should assess the direct effects of products of heated flavorants on agonist-induced human platelet aggregation.

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Author contributions

AR generated, analyzed, and interpreted the biphasic responses that are presented in the data as well as co-wrote the manuscript. TK planned and conducted experiments, generated and analyzed the data, and co-wrote the manuscript. PL, SD, AB, SS and DJC interpreted data and edited the manuscript. DJC is the guarantor of this work. As such, he has full access to all data and takes responsibility for the integrity and accuracy of the data.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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References

- [1] A.S. Gentzke, M. Creamer, K.A. Cullen, B.K. Ambrose, G. Willis, A. Jamal, B. A. King, Vital signs: Tobacco product use among middle and high school students - United States, 2011-2018, *MMWR Morb. Mortal. Wkly. Rep.* 68 (2019) 157–164.
- [2] E. Park-Lee, C. Ren, M.D. Sawdey, A.S. Gentzke, M. Cornelius, A. Jamal, K. A. Cullen, Notes from the field: e-cigarette use among middle and high school students - National Youth Tobacco Survey, United States, 2021, *MMWR Morb. Mortal. Wkly. Rep.* 70 (2021) 1387–1389.
- [3] P.A. Tierney, C.D. Karpinski, J.E. Brown, W. Luo, J.F. Pankow, Flavour chemicals in electronic cigarette fluids, *Tob. Control* 25 (2016) e10–e15.
- [4] S.H. Zhu, J.Y. Sun, E. Bonnevie, S.E. Cummins, A. Gamst, L. Yin, M. Lee, Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation, *Tob. Control* 23 (Suppl 3) (2014) iii3-9.
- [5] E.E. Omaiye, K.J. McWhirter, W. Luo, P.A. Tierney, J.F. Pankow, P. Talbot, High concentrations of flavor chemicals are present in electronic cigarette refill fluids, *Sci. Rep.* 9 (2019) 2468.
- [6] R.Z. Behar, B. Davis, Y. Wang, V. Bahl, S. Lin, P. Talbot, Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids, *Toxicol. Vitr.* 28 (2014) 198–208.
- [7] T. Muthumalage, M. Prinz, K.O. Ansah, J. Gerloff, I.K. Sundar, I. Rahman, Inflammatory and oxidative responses induced by exposure to commonly used e-cigarette flavoring chemicals and flavored e-liquids without nicotine, *Front Physiol.* 8 (2017) 1130.
- [8] W.H. Lee, S.G. Ong, Y. Zhou, L. Tian, H.R. Bae, N. Baker, A. Whitlatch, L. Mohammadi, H. Guo, K.C. Nadeau, M.L. Springer, S.F. Schick, A. Bhatnagar, J. C. Wu, Modeling cardiovascular risks of e-cigarettes with human-induced pluripotent stem cell-derived endothelial cells, *J. Am. Coll. Cardiol.* 73 (2019) 2722–2737.
- [9] J.L. Fetterman, R.M. Weisbrod, B. Feng, R. Bastin, S.T. Tuttle, M. Holbrook, G. Baker, R.M. Robertson, D.J. Conklin, A. Bhatnagar, N.M. Hamburg, Flavorings in tobacco products induce endothelial cell dysfunction, *Arterioscler. Thromb. Vasc. Biol.* 38 (2018) 1607–1615.
- [10] G.R. Wang, Y. Zhu, P.V. Halushka, T.M. Lincoln, M.E. Mendelsohn, Mechanism of platelet inhibition by nitric oxide: in vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase, *Proc. Natl. Acad. Sci. USA* 95 (1998) 4888–4893.
- [11] E. Gkaliagkousi, V. Corrigan, S. Becker, P. de Winter, A. Shah, C. Zamboulis, J. Ritter, A. Ferro, Decreased platelet nitric oxide contributes to increased circulating monocyte-platelet aggregates in hypertension, *Eur. Heart J.* 30 (2009) 3048–3054.
- [12] H. Qasim, Z.A. Karim, J.C. Silva-Espinoza, F.T. Khasawneh, J.O. Rivera, C.C. Ellis, S.L. Bauer, I.C. Almeida, F.Z. Alshbool, Short-term e-cigarette exposure increases the risk of thrombogenesis and enhances platelet function in mice, *J. Am. Heart Assoc.* (2018) 7.
- [13] G.V. Born, Aggregation of blood platelets by adenosine diphosphate and its reversal, *Nature* 194 (1962) 927–929.
- [14] D.H. Namm, A.S. Tadepalli, J.A. High, Species specificity of the platelet responses to 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine, *Thromb. Res.* 25 (1982) 341–350.
- [15] A. Pelagalli, P. Lombardi, D. d'Angelo, R. Della Morte, L. Avallone, N. Staiano, Species variability in platelet aggregation response to different agonists, *J. Comp. Pathol.* 127 (2002) 126–132.
- [16] J.G. Allen, S.S. Flanigan, M. LeBlanc, J. Vallarino, P. MacNaughton, J.H. Stewart, D.C. Christiani, Flavoring chemicals in e-cigarettes: diacetyl, 2,3-pentanedione, and acetoin in a sample of 51 products, including fruit-, candy-, and cocktail-flavored e-cigarettes, *Environ. Health Perspect.* 124 (2016) 733–739.
- [17] G. Wolkart, A. Kollau, H. Stessel, M. Russwurm, D. Koesling, A. Schrammel, K. Schmidt, B. Mayer, Effects of flavoring compounds used in electronic cigarette refill liquids on endothelial and vascular function, *PLoS One* 14 (2019), e0222152.
- [18] C. Girvalaki, M. Tzatzarakis, C.N. Kyriakos, A.I. Vardavas, P.D. Stivaktakis, M. Kavvalakis, A. Tsatsakis, C. Vardavas, Composition and chemical health hazards of the most common electronic cigarette liquids in nine European countries, *Inhal. Toxicol.* 30 (2018) 361–369.
- [19] M.A. Nystoriak, P.J. Kilfoil, P.K. Lorkiewicz, B. Ramesh, P.J. Kuehl, J. McDonald, A. Bhatnagar, D.J. Conklin, Comparative effects of parent and heated cinnamaldehyde on the function of human iPSC-derived cardiac myocytes, *Toxicol. Vitr.* 61 (2019), 104648-104648.
- [20] S.E. Soulet, M. Duquesne, J. Toutain, C. Pairaud, H.E. Lalo, Influence of coil power ranges on the e-liquid consumption in vaping devices, *Int. J. Environ. Res Public Health* 15 (2018) 1853–1867.
- [21] D.J. Conklin, M.A. Ogunwale, Y. Chen, W.S. Theis, M.H. Nantz, X.A. Fu, L.C. Chen, D.W. Riggs, P. Lorkiewicz, A. Bhatnagar, S. Srivastava, Electronic cigarette-generated aldehydes: the contribution of e-liquid components to their formation and the use of urinary aldehyde metabolites as biomarkers of exposure, *Aerosol Sci. Technol.* 52 (2018) 1219–1232.
- [22] A. Rasheed, G.M. Laekeman, A.J. Vlietinck, J. Janssens, G. Hatfield, J. Totte, A. G. Herman, Pharmacological influence of nutmeg and nutmeg constituents on rabbit platelet function, *Planta Med.* 50 (1984) 222–226.
- [23] S.A. Saeed, A.H. Gilani, Antithrombotic activity of clove oil, *J. Pak. Med. Assoc.* 44 (1994) 112–115.

- [24] K.C. Srivastava, Antiplatelet principles from a food spice clove (*Syzygium aromaticum* L) [corrected], *Prostaglandins Leukot. Ess. Fat. Acids* 48 (1993) 363–372.
- [25] S.A. Saeed, R.U. Simjee, G. Shamim, A.H. Gilani, Eugenol: a dual inhibitor of platelet-activating factor and arachidonic acid metabolism, *Phytomedicine* 2 (1995) 23–28.
- [26] A. Bennett, I.F. Stamford, I.A. Tavares, S. Jacobs, F. Capasso, N. Mascolo, G. Autore, V. Romano, Di, The biological activity of eugenol, a major constituent of nutmeg (*Myristica fragrans*): Studies on prostaglandins, the intestine and other tissues, *Phytother. Res.* 2 (1988) 124–130.
- [27] L. Zhou, A.H. Schmaier, Platelet aggregation testing in platelet-rich plasma: description of procedures with the aim to develop standards in the field, *Am. J. Clin. Pathol.* 123 (2005) 172–183.
- [28] L. Perez-Campos-Mayoral, E. Pérez-Campos, E. Zenteno, A. Majluf-Cruz, E. Perez-Ortega, D. Matias-Pérez, F.J. Rodal-Canales, R. Martínez-Cruz, S. Pina-Canseco, M. A. Reyes Franco, G. Mayoral Andrade, P. Hernández, B. Gallegos, Better detection of platelet aggregation in patients with metabolic syndrome using epinephrine and ADP, *Diabetol. Metab. Syndr.* 6 (2014), 93–93.
- [29] M. Takenaga, A. Hirai, T. Terano, Y. Tamura, H. Kitagawa, S. Yoshida, In vitro effect of cinnamic aldehyde, a main component of Cinnamomi Cortex, on human platelet aggregation and arachidonic acid metabolism, *J. Pharm.* 10 (1987) 201–208.
- [30] J. Huang, S. Wang, X. Luo, Y. Xie, X. Shi, Cinnamaldehyde reduction of platelet aggregation and thrombosis in rodents, *Thromb. Res.* 119 (2007) 337–342.
- [31] W.-Y. Lin, C.-M. Teng, I.-L. Tsai, I.-S. Chen, Anti-platelet aggregation constituents from *Gynura elliptica*, *Phytochemistry* 53 (2000) 833–836.
- [32] M. Murayama, K.K. Kumaroo, Inhibitors of ex vivo aggregation of human platelets induced by decompression, during reduced barometric pressure, *Thromb. Res.* 42 (1986) 511–516.
- [33] H.M. Park, J.H. Lee, J. Yaoyao, H.J. Jun, S.J. Lee, Limonene, a natural cyclic terpene, is an agonistic ligand for adenosine A(2A) receptors, *Biochem Biophys. Res Commun.* 404 (2011) 345–348.
- [34] H.C. Erythropel, S.V. Jabba, T.M. DeWinter, M. Mendizabal, P.T. Anastas, S. E. Jordt, J.B. Zimmerman, Formation of flavorant-propylene glycol adducts with novel toxicological properties in chemically unstable e-cigarette liquids, *Nicotine Tob. Res.* 21 (2019) 1248–1258.
- [35] S.V. Jabba, A.N. Diaz, H.C. Erythropel, J.B. Zimmerman, S.E. Jordt, Chemical adducts of reactive flavor aldehydes formed in e-cigarette liquids are cytotoxic and inhibit mitochondrial function in respiratory epithelial cells, *Nicotine Tob. Res.* 22 (2020) S25–S34.
- [36] H.C. Erythropel, Garcia, D.S. Torres, J.G. Woodrow, T.M. de Winter, M.M. Falinski, P.T. Anastas, S.S. O'Malley, S. Krishnan-Sarin, J.B. Zimmerman, Quantification of flavorants and nicotine in waterpipe tobacco and mainstream smoke and comparison to e-cigarette aerosol, *Nicotine Tob. Res.* 23 (2021) 600–604.