SUPPLEMENTARY MATERIALS

Qiu et al., Increased vulnerability to atrial and ventricular arrhythmias caused by different types of inhaled tobacco or marijuana products

Full Methods

Animals. Sprague-Dawley rats, 8-10 weeks old, mixed gender, were used for this study. Animal procedures were approved and monitored by the Institutional Animal Care Use Committee of the University of California, San Francisco. The research reported in this paper adhered to the ARRIVE guidelines for reporting animal research and the National Academies of Sciences, Engineering, and Medicine Guide for the Care and Use of Laboratory Animals.

Group sizes varied from 5-16 depending on the experiment; a situation resulting from complications of the COVID-19 lab shutdowns although sufficient power was still achieved. To elaborate, our multiple planned outcomes (in vivo measurements of SBP, cardiac function, and HRV; ex vivo measurements susceptibility to VT and AF; and histological evaluations) were planned for n=6 or n=8/group based on power calculations using an α of 0.05, two-tailed testing, and power of 0.8. What had been planned as a single large 8-week experiment with all rats undergoing all measurements was allowed to proceed during the shutdown with a single researcher authorized to be in the lab, but some planned electrophysiological endpoints were not practical under those circumstances. The experiment was completed without those endpoints, and then the experiment was repeated later to enable the missing endpoints to be studied. Functional data was collected during both experiments so all of it was combined for those analyses. Occasional animal mortality resulted in additional minor differences in final group size.

Smoke/aerosol generation and animal exposure. To mimic human active smoking/vaping, conscious rats in restrainers (DecapiCones, DC 200, Braintree Scientific, Inc., Braintree, MA) were exposed to pulsatile smoke/aerosol commencing after at least 3 days of acclimation to the restrainers, by which time the rats tolerated being held, as determined by breathing smoothly and not struggling. Each rat was exposed 5 days/week for 2 months, one session/day, with each session consisting of 10 cycles spread over 5 min, to approximate the consumption of a single cigarette or a single vaping session. A Universal Vaping Machine (Gram Research, Oakland, CA) was used for the generation of 35 mL smoke or aerosol per puff over 2 seconds, with rats in the nosecones for 5 seconds followed by 25 seconds of room air exposure as we described previously.[17] Separate sets of nosecones, connecting tubes, valves, and syringe pump were used for each exposure condition to avoid cross-contamination of products; four rats were exposed simultaneously via a 4-branch adapter. Nosecone undiluted smoke exposure instead of chamber exposure mimics human smoking, prevents post-exposure continued absorption due to licking material adhered to the fur, and enables immediate switching between smoke/aerosol and air. Two out of 18 initial animals in the tobacco cigarette group died on days 1 and 14 of exposure and were replaced; no other mortalities occurred.

The following commercial products and group sets were used: tobacco cigarettes (Marlboro Reds, batch number: V284 Y81B5), HTPs (IQOS, an IQOS 3.0 Multi device from Germany using Russian Parliament branded HeatSticks, batch number zz0362/718), e-cigs (JUUL, Virginia Tobacco flavor, 5% nicotine, batch number: G0811CG-1), marijuana (~10% THC, supplied in bulk from the University of Mississippi via RTI International, contracted through the National Institute on Drug Abuse, and rolled into cigarettes in our lab), and "Placebo marijuana" (<.01% THC, supplied as pre-rolled cigarettes from Univ. of Mississippi via RTI). All required federal, state, and institutional approvals for acquisition and possession of marijuana and exposure of rodents were obtained.[18] Air exposure was used as control. Group size was 8-16.

Conscious systolic blood pressure (SBP) measurement. Conscious SBP was obtained from rats in rodent restrainers (ECU, Braintree Scientific, Inc., USA), using a non-invasive blood pressure system (ML125/R NIBP, ADInstruments, Sydney, Australia) via a tail cuff and pulse transducer in conjunction with a PowerLab system and analyzed by LabChart 5. At least three days pre-adaptability training to restrainer was necessary, 30 minutes/day, in order to obtain calm conscious SBP. We measured conscious SBP on the first exposure day and the end of the 2nd, 4th, 6th, and 8th week (mimicking one cigarette or vaping session per day) to determine progressively chronic effects. On each measurement day, SBP was measured twice, both before and after that day's single exposure, to determine that day's acute effect. After 3 days of training for acclimation to restraint, rats tolerated being held, as determined by breathing smoothly and not struggling.

Echocardiography. Echocardiography was performed at baseline and the 4th and 8th weeks, using a preclinical echocardiographic system (Vevo 3100LT, FUJIFILM VisualSonics, Toronto, Canada) equipped with a 25-MHz MX250D mechanical transducer. Parasternal long-axis and 3 levels of short-axis views (at distal, mid-papillary muscle, and apex level) were acquired to calculate left ventricular function based on the cylinder-hemiellipsoid model using modified Simpson's rule.[22] LV mass was calculated from the M mode of the parasternal short-axis view at the mid-papillary muscle level.

ECG telemetry for heart rate variability (HRV). ECG radiotelemetry (DSI, Harvard Bioscience, Inc., Dallas, TX) was used to collect 24-hour ECG for analyzing HRV in conscious and free-living rats. Analgesic (Buprenex, 0.05 mg/kg) was injected once before surgery. Rats were anesthetized by 1.5% isoflurane. An ECG telemetry device (CTA F-40, DSI) was implanted subcutaneously in the dorsal region with the tunneled placement of the two ECG leads in a modified Lead II position where the negative was placed over the right pectoral muscle and the positive at the left caudal rib region about 2 cm to the left of the xiphoid process. Rats were then housed in a noise cancellation room at least 7 days before data collection to allow wound healing, pain relief, and environmental adaptation. Housing in the noise cancellation room allowed us to record animal physiology without interference from human activity. ECG signal was collected after the 8th week of exposure. RR intervals were extracted and HRV was analyzed through Ponemah physiological platform (P3, version 6.50, DSI) based on time and frequency domain methods. Parameters are shown in the Supplementary Table 1.

Supplementary Table S1. HRV variability analysis setting.

Frequency Domain			
Interpolation		Calculation	
Rate	50 Hz	Segment Duration	1 min
Method	Quadratic	Periodogram Resolution	Max
Tolerance	20 ms	Windowing Method	Hamming
		Overlapping sub-series	2
		Frequency Axis Range	0–5 Hz
		☑ Enable Detrend ☑Enable Suppress Mean ☑ Normalized Result	
		☑ Enable Frequency Bins	
		Ultra-Low frequency (ULF) Very Low Frequency (VLF) Low Frequency (LF) High Frequency (HF)	0–0.05 0.05–0.25 0.25–1.00 1.00–3.00

Time Domain	
Reporting Period	2 min
Reporting Start Time	0
pNNx – x Value	9 ms
☑ Enable Outlier Removal	
Maximum Standard Deviation	3.0

Ex vivo heart optical mapping for simultaneous membrane potential and intracellular calcium. Eight weeks post exposure, ex vivo heart optical mapping was performed as described previously to test the susceptibility to arrhythmias originating from left and right atria and ventricles and to evaluate their electrophysiological characteristics.[19]

The isolated heart was placed in a Langendorff system and perfused with 37˚C modified-Tyrode solution. Blebbistatin (BML-EI315-0025, Enzo Life Sciences, Inc., Farmingdale, NY) was then added into the solution at 15 μmol/L and perfused through the heart. The voltage-sensitive dye RH237 (1 μmol/L dissolved in dimethyl sulfoxide, S1109, Thermo Fisher, Waltham, MA) was then added into 10 mL Tyrode solution and impulsively injected into the perfusion, followed by injection of a mixed solution of the intracellular calcium indicator Rhod-2 (5 μmol/L in dimethyl sulfoxide, R1244, Thermo Fisher) and cellular permeability promoter Pluroic F-127 (P3000MP, Thermo Fisher) in 10 mL Tyrode solution, to obtain transmembrane potential and calcium transient signals. Fluorescent optical maps were obtained by a CCD camera (MiCAM02, SciMedia) and analyzed with OMproCCD software.

The action potential duration at 80% repolarization (APD_{80}) and calcium transient duration at 80% repolarization ($CATD_{80}$) were measured after a series of 20×S1 pacing trains at the pacing cycle lengths (PCL) of 150, 130, 120, 110, 100, 90, 80, and 70 ms. The effective refractory period (ERP) and the susceptibility to both AF and VT were tested via programmed stimulations including extra-stimuli and overdrive pacing. AF was diagnosed as fast and irregular beating lasting more than 2 seconds, whereas VT was determined as at least 6 non-driven consecutive ventricular premature beats.[23,24] After these procedures, the heart was weighed and embedded in O.C.T compound (Sakura Finetek, Inc., Torrance, CA) for the following histological analyses.

Histology. Heart coronal or transverse cryosections (10 μm) were cut using a LEICA CM1860 cryostat (Leica Biosystems, Wetzlar, Germany) and mounted on super frost slides (Fisher Scientific). Sirius red-fast green staining was used to assess fibrosis. Ten images were randomly acquired with a 20× objective using a Nikon E800 microscope attached to a Retiga R6 camera (Teledyne Photometrics,

Tucson, AZ) with Q-capture Pro 7 software. The percentage of the total tissue area that was stained red, reflecting the degree of fibrosis, was quantified using Fiji Image J.[28] Gaps in the tissues were excluded from calculation.

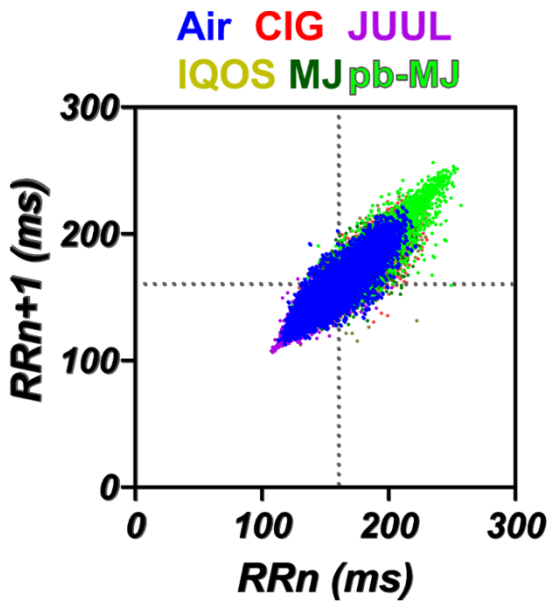
Intrinsic cardiac nervous system (ICNS) as the autonomic nerve system inside the heart is vital for cardiac function and arrhythmogenesis.[25] To visualize ICNS, we performed immunofluorescence staining of tyrosine hydroxylase (TH, used as sympathetic nerve marker) and choline acetyltransferase (ChAT, used as parasympathetic nerve marker). Hearts were fixed before staining as previously described.[21] Tissue was cut longitudinally along the approximate coronal plane as 15 μm frozen sections, mounted, and dried at 38°C on a slide warmer (XH-2001, Ted Pella, Inc.) for 90 minutes. Sections were rinsed 3 times with phosphate buffered saline (PBS). To block non-specific antibody binding, tissue sections were first incubated for 2 h in 5% normal goat serum (G9023, Millipore Sigma) in PBS at room temperature in a humid box and then washed with PBS three times in 10 min and incubated in a mixture of double primary antibodies including rabbit anti-TH (1:200, AB152, Millipore Sigma) and mouse anti-ChAT (1:200, AMAB91130, Millipore Sigma) for 48 h. The area of tissue was marked with Pap pen (ab2601, Abcam, Cambridge, UK). Antibody diluent was made by 0.3% (v/v) Triton X-100 (T8787, Millipore Sigma), 2% (w/v) polyvinylpyrolidone (P5288, Millipore Sigma), 2% (w/v) bovine serum albumin (5217, Tocris Bioscience, UK) in PBS. To detect primary antibodies raised in mouse (anti-ChAT) and rabbit (anti-TH), tissues were incubated in antisera with AlexaFluor 555 Donkey anti-mouse IgG (H+L) (1:1500 in PBS with 0.3% (v/v) Triton X-100, A31570, Invitrogen, Grand Island, NY) and AlexaFluor 488 Donkey anti-rabbit IgG (H+L) (1:1500, A32790, Invitrogen) for 2 hours at room temperature followed by washing in PBS 3 times in 10 mins. To identify cell nuclei, tissue sections were incubated in 300 nM 4',6-diamidino-2-phenylindole (62248, Thermo Fisher) in PBS for 10 minutes and rinsed 3 times in PBS, and mounted in ProLong™ Gold Antifade Mountant (P10144, Thermo Fisher). Sections were imaged with a 20× objective using Micro-Manager 2.0 gamma. The exposure time needed to be adjusted until the dimmest regions were discerned and the brightest regions were not oversaturated. Ten views were taken randomly from subepicardium, midmural, and subendocardium for each section to calculate mean optical area that represented the average intensity level using Fiji ImageJ.

In order to quantify microvessels including both capillaries and small precapillary arterioles with a cross area of 10–314 μm^2,[26] slides were incubated with biotinylated Griffonia simplicifolia I lectin (GS-I, L2140, Sigma-Aldrich) and then labeled with Alexa Fluor 488 Streptavidin (S11223, Invitrogen) as we previously described.[27] For the analysis of microvessels, cardiac tissue was cut and collected from the transverse plane at papillary muscle level. Methods of image acquisition and analysis were similar to those done for fibrosis. Microvessel density (microvessel count per mm^2 of tissue area) and area percentage were calculated.

Serum enzyme-linked immunosorbent assay (ELISA). After 8 weeks of exposure, blood was collected through the tail artery and then serum was isolated by 3000 rpm centrifugation for 20 mins. Fibrotic biomarkers including galectin-3 (Gal-3), matrix metalloproteinase-9 (MMP-9) and its endogenous tissue inhibitor-1 (TIMP1), and blood pressure regulators including the total angiotensin

and norepinephrine, were measured using corresponding ELISA kits including ERLGALS3 for Gal-3 and ERTIMP1 for TIMP1, from Invitrogen, RMP900 for MMP-9 from R&D Systems, and ab288178 for angiotensin and ab287789 for norepinephrine from Abcam, following their instructions.

Data statistics and graphing. Data are shown as mean ± SD. The inducibility of arrhythmias as categorical variables were described as percentages and analyzed by Fisher exact test. One-way, two-way ANOVA, or Kruskal–Wallis test were used to compare differences among groups based on the outcome of the normality and homogeneity of variances tests. Statistics and histograms were done by GraphPad Prism v9 (San Diego, USA) and some statistic, i.e., the arrhythmias inducibility, was done by Stata 13 (StataCorp, USA). $P < 0.05$ was required for significance.



Supplementary Fig.1 Poincaré plot of 24-h NN intervals.