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Male Mice have Increased Thrombotic Potential: Sex Differences in a Mouse Model of Venous Thrombosis

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Abstract

Introduction—Our objectives were to characterized sex differences during venous thrombosis using the electrolytic inferior vena cava model of the disease.

Materials and Methods—Male and female C57BL/6 mice (6–8 weeks) underwent inferior vena cava thrombosis. Time points included 6 hours, day 2, day 6, and day 14 post surgery along with surgically naïve true controls and surgical shams. Analyses included thrombus weight, vein wall morphometrics, and vein wall protein and gene expression for P-selectin, interleukin-1 β , and tumor necrosis factor- α ; hematology, soluble P-selectin, and plasma microparticle tissue factor activity assays.

Results—Male venous thrombi were significantly larger than females at days 2 (13.1 ± 1.0 vs. $6.8 \pm 0.5 \times 10^{-3}$ grams, $p < 0.01$), 6 (10.4 ± 0.8 vs. $5.4 \pm 0.5 \times 10^{-3}$ grams, $p < 0.01$) and 14 (6.3 ± 0.5 vs. $4.1 \pm 0.3 \times 10^{-3}$ grams, $p < 0.01$). Both male and female mice exhibited significantly increased vein wall P-selectin at 6 hours, vs. true controls ($p < 0.05$). Males had increased vein wall interleukin-1 β , versus females, at 6 hours (180.926 ± 24.596 vs. 60.417 ± 10.478 pg/mL, $p < 0.05$) and day 6 (76.966 ± 13.081 vs. 33.834 ± 4.198 pg/mL, $p < 0.01$). Males showed decreased tumor necrosis factor- α expression (-66%) at 6 hours. Females had increased tumor necrosis factor- α expression at 6 hours ($+541\%$) and day 6 ($+539\%$). Both sexes demonstrated decreased peripheral platelets at 6 hours ($p < 0.05$), coinciding with thrombogenesis. Plasma P-selectin increased in both sexes, versus controls, through day 6 ($p < 0.05$).

Conclusions—Males had significantly larger venous thrombi than females. Sex differences in vascular anatomy and response to inflammation may influence thrombus formation in our mouse thrombosis model.

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Keywords

Venous Thrombosis; Sex Differences; Animal model; Inflammation; Thrombogenesis; Thrombus resolution

Venous thromboembolism (VTE) includes both deep vein thrombosis (DVT) and pulmonary embolism (PE). In the United States (U.S.), the high morbidity and mortality rates make VTE a serious health concern. After heart disease and stroke, VTE is the most common vascular disease (1). In the U.S. alone, there is an estimated 900,000 people affected each year, with 300,000 deaths occurring annually (2–3). Venous thrombosis results from a combination of hypercoagulability conditions, blood flow stasis, and endothelial injury (4).

Studies have provided evidence that there are sex-specific differences in pharmacokinetics, pharmacodynamics, and physiology for a number of drugs (5). In 1994 the FDA created the Office of Women's Health (OWH). This program has sought out research projects in sex differences in the areas of cardiovascular diseases, autoimmune diseases, and the safety and efficacy of FDA regulated products (6). In 1998, the FDA mandated that new drug applications include data on safety and effectiveness by sex (7).

Recently, our laboratory developed a mouse electrolytic inferior vena cava (IVC) model (EIM) of DVT. This model consistently induces IVC thrombogenesis in the presence of continuous blood flow (8). This concept of continuous flow mimics the condition of a partial occlusive DVT in humans. The aim of this study was to determine if sex influences non-occlusive venous thrombogenesis in our animal model of the disease. Our hypothesis was that male and female mice have different inflammatory responses and physiological factors that would directly affect venous thrombogenesis.

Materials and Methods

Mice

For this study, 6–8 week old male (n=130, mean body weight=22.0g) and female (n=130, mean body weight 18.4g) C57BL/6 mice were used (Charles River Laboratories, Portage, Michigan).

Animal Model

We used the electrolytic inferior vena cava model (EIM) to induce thrombogenesis(8). Mice were anesthetized with an inhalation mixture of 2% isoflurane gas and oxygen (100%). The IVC was exposed via a midline laparotomy and blunt dissection. All side branches, inferior to the level of the left renal vein but superior to the iliac veins (excluding the right uterine vein in female mice), were ligated using 7-0 Prolene (Ethicon, Inc, Somerville, NJ). All back IVC branches remained patent. In the female mice, the right uterine vein was kept patent in order to enable the reproductive system to maintain a normal hormonal balance at chronic time points [Figure 1]. A 30 gauge silver coated copper wire (KY-1-GRN, Electrospec, Dover, NJ, USA) was inserted into a 25 gauge, 5mm stainless steel needle which was then placed into the caudal IVC and positioned against the anterior wall (anode). Another needle, with attached copper wire, was placed subcutaneously completing the circuit (cathode). A current of 250 μ Amps over 15 minutes was applied using a Grass S48 square wave stimulator and a constant current unit (Grass Technologies, An Astro-Med, Inc., West Warwick, RI). After the required amount of time, needles were removed, the abdominal muscle and skin were closed, and the mouse recovered. For the sham group, the IVC branches were ligated, but current was not applied, and IVCs were harvested 15 minutes after needle placement. True controls (TC), that were surgically naïve animals, were also

utilized. The EIM does not produce a thermal injury in order to initiate thrombogenesis; instead, the current results in formation of free radicals which erode the IVC endothelial surface, thus promoting a thrombogenic environment and subsequent thrombogenesis (8).

Male and female mice were grouped by sex and had circulating or tissue parameters evaluated at the following time points: 6 hours, day 2, day 6, and day 14 post EIM. Animals underwent the following analyses: thrombus weight, vein wall protein determination by enzyme-linked immunosorbent assay (ELISA) and gene expression by quantitative real time PCR (qRT-PCR) for P-selectin, interleukin-1 β (IL-1 β), and tumor necrosis factor-alpha (TNF- α), vein wall morphometrics, hematology, soluble P-selectin (sP-Sel), and plasma microparticle tissue factor activity (MP-TF) [Table 1].

Thrombus weight (TW)

In brief, groups of mice were analyzed for (wet) thrombus weight, at the time of euthanasia, in which the IVC thrombus and its associated vein wall were removed and weighed in grams (g) (9).

Vein wall protein analysis

The vein wall was separated from the thrombus and then immediately frozen in liquid nitrogen (-196°C) and stored at -70°C until analyzed. To prepare for ELISA plate loading, vein walls, separated from their respective thrombi, were homogenized in 400 μL Complete Lysis Buffer (Roche Indianapolis, IN), sonicated for 30 seconds and then spun down at 15,000 RPM for 3 minutes. Vein wall tissue sections were evaluated for P-selectin (≤ 50.0 pg/mL sensitivity), IL-1 β (≤ 3.0 pg/mL sensitivity), and TNF- α (≤ 5.1 pg/mL sensitivity), using a direct sandwich ELISA (R&D Systems, Minneapolis, MN). Protein levels within each group were reported as mean $\pm$ standard error of the mean (SEM) pg/mL.

Quantitative (real-time) polymerase chain reaction (qRT-PCR)

Gene expression of β -actin, CD-62 (P-selectin), IL-1 β , and TNF- α were determined as follows: Vein wall segments, separated from their respective thrombi, were placed in a 15 mL conical tube containing 1.5 mL of TRIzol (Invitrogen, Carlsbad, CA) and then homogenized using a standard protocol. The concentration of total RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Five μg of vein total RNA was reverse transcribed using-MLV as the reverse transcribing enzyme and oligo (dT) as the primer. The cDNA was determined using a Nanodrop spectrophotometer. cDNA was diluted to 22 ng/ μL and used as a template for Real-time PCR reaction. Real time PCR was set up as a 25 μL reaction using 12.5 μL of 2 $\times$ RT2 Real-Time Sybr Green PCR Master Mix, 1.0 μL of 22 ng/ μL cDNA, 1.0 μL of RT2 qPCR Selp primer band size 107, reference position 2337, 1.0 μL of RT2 qPCR IL-1 β primer (band size 156, reference position 1080), 1.0 μL of RT2 qPCR TNF- α primer (band size 93, reference position 879) and β -actin (Actb, band size 154, reference position 182) primers from SABiosciences (Frederick, MD). The reaction was run at 95°C for 10 min via 40 cycles of (95°C , 15 sec 60°C , 60 sec) in a Corbett Rotocycler (Corbett Research Mortlake, New South Wales, Australia). Primers and sequences were derived from relative mRNA expression was calculated by the formula $2^{-(\text{Ct target gene} - \text{Ct reference gene})}$ and cycle lengths used within the exponential phase of the PCR.

Vein wall morphometrics

For histology samples only, the IVC along with the aorta, were removed, paraffin-embedded, sectioned onto slides, and stained with hematoxylin and eosin (H&E). Slides were examined under oil immersion light microscopy. Five representative high-power fields

(5HPFs) were examined under high power (100×) around the vein wall and cell counts were analyzed. Cells were identified as polymorphonuclear cells (PMN), monocytes (MO), or lymphocytes (LYM) on the basis of standard histological criteria including nuclear size, cytoplasmic content, and total cell size in a blinded fashion by a board-certified veterinary pathologist. Results from the 5HPFs were added together and the mean $\pm$ SEM calculated for each vein segment studied (9–10).

Hematology

Five hundred μ L of blood was drawn, via cardiocentesis, and placed in EDTA microtainer tubes (Becton Dickinson, and Co. Franklyn Lakes, NJ) for a complete blood panel (20 μ L) using the CDC Technologies HEMAVET® Multispecies Hematology Analyzer (Drew Scientific, Inc. Oxford, CT).

Mouse soluble P-selectin (sP-Sel)

After hematology samples were run, the same EDTA sample was processed, according to the manufacturer's suggestions for this soluble P-selectin ELISA kit (R&D Systems Minneapolis, MN), to measure circulating sP-Sel protein levels (0.007 – 0.018 ng/mL sensitivity). All samples were run in duplicate and the results were normalized to total protein using the standard BCA assay (Pierce Rockford, IL).

Microparticle Tissue Factor (MP-TF) Activity Assay

Mouse whole blood (500 μ L) was collected via cardiocentesis using a syringe primed with 0.1 mL of sodium citrate 3.2%. Two hundred μ L of platelet poor plasma (PPP) was obtained by centrifuging blood at 1,500 g for 25 minutes. The plasma was then transferred from the original eppendorf into a clean eppendorf and centrifuged once more for 2 minutes at 15,000 g to remove contaminating cells. Samples were thawed, then immediately frozen in liquid nitrogen (-196°C) and sent to Dr. Nigel Mackman, University of North Carolina at Chapel Hill (UNC-CH) for analysis. At UNC-CH, samples were then incubated with the rat inhibitory anti-mouse TF antibody (1H1, 100 μ g/mL), the mouse anti-human TF antibody (HTF-1, 10 μ g/mL) or IgG controls for 15 minutes at room temperature. Mouse FVIIa was used. FXa generation was stopped by the addition of 25 μ L of 25 mM EDTA buffer. Finally, 25 μ L of the chromogenic substrate Pefachrome FXa 8595 (4 mM) was added and the mixture incubated at 37 $^{\circ}\text{C}$ for 15 min. Absorbance at 405 nm was measured using a VERSAmax microplate reader (Molecular Devices, Inc. Sunnyvale, CA). The MP-TF activity of the sample was calculated by reference to a standard curve generated using recombinant human relipidated TF (0–14 pg/mL, Dade Innovin). The MP-TF activity was reported in (pg/mL)(11).

Animal use and statistical analysis

This study was approved by the University of Michigan Committee on Use and Care of Animals and conducted in accordance with The Guide for the Care and Use of Laboratory Animals (12). All animals were healthy and free of specific pathogens. The university's animal program and facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Statistical analysis included the mean $\pm$ SEM for each group. Statistical significance between time points and sexes was calculated using unpaired t-test with Welch's Correction (GraphPad Software, Inc., La Jolla, CA). Significance was defined as $p \leq 0.05$.

Results

Thrombus Weight (TW)

There were no significance differences noted in vein wall weights between non-thrombosed TC and shams in both male mice (1.8 ± 0.1 vs. $1.7 \pm 0.2 \times 10^{-3}$ grams, $p=0.7582$) and females (1.6 ± 0.2 vs. $1.6 \pm 0.04 \times 10^{-3}$ grams, $p=0.7370$), respectively. Males showed significant increases in TW at 6 hours, versus male TC (8.4 ± 0.7 vs. $1.8 \pm 0.1 \times 10^{-3}$ grams, $p<0.0001$). Peak TW in males was observed at day 2, versus TC (13.1 ± 1.0 vs. $1.8 \pm 0.1 \times 10^{-3}$ grams, $p<0.0001$). This was followed by significant decreases in TW both between days 2 and 6 (13.1 ± 1.0 vs. $10.4 \pm 0.08 \times 10^{-3}$ grams, $p=0.0477$), and between days 6 and 14 (10.4 ± 0.08 vs. $6.3 \pm 0.5 \times 10^{-3}$ grams, $p=0.0002$). [Figure 2A]

Females had significant TW increases at 6 hours, versus female TC (6.7 ± 0.6 vs. $1.6 \pm 0.2 \times 10^{-3}$ grams, $p<0.001$). Peak TW, in females, was observed in the 6 hours and day 2 groups (6.7 ± 0.6 and $6.8 \pm 0.5 \times 10^{-3}$ grams, respectively). The females showed a decrease in TW between days 2 and 6 (6.8 ± 0.5 vs. $5.4 \pm 0.5 \times 10^{-3}$ grams, ns), followed by a significant decrease between days 6 and 14 (5.4 ± 0.5 vs. $4.1 \pm 0.3 \times 10^{-3}$ grams, $p=0.0466$). [Figure 2B]

Male IVC thrombi were significantly larger than female thrombi, at day 2 (13.1 ± 1.0 vs. $6.8 \pm 0.5 \times 10^{-3}$ grams, $p=0.0006$), day 6 (10.4 ± 0.8 vs. $5.4 \pm 0.5 \times 10^{-3}$ grams, $p<0.0001$) and day 14 (6.3 ± 0.5 vs. $4.1 \pm 0.3 \times 10^{-3}$ grams, $p=0.0073$).

Vein Wall Protein Analysis (ELISA)

P-selectin—Male mice had increased vein wall P-selectin protein at 6 hours, versus male TC (11819.860 ± 649.071 vs. 6384.445 ± 1279.758 pg/mL, $p=0.0323$). This was followed by a significant decline between day 2 and day 6 (**11094.0 ± 532.9** pg/mL vs. 7280.340 ± 749.401 , **$p=0.0016$**).

P-selectin vein wall protein levels in females peaked significantly at 6 hours, versus female TC (13884.990 ± 680.615 vs. 5804.777 ± 490.957 pg/mL, $p=0.0024$). This was followed by significant declines between 6 hours and day 2 (13884.990 ± 680.615 vs. 9747.589 ± 555.449 pg/mL, $p=0.0053$), days 2 and 6 (9747.589 ± 555.449 vs. 7617.925 ± 782.402 pg/mL, $p=0.0449$), and days 6 and 14 (7617.925 ± 782.402 vs. 5305.196 ± 761.212 pg/mL, $p=0.05$).

Interleukin-1 β (IL-1 β)—Peak IL-1 β vein wall protein levels were observed in male mice at 6 hours with a significant increase, versus TC (180.926 ± 24.596 vs. 96.642 ± 21.406 pg/mL, $p=0.0272$). This was followed by a significant decline between 6 hours and day 2 (180.926 ± 24.596 vs. 51.370 ± 6.336 pg/mL, $p=0.0003$). Significant IL-1 β increases were observed between days 2 and 14 (51.370 ± 6.336 vs. 90.230 ± 14.452 pg/mL, $p=0.0299$). Female mice showed no significant changes, versus female TC, in IL-1 β levels. Male mice had significantly higher levels of IL-1 β , versus females, at 6 hours (180.926 ± 24.596 vs. 60.417 ± 10.478 pg/mL, $p=0.0012$) and day 6 (76.966 ± 13.081 vs. 33.834 ± 4.198 pg/mL, $p=0.0358$).

Tumor necrosis factor- α (TNF- α)—Male mice had a significant decrease in vein wall TNF- α protein at 6 hours, versus male controls (10.673 ± 2.377 vs. 73.031 ± 21.455 pg/mL, $p=0.0342$), followed by a return to baseline production levels at day 14. In female mice there was a trend in decreased vein wall TNF- α protein at 6 hours, versus female TC, followed by a return to baseline values at day 14. Male mice had significantly higher levels of vein wall protein, versus females, on day 2 (26.840 ± 5.124 vs. 8.770 ± 1.867 pg/mL, $p=0.0132$).

Quantitative (real-time) polymerase chain reaction (qRT-PCR)

P-selectin—Male vein wall P-selectin gene expression demonstrated peaks at 6 hours (+303.5%) and at day 14 (+182.0%) thrombosis, versus male TC. Vein wall P-selectin gene expression was significantly up-regulated at 6 hours, versus male TC (2.0 ± 1.3 vs. 0.50 ± 0.3 gene/ β -actin, $p=0.037$). In female mice there were trends of up-regulated P-selectin gene expression at 6 hours (+1694%) and 6 days (+2624%), versus female TC. [Figure 3A]

Interleukin-1 β (IL-1 β)—In male mice, IL-1 β showed peak gene expression at 6 hours (1510 % increase vs. TC). IL-1 β gene expression was significantly up-regulated at day 14, versus male TC (4.13 ± 1.0 vs. 0.65 ± 0.35 gene/ β -actin, $p<0.05$). In female mice, IL-1 β gene expression exhibited an increased gene expression at 6 hours (+673.9 %) and once again at day 6 (+512.2 %). A significant increase in IL- β expression was noted at 6 hours, versus TC (2.44 ± 0.57 vs. 0.31 ± 0.30 gene/ β -actin, $p<0.05$). [Figure 3B]

Tumor necrosis factor- α (TNF- α)—A trend of decreased TNF- α gene expression at 6 hours (−66%) in male mice versus male TC was seen. Female mice exhibited trends of increased TNF- α at 6 hours (+541%) and at day 6 (+539%), post thrombosis. [Figure 3C]

Vein Wall Morphometrics

In both sexes, vein wall inflammatory cell populations showed an acute to chronic inflammatory response with venous thrombi present. This inflammatory response was characterized by an acute increase in polymorphonuclear (PMN) cells followed by sub-acute and chronic increases in monocyte populations.

Male mice exhibited significant increases in vein wall PMN at day 2 (74 ± 20 vs. 4 ± 1 Cells/5HPF, $p=0.0268$), day 6 (27 ± 7 vs. 4 ± 1 Cells/5HPF, $p=0.0260$), and day 14 (15 ± 3 vs. 4 ± 1 Cells/5HPF, $p=0.0367$), versus TC animals. Peak vein wall PMN counts were observed at 6 hours (91 ± 36) and then declined steadily through the follow up time points. [Figure 4A]

Female mice had a significant increase in vein wall PMN counts at 6 hours, versus female TC (112 ± 26 vs. 11 ± 4 Cells/5HPF, $p=0.0187$). [Figure 4B] No significant differences were observed between male and female mice at any time point for vein wall PMN counts.

Male mice showed significant increases in vein wall MO counts at days 2 (23 ± 3 vs. 3 ± 1 Cells/5HPF, $p=0.0028$), 6 (59 ± 17 vs. 3 ± 1 Cells/5HPF, $p=0.0304$), and 14 (67 ± 11 vs. 3 ± 1 Cells/5HPF, $p=0.0048$), versus male TC. [Figure 4A]

Female mice followed similar recruitment pattern of vein wall MO counts as male mice. In female mice, a significant increase in vein wall MO counts was found at day 6, versus female TC (124 ± 31 vs. 8 ± 1 Cells/5HPF, $p=0.0204$). [Figure 4B]

Female TC had significantly higher vein wall MO counts, versus male TC (8 ± 1 vs. 3 ± 1 Cells/5HPF, $p=0.0047$). Compared to male mice, females demonstrated a two fold increase in vein wall monocytes at day 6 (124 ± 31 vs. 59 ± 17 , cells/5HPFs, ns), and significantly smaller venous thrombi at this time point ($p<0.0001$).

Male mice exhibited significant increases in vein wall LYM counts at day 14, versus male TC (51 ± 5 vs. 25 ± 5 Cells/5HPF, $p=0.0074$). [Figure 4A]

Female mice had a significant increase in vein wall LYM counts at day 6, versus female TC (50 ± 11 vs. 12 ± 2 Cells/5HPF, $p=0.0408$). [Figure 4B]

No significant differences in vein wall LYM counts were noted between male and female mice when direct group comparisons were made.

Male mice showed significant increases in vein wall total inflammatory cells, versus male TC, at days 2 (120 ± 22 vs. 31 ± 6 Cells/5HPF, $p=0.0174$), 6 (129 ± 35 vs. 31 ± 6 Cells/5HPF, $p=0.0501$), and 14 (134 ± 13 vs. 31 ± 6 Cells/5HPF, $p=0.0009$). [Figure 4A]

In female mice, significant increases in total inflammatory cells were found at 6 hours (134 ± 26 vs. 32 ± 4 Cells/5HPF, $p=0.0182$), day 2 (70 ± 9 vs. 32 ± 4 Cells/5HPF, $p=0.0134$) and day 6 (223 ± 27 vs. 32.00 ± 4 Cells/5HPF, $p=0.0023$), versus female TC. [Figure 4B] No significant differences in vein wall total inflammatory cells were found between male and female mice.

Hematology

Peripheral white blood cell (WBC) counts—Peripheral WBC counts in male mice significantly declined at 6 hours, versus male TC (3.56 ± 0.70 vs. 10.53 ± 1.21 K/ μ L, $p=0.0025$). Female mice exhibited the same pattern of WBC decline in the circulation. Male TC had significantly higher WBC counts, versus female TC (10.53 ± 1.21 vs. 5.29 ± 0.37 K/ μ L, $p=0.0475$). On day 14, females had significantly higher WBC counts than males (14.32 ± 0.78 vs. 8.89 ± 1.21 K/ μ L $p=0.0289$). [Table 2]

Peripheral neutrophils (NE)—In male mice there was a significant difference in NE counts between TC and shams (2.01 ± 0.32 vs. 0.32 ± 0.07 K/ μ L, $p=0.0067$). Also, a significant increase in NE counts at day 14 were observed, versus male TC (4.43 ± 0.22 vs. 2.01 ± 0.32 K/ μ L, $p=0.0008$). [Table 2]

Female animals had a significant increase in NE counts at day 14, versus female TC (2.95 ± 0.19 vs. 1.17 ± 0.38 K/ μ L, $p=0.0083$). In comparing the sexes, males had significantly higher NE counts versus females at day 2 (2.25 ± 0.26 vs. 0.76 ± 0.11 K/ μ L, $p=0.0014$) and day 14 (4.43 ± 0.22 vs. 2.95 ± 0.19 K/ μ L, $p=0.0123$). [Table 2]

Peripheral monocytes (MO)—Males had significant declines in MO counts in shams (0.05 ± 0.01 K/ μ L, $p=0.0046$), at 6 hours (0.23 ± 0.05 K/ μ L, $p=0.0282$), and on day 2 (0.18 ± 0.01 K/ μ L, $p=0.0162$), versus male TC (0.47 ± 0.07 K/ μ L). Female mice had a significant increase in MO counts on day 14, versus female TC (0.63 ± 0.04 vs. 0.23 ± 0.06 K/ μ L, $p=0.0025$). Also, female mice had significantly higher MO counts than males at day 14 (2.96 ± 0.19 vs. 0.36 ± 0.08 K/ μ L, $p=0.0201$). [Table 2]

Circulating red blood cells (RBC)—Males, in the sham group, had a significant difference in circulating RBC, versus male TC (10.34 ± 0.10 vs. 8.66 ± 0.35 K/ μ L, $p=0.0025$). Females had significant RBC count increases in the sham group (9.67 ± 0.11 vs. 7.78 ± 0.21 K/ μ L, $p=0.0005$) and at 6 hours (9.43 ± 0.27 K/ μ L, $p=0.0019$), versus female TC. In group comparisons between the sexes, male sham animals exhibited significantly higher RBC counts than female shams (10.34 ± 0.10 vs. 9.67 ± 0.11 K/ μ L, $p=0.0019$). [Table 2]

Circulating platelets (PLT)—Both sexes displayed significant acute decreases in circulating PLT which coincided with active thrombogenesis. Male animals had significant decreases in PLT at 6 hours (340 ± 63 vs. 734 ± 74 K/ μ L, $p=0.0049$) and 2 days (395 ± 89 vs. 734 ± 74 K/ μ L, $p=0.0233$), versus male TC. There was a significant increase in circulating PLT in male mice at day 6, versus male TC (1025 ± 62 vs. 734 ± 74 K/ μ L, $p=0.0197$).

Female mice displayed a significant decrease in circulating PLT at 6 hours, versus female TC (393 ± 54 vs. 621 ± 11 K/ μ L, $p=0.0140$). In direct gender comparisons, males had

significantly higher PLT counts than females at day 6 (1025 ± 62 vs. 820 ± 72 K/ μ L, $p=0.0230$). [Table 2]

Mouse Soluble P-Selectin (sP-Sel)

Male mice demonstrated significant increases in sP-Sel at 6 hours (401.148 ± 18.060 vs. 162.161 ± 10.939 pg/mL, $p<0.0001$), day 2 (259.638 ± 13.810 vs. 162.161 ± 10.939 pg/mL, $p=0.0002$), and day 6 (333.091 ± 31.930 vs. 162.161 ± 10.939 pg/mL, $p=0.0005$), versus male TC. Peak sP-Sel levels were observed in male mice at 6 hours (401.148 ± 18.060 pg/mL) and 6 days (333.091 ± 31.930 pg/mL), versus male TC (162.161 ± 10.939 pg/mL). [Figure 5A]

Females had increased sP-Sel levels in shams (166.255 ± 10.618 vs. 133.102 ± 5.0192 pg/mL, $p=0.0370$), at 6 hours (424.611 ± 34.463 vs. 133.102 ± 5.019 pg/mL, $p<0.0001$), and on days 2 (238.725 ± 11.539 vs. 133.102 ± 5.019 pg/mL, $p<0.0001$), 6 (263.868 ± 20.546 vs. 133.102 ± 5.0192 pg/mL, $p=0.0003$) and 14 (201.152 ± 21.211 vs. 133.102 ± 5.019 pg/mL, $p=0.0097$), versus female TC. [Figure 5B]

When comparing males to females, there was a significant increase in sP-Sel levels at day 6 (333.091 ± 31.930 vs. 263.868 ± 20.546 pg/mL, $p=0.0362$).

Microparticle Tissue Factor (MP-TF) Activity Assay

Male mice showed significant increases in MP-TF in shams (4.09 ± 1.10 vs. 0.47 ± 0.18 pg/mL, $p=0.0175$) and at 6 hours (4.96 ± 1.13 vs. 0.47 ± 0.18 pg/mL, $p=0.0044$), versus male TC. Peak levels of MP-TF were observed at 6 hours and day 6. [Figure 6A]

In females, an increase in MP-TF was seen at 6 hours, versus female TC (6.92 ± 1.60 vs. 2.21 ± 0.45 pg/mL, $p=0.0216$). [Figure 6B]

No significant differences were observed between male and female mice at any time point for MP-TF activity.

Discussion

Deep vein thrombosis is a serious health care problem that remains prevalent in our society despite all of the advances made in medical treatments throughout the past 25 years (13–14). It has been reported that the annual number of pulmonary embolism related deaths may exceed myocardial infarction and stroke related deaths in the United States (1).

There have been several patient studies investigating the differences in VTE among genders (15). Some of these published studies found a similar incidence of first VTE among the genders, while others have reported that females might be protected by their hormones for VTE (15–17). One mouse study found that male mice were more susceptible to thrombosis because of differences in sex-specific growth hormone secretion patterns. (18) Our current study paralleled this finding with the male mice having significantly larger thrombi than female mice.

We measured protein and gene expression of P-selectin, IL-1 β , and TNF- α in the IVC vein wall pre and post thrombotic stimulus (EIM). The role of P-selectin has been well characterized in male mice however the role of IL-1 β and TNF- α , in this disease, has rarely been addressed. Levels of P-selectin and IL-1 β vein wall protein peaked at 6 hours in both sexes. P-selectin, which is located in the α -granules of platelets, and the Weibel-Palade bodies of endothelial cells, plays a key role in leukocyte and platelet interactions, post vascular stimulation (9,19–20).

A previous study using the IVC ligation model with P-selectin knockout mice demonstrated P-selectin to be important in early neutrophil vein wall extravasation (9). IL-1 β and TNF- α are both pro-inflammatory cytokines. IL-1 β is produced by dendritic cells and macrophages and TNF- α is produced by endothelial cells and macrophages. IL-1 β and TNF- α are important mediators of the acute phase response to inflammation. Elevations in both vein wall P-selectin and IL-1 β proteins, in the mice, suggest an inflammatory response to endothelial activation leading to thrombogenesis.

As the thrombus resolves, pro-inflammatory factors, including IL-1 β and TNF- α are released (22). Both sexes were observed to have peaks in vein wall IL-1 β protein at 6 hours and at 14 days, post thrombosis. In both sexes, vein wall TNF- α protein was significantly decreased at 6 hours and then returned to baseline at day 14. Future experiments in this laboratory will attempt to clarify the role that sex has on the response of the endogenous pyrogen, TNF- α , during venous thrombosis.

TNF- α is a potent mediator of inflammation that promotes monocyte and leukocyte recruitment. Little is known of this endogenous pyrogen's effects on an *in vivo* flow model of venous thrombosis. Of interest, in the current study, male mice exhibited a decrease in TNF- α gene expression at 6 hours while female mice exhibited increases in TNF- α gene expression at 6 hours and at day 6. These findings are in accordance with other reports which describe sex steroid dependant modulation of TNF- α production, suggesting that testosterone suppresses proinflammatory cytokines (21–24). One study in mice concluded that sexual dimorphism in experimental superantigen toxic shock results from increased systemic TNF- α in females and was reduced by tamoxifen pretreatment (21). Another study reported injection of bacterial endotoxin into castrated mice increased endogenous production of TNF- α , which was abrogated by testosterone replacement (25). Furthermore, clinical studies found that testosterone may suppress expression of proinflammatory cytokines, including TNF- α (22).

In this study, day 6 female had a two-fold increase (+109%) in monocyte vein wall counts vs. males (123.8 ± 31.00 vs. 59.20 ± 17.23 , cells/5HPFs, ns). Previous research has determined that monocytes facilitate thrombus resolution (26–27). Thus, increased vein wall monocyte extravasation in female mice, post thrombosis, may serve as a protective mechanism against venous thrombosis. The evaluation of female mice, that have had their ovaries removed and are then treated with testosterone, or neutered males, that no longer have high levels of testosterone to determine how hormonal differences play a role in DVT is warranted.

Post sham injury in both sexes produced a transient increase in the number of red blood cells that was significant. Overall, male mice acute erythrocytosis was increased versus female mice but with no biological effect noted. Peripheral platelets in our study declined significantly from baseline at 6 hours (both genders) and day 2 (males) and then peaked at day 6 (both genders). The initial decline in the circulating platelets showed an inverse relationship to thrombus weight. This suggests that platelets may be sequestered during thrombogenesis. An acute to chronic inflammatory response was demonstrated in the thrombosed IVC vein walls of both sexes. Early inflammation was observed with peak vein wall neutrophils at 6 hours followed by a peak in vein wall monocytes at day 6 (female) and 14 (male). This observation follows the previously described findings in a mouse model of complete stasis venous thrombosis (9).

Soluble P-selectin has been found in both mice and humans. Elevated levels of soluble P-selectin are found in thrombotic consumptive disorders (20,28). In this study, we noted significant increases in soluble P-selectin at 6 hours, days 2, 6 (both sexes) and 14 (females) versus non-thrombosed animals. Significant decreases in soluble P-selectin were detected in

both sexes at day 14 versus day 6 as the thrombi resolved. These findings may suggest that soluble P-selectin may be a good biomarker for the detection of venous thrombotic disorders in mice.

Recent studies have demonstrated that tissue factor bearing microparticles (MP) have a procoagulant role in thrombogenesis (29–33). Selectin upregulation is associated with the formation of microparticles. MPs derived from endothelial cells induce monocyte tissue factor antigen release and increased expression (29–30). Tissue factor is present in the sub-endothelium and in the circulation. EIM introduces a needle through the vein wall that likely influenced early MP-TF activity in the male sham group. MP-TF activity significantly increased at 6 hours in both sexes.

In conclusion, male mice had larger venous thrombi than age-matched female mice at all time points. Anatomically, female mice had uncompromised uterine venous flow to the IVC which may have limited venous thrombi progression. Males had significantly increased vein wall interleukin-1 β , versus females, at 6 hours and day 6. Males also showed decreased tumor necrosis factor- α expression at 6 hours, while females had increased tumor necrosis factor- α expression at 6 hours and day 6. Both male and female mice demonstrated similar vein wall inflammatory cell responses to thrombogenesis. This is the first study to evaluate sex differences in thrombus formation using a non-occlusive mouse flow model of venous thrombosis.

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Abbreviations

EIM	electrolytic inferior vena cava model
IVC	inferior vena cava
TW	thrombus weight
IL-1β	interleukin 1- β
TNF-α	tumor necrosis factor- α
sP-Sel	soluble P-selectin
MP-TF	plasma microparticle tissue factor
PMN	polymorphonuclear
NE	neutrophils
MO	monocytes
LYM	lymphocytes
qRT-PCR	quantitative real time polymerase chain reaction
ns	non-significant

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Figure 1. Mouse Venous Anatomy Slide

Mouse Venous Anatomy - Male on the left, female on the right. Similar anatomy was found in both genders of mice with the exception of the right uterine vein that usually drains its contents into the IVC. Arrow indicates the right uterine vein as it enters the inferior vena cava.

Figure 2A and Figure 2B. Male and Female Thrombus Weight Composite

Thrombus Weight Composite - stars indicate significant differences, versus true controls (n=10 for true control and sham groups, n=20 for each time point group). TC= True Controls, hr=hours, D=days, post EIM. Males had significantly higher thrombus weight at days 2, 6, and 14, versus females.

Figure 3A, Figure 3B, and Figure 3C. Quantitative (real time) PCR

Quantitative (real time) PCR - star indicate significant differences, versus true controls (n=5 for true control and sham groups, n=10 for each time point group). TC= True Controls, hr=hours, D=days past EIM.

Figure 4A and Figure 4B. Vein Wall Morphometrics Composite

Vein Wall Morphometrics - average cell counts for each time point; stars indicate significant differences, versus true controls (n=5 for true control and sham groups, n=10 for each time point group). TC= True Controls, hr=hours, D=days past EIM, PMN= polymorphonuclear cells, MO=monocytes, TIC= total inflammatory cells, LYM= lymphocytes. No significant differences were found for vein wall PMN, LYM, or TIC between the sexes. Female true controls had significantly higher vein wall MO, versus male true controls.

Figure 5A and Figure 5B. Soluble P-selectin

Plasma Soluble P-Selectin - stars indicate significant differences, versus true controls (n=5 for true control and sham groups, n=10 for each time point group). TC= True Controls, hr=hours, D=day. Males had significantly increased plasma soluble P-selectin at day 6, versus females.

Figure 6A and Figure 6B. Plasma Tissue Factor Microparticles (MP-TF) Composite
 Plasma Tissue Factor Microparticles (MP-TF) - stars indicate significant differences, versus true controls (n=5 for true controls and sham groups, n=10 for each time point group). TC= True Controls, hr=hours, D=days post EIM. No significant differences in MP-TF were noted between the sexes.

Table 1**Animal Groups**

Animal Groups, n= number of animals in each group. TC= True Control, D= days.

Group	n (per gender)	Tissue evaluation	Blood/Plasma evaluation
Control	5	Thrombus Weight & ELISA	sP-Selectin
Sham	5		
6hr	10		
2D	10		
6D	10		
14D	10		
Control	5	Thrombus Weight & qRT-PCR	Tissue Factor Microparticles
Sham	5		
6hr	10		
2D	10		
6D	10		
14D	10		
Control	5	Vein Wall Morphometrics	Hematology
Sham	5		
6hr	5		
2D	5		
6D	5		
14D	5		

Hematology Data

Table 2

Hematology Data, displayed as ranges and (mean), WBC= White Blood Cells, NE= Neutrophils, MO= Monocytes, LYM= Lymphocytes, RBC= Red blood cells. Bold numbers and arrows indicate a significant increase/decrease ($p \leq 0.05$), versus true controls.

C57BL/6 Male (6–8 weeks)							
	WBC (K/ μ L)	NE #/%	MO#/%	LYM#/%	RBC (M/ μ L)	HCT %	PLT (K/ μ L)
True Control	7.00 – 14.00 (10.53)	1.23 – 3.05 (2.01)/ 18.76	0.30 – 0.66 (0.47)/ 4.49	5.40 – 9.95 (7.75)/ 74.08	7.60 – 9.68 (8.66)	32.50 – 46.60 (37.34)	587 – 969 (734)
Sham	↓1.56 – 6.38 (3.012)	↓0.19 – 0.50 (0.32)/ 16.94	↓0.03 – 0.66 (0.05)/ 2.22	↓1.30 – 4.59 (2.35)/ 80.34	↓10.12 – 10.51 (10.34)	42.50 – 45.40 (43.85)	662 – 937 (828)
6 hours	↓1.48 – 5.18 (3.56)	0.50 – 2.78 (1.69)/ 44.92	↓0.10 – 0.37 (0.23)/ 7.48	↓0.78 – 2.55 (1.60)/ 46.59	5.19 – 9.87 (7.71)	22.00 – 48.80 (36.12)	↓189 – 554 (340)
Day 2	4.54 – 10.98 (7.224)	1.28 – 2.70 (2.25)/ 31.80	↓0.16 – 0.20 (0.18)/ 3.02	↓3.10 – 7.87 (4.73)/ 64.79	7.020 – 10.15 (8.37)	30.80 – 44.20 (36.54)	↓169 – 586 (395)
Day 6	5.94 – 21.46 (12.49)	1.35 – 8.07 (5.10)/ 41.81	0.10 – 1.12 (0.55)/ 3.75	3.03 – 11.34 (6.53)/ 52.67	8.06 – 9.51 (8.73)	33.80 – 38.80 (36.24)	↓884 – 1201 (1025)
Day 14	6.86 – 13.38 (8.89)	3.77 – 4.73 (4.43)/ 45.68	0.13 – 0.56 (0.36)/ 3.97	2.47 – 7.52 (4.55)/ 48.67	7.46 – 11.13 (8.98)	33.10 – 51.40 (40.16)	461 – 1052 (800)

C57BL/6 Females (6–8weeks)							
	WBC (K/ μ L)	NE #/%	MO#/%	LYM#/%	RBC (M/ μ L)	HCT %	PLT (K/ μ L)
True Control	4.26 – 6.04 (5.29)	0.59 – 2.59 (1.17)/ 16.85	0.10 – 0.33 (0.23)/ 4.41	3.37 – 8.14 (4.91)/ 76.00	7.33 – 8.58 (7.78)	31.10 – 37.60 (33.52)	589 – 634 (621)
Sham	↓2.68 – 5.34 (3.85)	0.33 – 0.86 (0.53)/ 13.39	0.05 – 0.24 (0.12)/ 2.91	2.25 – 4.17 (3.17)/ 82.97	↓9.39 – 9.88 (9.67)	↓39.40 – 43.00 (41.24)	482 – 833 (687)
6hours	↓3.32 – 3.82 (3.61)	0.96 – 3.07 (1.95)/ 46.43	0.07 – 0.52 (0.22)/ 5.47	↓0.98 – 3.26 (1.99)/ 46.34	↓8.62 – 9.99 (9.43)	↓36.90 – 42.40 (39.90)	↓240 – 525 (393)
Day 2	3.06 – 6.06 (4.42)	0.38 – 0.95 (0.756)/ 17.07	0.09 – 0.19 (0.14)/ 3.19	2.45 – 4.96 (3.52)/ 79.47	5.96 – 9.02 (7.66)	25.30 – 43.30 (34.60)	416 – 632 (551)
Day 6	4.22 – 7.62 (5.74)	0.90 – 2.52 (1.526)/ 25.90	0.28 – 0.33 (0.310)/ 5.718	2.63 – 5.30 (3.80)/ 66.44	6.70 – 8.61 (7.78)	32.80 – 35.30 (33.86)	617 – 949 (820)
Day 14	↓11.74 – 16.48 (14.32)	↓2.53 – 3.60 (2.95)/ 20.89	↓0.52 – 0.79 (0.63)/ 4.47	↓7.68 – 12.69 (10.19)/ 70.71	6.65 – 9.12 (7.68)	26.90 – 40.70 (32.10)	515 – 923 (723)