Colchicine Does Not Reduce Abdominal Aortic Aneurysm Growth in a Mouse Model

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1. Introduction

Surgical repair is the only current treatment for abdominal aortic aneurysms (AAA) [1]. In order to discover AAA drugs, a number of rodent models have been developed [1]. Previous animal studies have mainly used the angiotensin II, intraluminal elastase, or calcium chloride models [1]. These models simulate some but not all pathological characteristics of human AAA [1]. A major limitation of these models is that increase in aortic diameter is limited to 1-2 weeks meaning that they are not suited to testing the effect of drugs on growth of established aneurysms [1]. A novel mouse model in which AAA is induced by topical application of elastase to the infrarenal aorta (IRA) and
oral administration of 3-aminopropionitrile fumarate salt (E-BAPN) has recently been reported [2]. AAAs in this model have been reported to grow slowly for at least 100 days [2]. Aneurysms have many features of human AAA including focal expansion of all layers of the aortic wall, intimal hyperplasia and elastolysis, [6, 7].}

The effects of drugs on aneurysm growth. Two day after commencing the administration of colchicine or vehicle, AAAs were induced in 60 mice, and these mice were randomly assigned to receive either sham or AAA induction surgery. For aim one, the E-BAPN AAA model was validated through two experiments. A short-term study was conducted whereby 9 mice were randomly allocated to receive colchicine (n = 3, 0.2 mg/kg/d, Sigma, Victoria) or vehicle control (n = 3) for 7 days in order to examine acute effects of colchicine. Two days after commencing the administration of colchicine or vehicle, AAAs were induced and mice were culled 5 days later for RNA-sequencing analysis of aortic tissue (Supplementary figure 1 and 2). In a longer-term study, AAAs were induced in 60 mice, and these mice were randomly allocated 21 days later to receive colchicine (n = 28, 0.2 mg/kg/d) or vehicle control (n = 29) for a further 69 days (Supplementary figure 1 and 2).

2.2. Study Design. Sham and AAA induction surgeries were conducted when mice were eight weeks old [2]. Body weight was measured weekly. The study was divided into two aims.

For aim one, the E-BAPN AAA model was validated through two experiments. A short-term study was conducted where mice were culled 5 days after AAA induction with E-BAPN (n = 3) or sham surgery (n = 3), and the IRA was assessed for differences in gene expression with an RNA-sequencing analysis. In a longer-term study, mice were culled 90 days after AAA induction with E-BAPN (n = 29) or sham surgery (n = 13) to examine AAA growth, NLRP3 activity, and ILT formation (Supplementary figure 1 and 2).

2.2.1. Mice. Seven-week-old male C57BL/6J mice were purchased from the animal resources centre (Western Australia) and acclimatised for one week at the James Cook University animal facility. Mice were maintained on a 12 hour light/dark cycle, at a relative humidity of 55 ± 2% and a temperature of 23 ± 2°C. Prior to experiments, mice were maintained on normal laboratory chow and water ad libitum. Ethics approval was obtained from the James Cook University Animal Ethics Committee, and experiments were conducted according to the NHMRC Australian code for the care and use of animals for scientific purposes guidelines for animal care and maintenance (2013). Research was reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0 [12]. Cotton, shredded paper, and cardboard tubes were provided as environmental enrichment for all mice.
2.5. Measurement of Tissue IL-1β Concentration and Caspase-1 Activity. The primary outcome for aim one was IRA IL-1β concentration, which was measured using a fluorometric ELISA (Catalog number - ab229384, Abcam, Victoria) performed according to manufacturer’s instructions. Relative caspase-1 activity was detected using a Caspase-Glo 1 Inflammasome Assay (Catalog number - G9951, Promega, NSW) according to the manufacturer’s instructions and measured using a Polarstar Omega plate reader.

2.6. Ultrasound Assessment of IRA Diameter. The primary outcome for aim two was AAA growth measured over time from outer to outer wall in the anterior-posterior orthogonal plane during late systole using a 10 MHz linear ultrasound transducer probe (Esaote, Italy) attached to a MyLab 30 ultrasound machine (Esaote, Italy), as previously described [17]. Diameters were measured at baseline (day 0) and 7, 21, 28, 42, 63, and 80 days after AAA induction. The intraobserver and interobserver reproducibility of IRA diameter measurement was assessed from ten repeats, and the coefficients of variation (CoV) were 4.4% and 7.2%, respectively.

2.7. Other Methods. The methods used for assessment of AAA diameter by morphometry, rupture, aneurysm severity, histology, immunofluorescence, RT-PCR, and RNA-sequencing are included in supplementary materials.

2.8. Sample Size Calculations. The required sample size for aim one was estimated based on aortic tissue IL-1β reported in a previous study in the angiotensin II model (vehicle 32 ± 14 pg/100 μg protein, AAA 164 ± 115 pg/100 μg protein, mean ± standard deviation) [6]. Based on these values, at least 8 mice were required at the experimental endpoint to test if IL-1β was upregulated (80% power, alpha 0.05, two-tailed). For aim two, the sample size was based on testing the hypothesis that AAA growth would be 20% lower in mice receiving colchicine compared to vehicle control [18]. A previous study reported that control mice undergoing the same AAA induction surgery exhibited a mean increase in IRA diameter of 800 ± 160% after 100 days [2]. Assuming a similar outcome for the control group in the current study and equal variance in both groups, a minimum sample size of 17 mice per group would be required to test if colchicine reduced AAA growth (power 80%, alpha 0.05). Sample sizes were increased by 80% to account for potential losses during the longer-term experiments.

2.9. Statistical Analyses. Data were tested for normal distribution with D’agostino and Pearson’s test. Data that were normally distributed were analysed using unpaired two-tailed t-tests and presented as means ± standard error of means (SEM), and data not normally distributed were analysed using Mann-Whitney U tests and presented as median with interquartile range (IQR) and individual values. Intraluminal thrombus (ILT) presence was compared using Fisher’s exact test, and AAA rupture was compared using the Mantel-Cox (log-rank) test. AAA growth analyses included all ultrasound data obtained up until the point of death and was performed using random slope and random intercept linear mixed effects models. Group allocation was treated as a fixed effect; mouse and time were included as random effects. The test statistic was interaction of time and intervention. Model fit was assessed by examination of residual distribution and q-q-norm plots. Where necessary, the response variables were log transformed to adhere to model assumptions of linearity.

2.10. Bioinformatics. Log transformed data were used to calculate p values in cases where it improved model fit; however, nonlog transformed values were reported to make data easier to interpret. RNA-sequencing FastQ files were aligned to reference genome GRCh38.p13 using STAR [19] and underwent quality control, normalisation, and differential expression analysis using the bioconductor consensus DE package [20]. Preranked Gene Set Enrichment Analyses (GSEA) were performed with log fold change values using gene ontology (GO) biological pathways (Baderlabs) up to date as of 1 September 2021, and the results were visualised as enrichment maps using cytoscape 3.8.2 with enrichment map app. The genes were collapsed to human orthologs for all analyses and presented as gene symbols. Differences were considered statistically significant when p values were <.05, or when q values were <.1 for GSEA of RNA-sequencing data. Statistical analyses were performed using GraphPad Prism V.6, GSEA desktop 4.1.0, and R Studio 4.1.2.

3. Results

3.1. Characteristics of the E-BAPN Model. Increase in IRA diameter determined by ultrasound was significantly greater in mice receiving E-BAPN compared with sham operated mice over an 80 day period (mean difference, MD, 4.3 mm, 95% CI 3.3, 5.3, p < .001). Ex vivo morphometry data at day 90 showed significantly greater IRA diameter in E-BAPN compared to sham operated mice (median, 4.18 mm, IQR, 2.84-5.49 versus 0.60 mm, 0.51-0.84, p < .001, Figures 1(a) and 1(b)). After 90 days, AAs were present in all (100%) mice receiving E-BAPN, and no AAs were observed in the sham mice (Figure 1(c) and Supplementary figure 3, p < .001). Aortic elastin degradation was present in all (100%) mice receiving E-BAPN including severe fragmentation and areas of complete destruction in 12 mice (50%); however, no elastin degradation was detected in sham mice (Figure 1(d)). Seventeen mice (23%) from the experimental groups died between the start of the experiment and sacrifice. Six mice receiving E-BAPN died due to aortic rupture, including three ruptures in the suprarenal aorta and three in the IRA. Eleven mice died due to causes unrelated to aneurysm rupture (Supplementary table 1). A summary of key features of the E-BAPN model compared to other AAA models are shown in supplementary table 2.

3.2. Genes Differentially Expressed between E-BAPN AAA Mouse Model versus Sham Mice after 7 Days. Data of 15,354 known genes, including 8221 that were differentially expressed between E-BAPN AAA mice and sham mice, were included in pathway analyses. Out of 14,214 listed biological pathways, 893 were significantly upregulated and 277 were
significantly downregulated in E-BAPN AAA mice compared with sham controls \((q < 0.1)\). The GSEA showed that the most substantially upregulated pathways were inflammatory and most downregulated pathways were related to muscle contraction (Figure 2 and supplementary table 3). Heat maps of overall gene expression and within the most upregulated pathway (positive regulation of leukocyte activation) showed upregulation of proinflammatory genes including those related to the NLRP3 (supplementary figure 4). The potent neutrophil chemoattractant and activator granulocyte chemotactic protein 2 \((CXCL6; \text{fold difference, } FD: 10.35; p < 0.001)\) and IL-6 \((FD: 8.32; p < 0.001)\) were the most highly differentially expressed proinflammatory genes (Supplementary figure 4). The significant upregulation of inflammasome genes in AAA samples suggesting this pathway was an important target to limit growth of small AAAs (Supplementary table 4). In addition, there was a significant upregulation of 9 genes and significant downregulation of 6 genes expressed in myeloid cells in AAA samples compared to sham controls (Supplementary table 5).

3.3. IRA Caspase-1 Activity and IL-1β Concentration in E-BAPN AAA Mice Model versus Sham Mice after 90 Days. Aortic caspase-1 activity \((109, 83-155 \text{ relative luminosity units (RLU) versus } 45, 38-64.5 \text{ RLU}, p < .001, \text{Figure 3(a)})\) and IL-1β concentration \((15.67, 7.11-22.60 \text{ pg/mg protein versus } 6.87, 4.54-11.60 \text{ pg/mg protein, } p < .050, \text{Figure 3(b)})\) were greater in aortic tissue samples of E-BAPN AAA mice compared with sham controls at day 90.

3.4. Effect of Colchicine in E-BAPN AAA Mice versus Vehicle Treatment on Growth of Established AAAs Assessed over 80 Days. Colchicine did not significantly reduce AAA growth over an 80-day period compared to vehicle control as measured by ultrasound \((\text{MD} -0.1 \text{ mm, 95% CI -1.1, 0.86, } p =\)
There was no significant difference in ex vivo AAA diameters between mice receiving colchicine and mice receiving vehicle on day 90 (4.0 mm, 2.9-5.0 versus 4.2 mm, 2.8-5.5, \( p = .713 \), Figure 4(b)). AAAs were present in all mice and considered severe in 20 (90%) mice receiving colchicine compared with 21 (91%) mice in the control group on day 90 (Figure 4(c) and Supplementary figure 5).

Elastin degradation was present in all aortic samples of mice receiving either colchicine or vehicle control, with severe fragmentation and areas of complete destruction in 12 (53%) mice receiving colchicine compared with 12 (50%) in the vehicle control mice at the endpoint (Figure 4(d)). Aneurysm rupture occurred in one mouse from the colchicine group (4%) and three mice from the control group (10%, \( p = .730 \), Figure 4(e)). Of mice reaching the experimental endpoint, ILT was present in 9 of 22 (41%) mice receiving colchicine and 14 of 23 (61%) control mice (Figure 4(f), \( p = .238 \)).
3.5. Effect of Colchicine in E-BAPN AAA Mouse Model versus Vehicle Treatment on Inflammatory Pathway Expression in the IRA after 7 Days. Data of 15,354 known genes, including 620 that were differentially expressed between mice receiving colchicine and mice receiving vehicle control, were included in pathway analyses. Out of 14,214 listed biological...
pathways, one pathway, associated with oxidative demethylation, was significantly upregulated, and no pathways were downregulated (q < 0.1). The aortic expression of inflammasome genes was not downregulated in mice receiving colchicine by comparison to vehicle controls. These results suggest that colchicine did not block the inflammasome in the short term (Supplementary table 4).

3.6. Effect of Colchicine in E-BAPN AAA Mice versus Vehicle Treatment on Caspase-1 Activity and Inflammatory Cytokine Concentrations or RNA Expression after 90 Days. IRA caspase-1 activity was significantly lower in mice receiving colchicine compared with vehicle control (79, 63-106 RLU versus 109, 83-155 RLU, p = .047. Figure 5(a)). IRA IL-1β protein concentration (10.03, 4.25-14.73 pg/mg protein versus 15.67, 7.11-22.60 pg/mg protein, p = .174) was not significantly reduced in mice receiving colchicine compared with vehicle control (Figure 5(b)). Similarly, IL-1β (0.63, 0.16-1.58 fold change versus 0.96, 0.45-2.30 fold change, p = .397), TNF-α (0.47, 0.37-1.55 versus 1.25, 0.71-1.45, p = .380), IFN-γ (0.96, 0.57-1.28 versus 1.27, 0.68-1.59, p = .346), and IL-18 (0.91, 0.67-3.29 versus 1.11, 0.60-2.52, p = .828) RNA expression were not significantly reduced in mice receiving colchicine compared with controls (Figure 5(c)). IRA percentage area stained for CD3 T cells (0.81, 0.24-2.05% versus 0.23, 0.10-3.37%, p = .005), CD68 macrophages (1.09, 0.44-13.94% versus 0.67, 0.40-6.68%, p = .478), and collagen (12.06, 8.31-18.69% versus 8.69, 6.08-10.59%, p = .068, Figure 5(d)) were not significantly different between mice receiving colchicine and mice receiving vehicle control.

4. Discussion

This study suggests that the E-BAPN model is an effective new model for studying the longer-term effects of drugs on AAA growth [2]. This model exhibits all key characteristics of human AAA that the three main other mouse models do not (Supplementary table 2). In this study, we showed, like other AAA mouse models and in patients, that activity of the NLRP3 inflammasome mediator caspase-1 was elevated with concomitant increases in IL-1β concentrations within aneurysmal tissue [21]. This activation of the inflammasome was demonstrated in both short-term transcriptomic analyses and long-term biochemical analyses using aortic inflammation.
samples. Transcriptomic analyses using aortic samples also showed significant upregulation of other inflammatory pathways within the aortas of the E-BAPN model in keeping with prior research implicating inflammation in AAA pathogenesis (Supplementary table 3) [22, 23].

Treatment with colchicine for 80 days significantly reduced caspase-1 activity, a marker of NLRP3 inflammasome activity; however, this did not result in decreased aortic IL-1β concentration and did not significantly reduce AAA growth or rupture. Colchicine did not effectively block the upregulation of aortic inflammation in the short term based on the transcriptomic analyses suggesting that the blockade of inflammasome takes place over long-term treatment.

ILT is a consistent feature of human AAA and ILT presence, and size has been correlated with early rupture and increased AAA growth rates [24, 25]. Over half of control mice (61%) had ILT present, and these ILT had high abundance of CD3 T cells and CD68 macrophages compared to surrounding aortic tissue, similar to that reported for human AAA [26]. The occurrence of ILT and relative CD3 T cell and CD68 macrophage accumulation was not significantly different between mice receiving colchicine and vehicle control in the current study.

Low-dose colchicine (0.5-1.0 mg/day, comparable to current study based on body surface area translation) has been shown to reduce cardiovascular events in patients with coronary heart disease [11]. The dose of colchicine used in this study was chosen to mimic the low dose used in clinical trials and to minimise side effects and did not significantly reduce tissue IL-1β concentrations. Colchicine had a small effect on caspase-1 activity and significantly upregulated the oxidative demethylation pathway which appears to be related to colchicine metabolism [27]. A higher dose of colchicine could have potentially suppressed IL-1β concentrations in the long term and inflammasome in the short term which could have had different effects on AAA growth. However, higher doses of colchicine may not be feasible to translate to patients as a long-term treatment to limit AAA growth. The documented poor tolerance of patients to higher colchicine doses limits the clinical relevance of testing this [28].

The study design and AAA model used in this study have a number of strengths. Aneurysms produced by this methodology are true aortic dilatations as opposed to dissecting false aneurysms and share key features of the human disease including involvement of the IRA, presence of ILT, T cells and macrophages, decreased vascular smooth muscle cells and collagen deposition within the aortic wall, and increased risk of rupture [2, 26, 29]. The design of the current study also simulates the clinical disease, whereby colchicine was administered 21 days after aneurysms were established. The use of BAPN inhibits crosslinking of elastin and collagen, and currently, no intervention has been shown to limit AAA growth in this model [2, 30]. This is reflective of the negative findings of clinical trials, and it is possible that this model is more realistic to identify translatable therapies although this remains to be confirmed [1]. Limitations of this study should also be acknowledged. Colchicine did not effectively block all elements of the inflammasome. We based the dose of colchicine used in this study on that found to downregulate proinflammatory cytokines, limit the NLRP3 inflammasome, improve cardiac function, and increase survival after myocardial infarction in a past study [16]. The dose used was also calculated to be equivalent to that which has been shown to be safe and well tolerated in patients, effective in limiting cardiovascular events and used as a treatment for gout [14]. Transcriptomic analyses showed that a number of inflammasome-associated genes were significantly differentially expressed in the aneurysm model compared to sham controls, and the major over- and underexpressed genes are reported in supplementary table 4. However, colchicine only downregulated caspase-1 in the long term but not short term when compared to vehicle controls.

In conclusion, the E-BAPN model shows evidence of NLRP3 inflammasome activation. Despite this, administration of colchicine, an inhibitor of the NLRP3 inflammasome, did not limit AAA growth or inflammation observed in the E-BAPN model.

Data Availability

Data are deposited in the repository maintained by James Cook University. It will be available on request after approval from the university.

Disclosure

JG holds a practitioner fellowship from the NHMRC (1117601) and a senior clinical research fellowship from the Queensland Government. Funding sources were not involved in the design, collection, analysis or interpretation of data included in the manuscript. The authors are solely responsible for this paper and its final contents.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

James Phie was involved in the project administration, data curation, conceptualization, formal analysis, funding acquisition, investigation, methodology, validation, visualisation, writing, and editing. Jonathan Golledge was involved in the project administration, conceptualization, funding acquisition, investigation, methodology, supervision, writing, and editing. Shivshankar Thanigaimani was involved in the project administration, data curation, formal analysis, investigation, methodology, validation, and editing. Pacific Huynh was involved in the project administration, investigation, methodology, validation, and editing. Raghuveeran Anbalagan was involved in the project administration, methodology, data curation, and investigation. Matt field was involved in the methodology, data curation, formal analysis, and editing. Corey Moran was involved in the investigation, methodology, supervision, validation, and editing. Robert Kinobe was involved in the investigation, methodology,
supervision, and editing. Joseph V. Moxon was involved in the formal analysis, methodology, supervision, validation, and editing. Smriti M. Krishna was involved in the methodology, supervision, and editing.

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Supplementary Materials

Supplementary figure 1: experimental plan of all 6 groups included in the two experiments, including timing of surgery and intervention. Supplementary figure 2: modified consort flow diagram of mice included in the short-term and longer-term studies. Supplementary figure 3: photographs of aortas at study endpoint on day 90. (A) Aortas from sham mice. (B) Aortas from E-BAPN mice. Supplementary figure 4: heat maps of the most highly differentially expressed genes in sham and E-BAPN IRA on day 5. (A) Heat map of the top 10 most over and under expressed genes overall. (B) Heat map of the top 10 most over- and underexpressed genes in the positive regulation of leukocyte activation pathway. NLRP3 is highly differentially expressed indicating activation of the NLRP3 inflammasome on day 5. Supplementary figure 5: photographs of aortas at study endpoint on day 90. (A) Aortas from mice receiving E-BAPN and vehicle control. (B) Aortas from mice receiving E-BAPN and colchicine intervention. Supplementary figure 6: representative immunofluorescence images of control, colchicine treatment, and sham groups. Supplementary table 1: deaths during experimental period. Supplementary table 2: representation of the key features of human AAA that are present in animal models. Supplementary table 3: top 10 most upregulated and downregulated GO biological pathways in mice receiving E-BAPN compared with sham controls determined by GSEA. Supplementary table 4: table showing the inflammasome-associated genes in vehicle and colchicine IRA on day 7 compared to vehicle-treated mice. Supplementary table 5: the aortic expression of myeloid cell associated genes in mice receiving vehicle control and colchicine for 7 days by comparison to vehicle controls. (Supplementary Materials)

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