Common anti-hemostatic medications increase the severity of systemic infection by uropathogenic *Escherichia coli*

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Abstract

Uropathogenic *Escherichia coli* (UPEC) causes urinary tract infections that can result in sepsis. The hemostatic system is protective in the pyelonephritis stage of ascending UPEC infection, but the role of the hemostatic system has not been investigated during sepsis. Here we utilize a zebrafish-UPEC systemic infection model to visualize infection-induced coagulation and examine the effects of commonly prescribed anti-hemostatic medications on the infection severity. Treatment of systemically infected zebrafish with warfarin, aspirin, or ticagrelor reduced host survival, while stabilization of clots with aminocaproic acid increased host survival. Anti-hemostatic drug treatment increased UPEC burden. Our findings provide evidence that commonly prescribed anti-hemostatic medications may worsen the outcome of severe UPEC infection.

Keywords

Infection, coagulation, thrombocyte, uropathogenic *Escherichia coli*, zebrafish

Background

Uropathogenic *Escherichia coli* (UPEC) is the primary pathogen responsible for urinary tract infection (UTI), which is among the most common bacterial infections worldwide. UPEC most commonly initiates self-limiting cystitis inside the urinary tract, but can ascend the ureters to the kidneys as acute pyelonephritis and potentially progress into sepsis [1, 2].

The hemostatic system plays an evolutionarily conserved roles in both immune control of infections and the pathogenesis of infectious complications from a wide range of pathogens [3-5]. In the case of UPEC, treatment of rodent UPEC pyelonephritis models with the injectable anticoagulant heparin demonstrates an important role of coagulation in preventing the progression of pyelonephritis to sepsis via an α-hemolysin/renal epithelial cell CD147/Tissue Factor axis [6, 7].

54 The prevalence of urinary tract infections rises from ~10% in the general population of women to ~20% in
55 women over 65 [8]. Catheterization further increases the risk of urinary tract infections, with a 10-25% risk
56 of a catheter-associated urinary tract infection [8]. The rise in prevalence of chronic cardiovascular
57 conditions, such as atrial fibrillation [9], causes a strong correlation between age and the use of anti-
58 hemostatic medications which might compound the risk and severity of UPEC urosepsis in the elderly. Here,
59 we have used a zebrafish embryo model of systemic UPEC infection to study the interaction between
60 commonly prescribed anti-hemostatic medications and the severity of UPEC sepsis [10].
61
62 **Methods**
63 *Zebrafish husbandry*
64 Adult zebrafish were housed at the Centenary Institute (Sydney Local Health District AWC Approval 2017-
65 036). Zebrafish embryos were produced by natural spawning and raised at 28°C in E3 media.
66
67 *Zebrafish lines*
68 Wild type zebrafish are the AB background. Transgenic lines are: *Tg(fabp10a:fgb-EGFP)*^{mi4001} which was
69 used to visualize clot formation [11], and *Tg(-6.0itga2b:eGFP)*^{la2} which was used to visualize thrombocytes
70 [12].
71
72 *Infection of zebrafish embryos*
73 Aliquots of midlog-phase *Escherichia coli* UTI89 carrying the pGI6 plasmid, grown in LB broth supplemented
74 with 50 µg/ml spectinomycin, were frozen at -80°C for use in infection experiments [13]. Bacterial aliquots
75 were thawed and diluted with phenol red dye (0.5% w/v). 10-15 nL was injected into the caudal vein or
76 trunk of M-222 (tricaine)-anaesthetized 5 dpf embryos resulting in a standard infectious dose of
77 approximately 10,000 CFU. Embryos were recovered into E3 and housed at 32°C for up to three days.
78
79 *CFU recovery assay*

80 Groups of 5 zebrafish embryos were pooled and homogenized by pipetting through a P200 tip, and
81 syringing through 23 G and 28 G needles. Homogenate was serially diluted and plated of LB agar
82 supplemented with 50 µg/ml spectinomycin to select of UTI89 carrying the pGI6 plasmid.
83
84 *Drug treatments*
85 Embryos were treated by immersion exposure to vehicle control (DMSO or water as appropriate), 10 µg/ml
86 aspirin in 0.1% final DMSO, 20 µg/ml ticagrelor in 0.1% final DMSO, 33 µM warfarin in water, or 100 mM
87 amino caproic acid (ACA) in water immediately after infection. Drugs were refreshed after two days only for
88 the survival experiments lasting longer than two days, all other experiments were performed with a single
89 dose.
90
91 *Imaging*
92 Imaging was carried out on embryos anaesthetized in M-222 mounted in 0.75% low melting point agarose
93 on a Deltavision Elite fluorescence microscope for 24 hours. Editing and bacterial fluorescent pixel count
94 was carried out with Image J Software Version 1.51j [14].
95
96 *Wound hemostasis assay*
97 We transected the tails of M-222 anesthetized embryos with a scalpel at the ventral pigment gap. This
98 severs the dorsal aorta and posterior cardinal vein resulting in rapid hemostasis in control embryos.
99 Embryos were recovered to E3 prior to imaging at 2 hours post wounding.
100
101 *Statistics*
102 Survival analyses were performed by Log-rank tests in GraphPad Prism. Fluorescent pixel count analyses
103 were performed by Student's *t*-test or ANOVA in GraphPad Prism as appropriate. Error bars represent
104 standard error of the mean.
105
106 **Results**

107 *Coagulation is targeted to bloodborne UPEC in zebrafish embryos*

108 UPEC infection-induced clotting has observed in live rats [6, 7]. We infected *Tg(fabp10a:fgb-EGFP)*^{mi4001}

109 zebrafish embryos, where clots can be visualized by GFP fluorescence, with UTI89 UPEC carrying the pGI6

110 plasmid, allowing visualization of red fluorescent UPEC, and performed timelapse microscopy. We observed

111 the progressive formation of both arterial and venous clots in infected embryos in close proximity to

112 fluorescent UPEC (Figure 1A).

113

114 *Warfarin increases the severity of systemic UPEC infection in zebrafish embryos*

115 Inhibition of clotting with heparin has been reported to worsen UPEC infection in mice [6, 7]. We sought to

116 determine if commonly prescribed anticoagulants had a similar effect in our zebrafish model using

117 warfarin, a vitamin K antagonist and a commonly prescribed anticoagulant.

118

119 We have previously demonstrated that warfarin reduced mycobacterial infection-induced coagulation in

120 zebrafish embryos [4], here we demonstrate that warfarin reduced Fgb-GFP fluorescence around

121 fluorescent UPEC while aminocaproic acid (ACA) treatment stabilized clots (Figure 1B). Treatment with

122 warfarin decreased embryo survival following systemic UPEC infection while treatment with ACA

123 conversely increased embryo survival following systemic UPEC infection (Figure 1C). The decreased survival

124 of warfarin-treated embryos correlated with increased UPEC burden at 18 hours post infection (hpi) (Figure

125 1D).

126

127 *Zebrafish thrombocytes interact with UPEC*

128 Clotting and thrombosis are coordinated during infection-induced hemostasis [3, 4]. To determine if

129 zebrafish thrombocytes could play a role in controlling systemic UPEC infection, we infected *Tg(-*

130 *6.0itga2b:eGFP)*^{la2} embryos, where thrombocytes can be visualized by GFP expression, with UPEC- pGI6. We

131 observed transient interactions between zebrafish thrombocytes and clumps of UPEC but were unable to

132 determine if the bacteria were extracellular or intracellular (Figure 2A).

133

134 *Aspirin and ticagrelor increase the severity of systemic UPEC infection in zebrafish embryos*

135 Having shown a negative effect of inhibiting clotting, we next investigated the effect of commonly
136 prescribed anti-platelet medications on systemic UPEC infection. Aspirin and ticagrelor reduced
137 thrombocyte aggregation to a sterile wound (Figure 2B), but we were unable to quantify changes due to
138 the transient and mobile nature of the thrombocyte-UPEC interaction in the blood stream.

139

140 Inhibition of thrombocytes with either aspirin or ticagrelor reduced survival of infected embryos compared
141 to DMSO-treated control embryos (Figure 2C and 2D). Treatment with either aspirin or ticagrelor increased
142 UPEC burden at 6 hpi compared to DMSO-treated control embryos (Figure 2E).

143

144 **Discussion**

145 We have found that common anti-hemostatic medications worsen the survival of UPEC-infected zebrafish.
146 Anti-hemostatic drug use is highly prevalent in the elderly as a preventative measure against heart attack
147 and stroke. Alongside this, the risk of urinary tract infections also increases from middle to old age and
148 there is elevated risk of recurrent urinary tract infections in aged care settings [15]. Our data illustrates a
149 negative association between preventative anti-hemostatic medication usage and the severity of UPEC
150 sepsis.

151

152 Our results add to a growing body of literature that the hemostatic system, and specifically clotting, is
153 crucial to the early containment of blood born UPEC [6, 7]. Clinical trials of anticoagulants to treat sepsis-
154 induced coagulopathy have delivered inconsistent results, however an emerging theme is that early
155 coagulation is host protective while late coagulation drives pathology [16]. Subsequently, administration of
156 anticoagulants at early stage can be detrimental to the clearance of pathogen by the native immune system
157 [17]. Additionally, there is little evidence on the beneficial effect of anticoagulants on the overall
158 population, however, is beneficial for critically ill subgroups such as sepsis-induced disseminated
159 intravascular coagulation [16, 18].

160

161 Pathogen-specific effects may drive the heterogeneous host response to anticoagulant therapy. For
162 example, Factor XI aided the containment of *Klebsiella pneumoniae* and *Streptococcus pneumoniae* in mice,
163 while knockout of Factor XII protected mice against against *K. pneumoniae* but not *S. pneumoniae* [5]. The
164 genetic conservation of vertebrate coagulation factor function and the amenability of zebrafish to rapid
165 CRISPR-Cas9 mutagenesis suggest zebrafish could be a cost-effective platform to further delineate exact
166 components of the coagulation cascade which we have bluntly targeted with warfarin in this study.
167
168 The negative effects of the antiplatelet drugs aspirin and ticagrelor demonstrate a host-protective role of
169 zebrafish thrombocytes during systemic UPEC infection. Degranulation of activated mammalian platelets
170 releases important inflammatory mediators such as antimicrobial proteins, cytokines, and ADP/ATP which
171 can directly kill pathogens and activate cellular immunity [19]. Clinical trials with antiplatelets, especially
172 aspirin, have demonstrated an improvement in mortality of severe septic patients which, similar to anti-
173 coagulant therapy, may reflect an effect of timing [20]. A limitation of our study is that we were unable to
174 directly quantify thrombocyte-UPEC interactions and determine if the increased susceptibility of aspirin and
175 ticagrelor-treated embryos was due to localized or systemic changes to thrombocyte biology.
176
177 Our CFU recovery experiments confirm warfarin treatment results in increased UPEC growth in systemic
178 infection of zebrafish embryos. This was expected from the literature where heparin treatment facilitates
179 increased UPEC growth in mammals [6, 7]. In parallel, we observed increased UPEC burden in embryos
180 treated with anti-platelet medications. This suggests the hemostatic system either directly controls the
181 growth of UPEC in zebrafish or assists the zebrafish innate immune system in efforts to control systemic
182 UPEC infection.
183
184 An important limitation of our study is that we have not established equivalency of systemic UPEC infection
185 in zebrafish embryos with sepsis seen in mammals. Additionally, our method of infusing UPEC directly into
186 the bloodstream of zebrafish embryos removes key steps of the natural ascending infection route via
187 bladder colonization and pyelonephritis which account for the majority of UPEC morbidity. Zebrafish

188 embryos are also highly tolerant of coagulopathy, as seen in an antithrombin III-deficient mutant [21], and
189 this may cause unexplored differences in their use of the hemostatic system to control systemic infections.
190
191 The internal concentrations of drugs achieved by immersion exposure of zebrafish embryos to drugs needs
192 to be determined for individual substances, however previous reports have reported a range of 1-20% peak
193 absorption of small molecules [22, 23]. Our 10 µg/ml dose of aspirin delivered by immersion exposure is
194 likely to be at the low end of the range of peak human therapeutic plasma concentrations 2-20 µg/ml [24,
195 25], while our 20 µg/ml dose of ticagrelor is likely to be on the high end of the peak human ticagrelor
196 plasma concentration of 3.6 µg/ml for a 400 mg/day dose [26], and our 33 µM, roughly equivalent to 10
197 µg/ml, dose of warfarin is likely to be close to therapeutic human plasma levels <1 µg/ml [27, 28].
198
199 Future studies should investigate the interaction between the hemostatic system and the natural course of
200 UPEC infection in both animal models and by retrospective clinical chart review. The potential for an
201 association between aspirin and warfarin, two of the most commonly prescribed anti-hemostatic
202 medications, with catherization-associated UPEC infections involves a significant proportion of aged care
203 patients and may represent an important cause of excess morbidity.
204
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215

216 **Author contributions**

217 E.H., and S.H.O designed the experiments. V.T., E.H., and S.H.O performed the experiments. V.T., E.H., and

218 S.H.O wrote the paper. E.H., W.J.B., and S.H.O. supervised the project.

219

220 **Declaration of Interests**

221 The authors declare no competing interests.

222

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Figures and Figure Legends

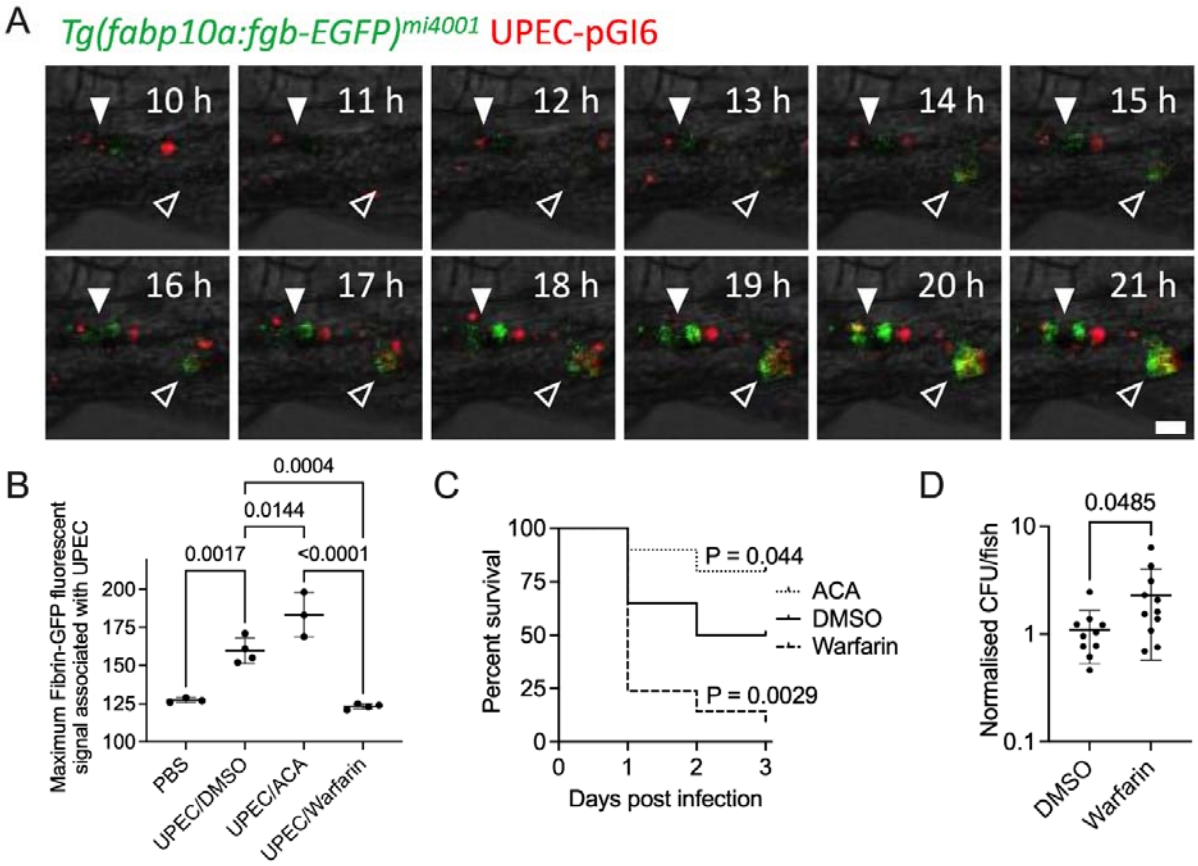

Figure 1: Clotting is host-protective against systemic UPEC infection.

(A) Intravital microscopy of Fgb-GFP clot formation around UPEC pGI6 (red) in a *Tg(fabp10a:fgb-EGFP)*^{mi4001} embryo. Filled arrowhead indicates site of arterial clot formation, hollow arrowhead indicates site of venous clot formation. Scale bar indicates 10 µm. (B) Maximal Fgb-GFP fluorescent intensity around fluorescent UPEC from 4 hpi embryos. Each data point represents one embryo. (C) Survival of UPEC-infected embryos treated with warfarin or ACA. n>=20 per group, data shown is from one experiment that is representative of two biological replicates. (D) UPEC CFU recovery from 18 hpi embryos, data shown is pooled from two independent experiments.

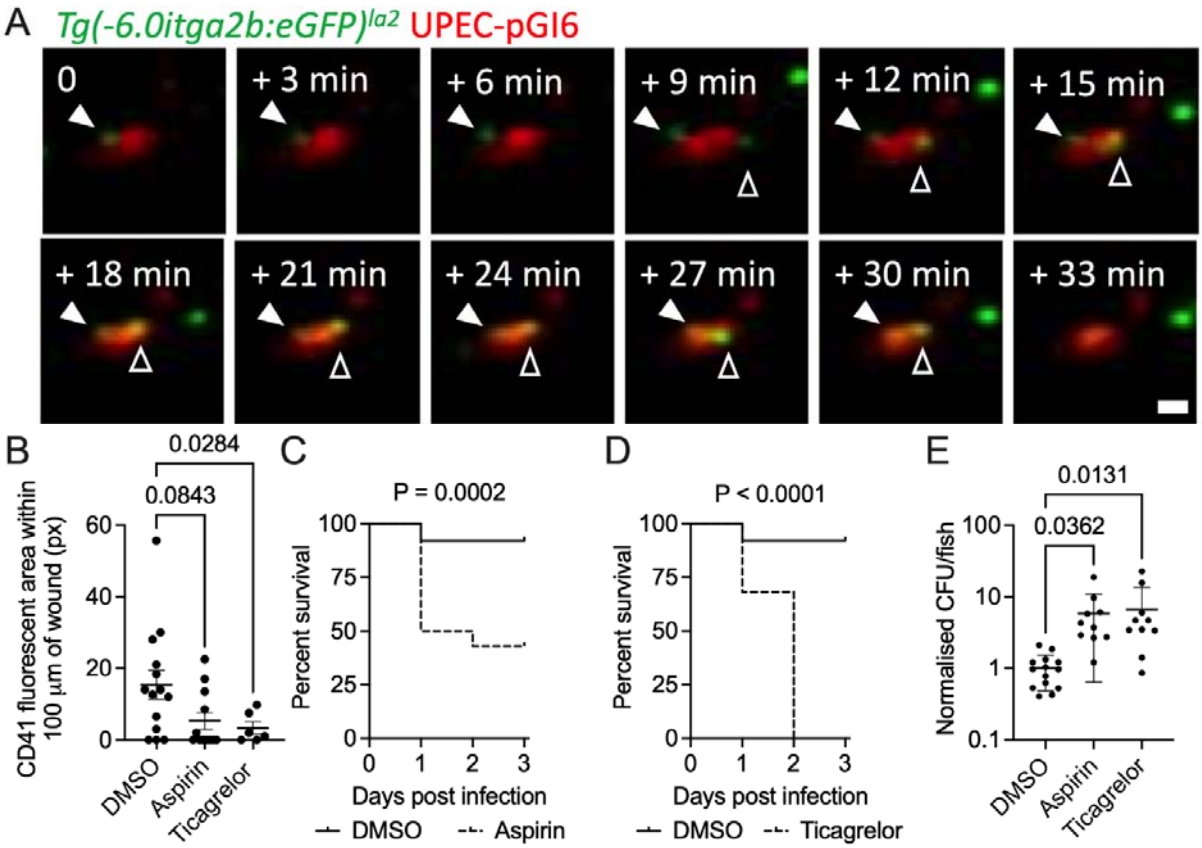

Figure 2: Thrombocyte activation is host-protective against systemic UPEC infection.

(A) Intravital microscopy of green thrombocytes interacting with UPEC pGI6 (red) in a *Tg(-6.0itga2b:eGFP)*^{la2} embryo. Filled arrowhead indicates first thrombocyte that interacts with clump of UPEC for 30 minutes, hollow arrowhead indicates a second thrombocyte that interacts with the same clump of UPEC for 21 minutes. Unindicated green cells are thrombocytes in circulation. t0 is approximately 1 hour post infection.

299 Scale bar indicates 10 µm. (B) CD41 (thrombocyte) fluorescent area at 2 hours post wounding. (C) Survival
300 of UPEC-infected embryos treated with aspirin. n>=25 per group, data shown is from one experiment that
301 is representative of two biological replicates. (D) Survival of UPEC-infected embryos treated with ticagrelor.
302 n=25 per group, data shown is from one experiment that is representative of two biological replicates. (E)
303 CFU recovery from 6 hpi embryos, data shown is pooled from two independent experiments.
304