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

Vi Tran[1], Elinor Hortle[1,2]†, Warwick J Britton[1,3], Stefan H Oehlers[1,2]†*

1 Tuberculosis Research Program at the Centenary Institute, The University of Sydney, Camperdown NSW Australia

2 The University of Sydney, Faculty of Medicine and Health & Marie Bashir Institute, Camperdown NSW Australia

3 Department of Clinical Immunology, Royal Prince Alfred Hospital, Camperdown, NSW 2050 Australia

† Co-corresponding author email: e.hortle@centenary.org.au

* Corresponding author email: stefan.oehlers@sydney.edu.au

Financial declaration: This work was supported by the University of Sydney Fellowship G197581; NSW Ministry of Health under the NSW Health Early-Mid Career Fellowships Scheme H18/31086; and the Kenyon Family Inflammation Award 2017 (S.H.O.), Kenyon Family Inflammation Award 2019 (E.H.).

The authors have no conflicts of interest to declare.

Corresponding author

Stefan Oehlers

Tuberculosis Research Program, Centenary Institute

Locked Bag 6

Newtown NSW 2042

Australia

Tel: +61 2 9565 6114

Fax : +61 2 9565 6101

Email: stefan.oehlers@sydney.edu.au

Abstract

Uropathogenic *Escherichia coli* (UPEC) causes urinary tract infections that can result in sepsis. Hemostasis is protective in the pyelonephritis stage of ascending UPEC infection, but the role of hemostasis 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].

Hemostasis plays an evolutionarily conserved roles in both immune control of infections and the pathogenesis of infectious complications from a wide range of pathogens [3, 4]. 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 [5, 6].

Elderly patients undergoing long term catheterization are at high risk of UPEC infections. These patients are also likely to be taking anti-hemostatic medications for the management of chronic cardiovascular

conditions which might compound their risk and severity of severe UPEC infections. Here, we have used a zebrafish embryo model of systemic UPEC infection to study the interaction between commonly prescribed anti-hemostatic medications and the severity of UPEC sepsis [7].

Methods

Zebrafish husbandry

Adult zebrafish were housed at the Centenary Institute (Sydney Local Health District AWC Approval 2017-036). Zebrafish embryos were produced by natural spawning and raised at 28°C in E3 media.

Zebrafish lines

Wild type zebrafish are the AB background. Transgenic lines are: *Tg(fabp10a:fgb-EGFP)*^{mi4001} which was used to visualize clot formation [8], and *Tg(-6.0itga2b:eGFP)*^{la2} which was used to visualize thrombocytes [9].

Infection of zebrafish embryos

Aliquots of midlog-phase *Escherichia coli* UTI89 carrying the pGI6 plasmid, grown in LB broth supplemented with 50 µg/ml spectinomycin, were frozen at -80°C for use in infection experiments [10]. Bacterial aliquots were thawed and diluted with phenol red dye (0.5% w/v). 10-15 nL was injected into the caudal vein or trunk of M-222 (tricaine)-anaesthetized 5 dpf embryos resulting in a standard infectious dose of approximately 10,000 CFU. Embryos were recovered into E3 and housed at 32°C for up to three days.

CFU recovery assay

Groups of 5 zebrafish embryos were pooled and homogenized by pipetting through a P200 tip, and syringing through 23 G and 28 G needles. Homogenate was serially diluted and plated of LB agar supplemented with 50 µg/ml spectinomycin to select of UTI89 carrying the pGI6 plasmid.

Drug treatments

Embryos were treated with vehicle control (DMSO or water as appropriate), 10 µg/ml aspirin, 20 µg/ml ticagrelor, 33 µM warfarin, or 100 mM amino caproic acid (ACA) immediately after infection.

Imaging

Imaging was carried out on embryos anaesthetized in M-222 mounted in 0.75% low melting point agarose on a Deltavision Elite fluorescence microscope for 24 hours. Editing and bacterial fluorescent pixel count was carried out with Image J Software Version 1.51j [11].

Wound hemostasis assay

We transected the tails of M-222 anesthetized embryos with a scalpel at the ventral pigment gap. This severs the dorsal aorta and posterior cardinal vein resulting in rapid hemostasis in control embryos. Embryos were recovered to E3 prior to imaging at 2 hours post wounding.

Statistics

Survival analyses were performed by Log-rank tests in GraphPad Prism. Fluorescent pixel count analyses were performed by Student's *t*-test or ANOVA in GraphPad Prism as appropriate. Error bars represent standard error of the mean.

Results

Coagulation is targeted to bloodborne UPEC in zebrafish embryos

UPEC infection-induced clotting has observed in live rats [5, 6]. We infected *Tg(fabp10a:fgb-EGFP)*^{mi4001} zebrafish embryos, where clots can be visualized by GFP fluorescence, with UTI89 UPEC carrying the pGI6 plasmid, allowing visualization of red fluorescent UPEC, and performed timelapse microscopy. We observed the progressive formation of both arterial and venous clots in infected embryos in close proximity to fluorescent UPEC (Figure 1A).

Warfarin increases the severity of systemic UPEC infection in zebrafish embryos

Inhibition of clotting with heparin has been reported to worsen UPEC infection in mice [5, 6]. We sought to determine if commonly prescribed anti-coagulants had a similar effect in our zebrafish model using warfarin, a vitamin K antagonist and a commonly prescribed anti-coagulant.

We have previously demonstrated that warfarin reduced mycobacterial infection-induced coagulation in zebrafish embryos [4], here we demonstrate that warfarin reduced Fgb-GFP fluorescence around fluorescent UPEC while aminocaproic acid (ACA) treatment stabilized clots (Figure 1B). Treatment with warfarin decreased embryo survival following systemic UPEC infection while treatment with ACA conversely increased embryo survival following systemic UPEC infection (Figure 1C). The decreased survival of warfarin-treated embryos correlated with increased UPEC burden at 18 hours post infection (hpi) (Figure 1D).

Zebrafish thrombocytes interact with UPEC

Clotting and thrombosis are coordinated during infection-induced hemostasis [3, 4]. To determine if zebrafish thrombocytes could play a role in controlling systemic UPEC infection, we infected *Tg(-6.0itga2b:eGFP)*^{la2} embryos, where thrombocytes can be visualized by GFP expression, with UPEC- pGI6. We observed transient interactions between zebrafish thrombocytes and clumps of UPEC but were unable to determine if the bacteria were extracellular or intracellular (Figure 2A).

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

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

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

Discussion

We have found that common anti-hemostatic medications worsen the survival of UPEC-infected zebrafish. Anti-hemostatic drug use is highly prevalent in the elderly as a preventative measure against heart attack and stroke. Alongside this, the risk of urinary tract infections also increases from middle to old age and there is elevated risk of recurrent urinary tract infections in aged care settings [12]. Our data illustrates a negative association between preventative anti-hemostatic medication usage and the severity of UPEC sepsis.

Our results add to a growing body of literature that hemostasis, and specifically clotting, is crucial to the early containment of blood born UPEC [5, 6]. Clinical trials of anticoagulants to treat sepsis-induced coagulopathy have delivered inconsistent results, however an emerging theme is that early coagulation is host protective while late coagulation drives pathology [13]. Subsequently, administration of anticoagulants at early stage can be detrimental to the clearance of pathogen by the native immune system [14]. Additionally, there is little evidence on the beneficial effect of anticoagulants on the overall population, however, is beneficial for critically ill subgroups such as sepsis-induced disseminated intravascular coagulation [13, 15].

The negative effects of the antiplatelet drugs aspirin and ticagrelor demonstrate a host-protective role of zebrafish thrombocytes during systemic UPEC infection. Degranulation of activated mammalian platelets releases important inflammatory mediators such as antimicrobial proteins, cytokines, and ADP/ATP which can directly kill pathogens and activate cellular immunity [16]. Clinical trials with antiplatelets, especially aspirin, have demonstrated an improvement in mortality of severe septic patients which, similar to anti-coagulant therapy, may reflect an effect of timing [17]. A limitation of our study is that we were unable to

directly quantify thrombocyte-UPEC interactions and determine if the increased susceptibility of aspirin and ticagrelor-treated embryos was due to localized or systemic changes to thrombocyte biology.

Our CFU recovery experiments confirm warfarin treatment results in increased UPEC growth in systemic infection of zebrafish embryos. This was expected from the literature where heparin treatment facilitates increased UPEC growth in mammals [5, 6]. In parallel, we observed increased UPEC burden in embryos treated with anti-platelet medications. This suggests the hemostatic system either directly controls the growth of UPEC in zebrafish or assists the zebrafish innate immune system in efforts to control systemic UPEC infection.

An important limitation of our study is that we have not established equivalency of systemic UPEC infection in zebrafish embryos with sepsis seen in mammals. Additionally, our method of infusing UPEC directly into the bloodstream of zebrafish embryos removes key steps of the natural ascending infection route via bladder colonization and pyelonephritis which account for the majority of UPEC morbidity.

The internal concentrations of drugs achieved by immersion exposure of zebrafish embryos to drugs needs to be determined for individual substances, however previous reports have reported a range of 1-20% peak absorption of small molecules [18, 19]. Our 10 µg/ml dose of aspirin delivered by immersion exposure is likely to be at the low end of the range of peak human therapeutic plasma concentrations 2-20 µg/ml [20, 21], while our 20 µg/ml dose of ticagrelor is likely to be on the high end of the peak human ticagrelor plasma concentration of 3.6 µg/ml for a 400 mg/day dose [22], and our 33 µM, roughly equivalent to 10 µg/ml, dose of warfarin is likely to be close to therapeutic human plasma levels <1 µg/ml [23, 24].

Future studies should investigate the interaction between hemostasis and the natural course of UPEC infection in both animal models and by retrospective clinical chart review. The potential for an association between aspirin and warfarin, two of the most commonly prescribed anti-hemostatic medications, with

catherization-associated UPEC infections involves a significant proportion of aged care patients and may represent an important cause of excess morbidity.

Funding

This work was supported by the University of Sydney Fellowship [grant number G197581]; NSW Ministry of Health under the NSW Health Early-Mid Career Fellowships Scheme [grant number H18/31086]; Centenary Institute Booster Grant to E.H.; and the Kenyon Family Inflammation Award 2017 to S.H.O., Kenyon Family Inflammation Award 2019 to E.H..

The authors have no conflicts of interest to declare.

Acknowledgements

We thank Associate Professor Iain Duggin for the UPEC strain and plasmid; Dr Angela Kurz and Sydney Cytometry for assistance with imaging equipment; and Dr Vivien Chen for helpful discussion of results.

Author contributions

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

Declaration of Interests

The authors declare no competing interests.

References

1. Wiles, T.J., R.R. Kulesus, and M.A. Mulvey, *Origins and virulence mechanisms of uropathogenic Escherichia coli.* Exp Mol Pathol, 2008. **85**(1): p. 11-9.
2. Ulett, G.C., et al., *Uropathogenic Escherichia coli virulence and innate immune responses during urinary tract infection.* Curr Opin Microbiol, 2013. **16**(1): p. 100-7.
3. Frick, I.M., L. Bjorck, and H. Herwald, *The dual role of the contact system in bacterial infectious disease.* Thromb Haemost, 2007. **98**(3): p. 497-502.
4. Hortle, E., et al., *Thrombocyte Inhibition Restores Protective Immunity to Mycobacterial Infection in Zebrafish.* J Infect Dis, 2019. **220**(3): p. 524-534.
5. Melican, K., et al., *Bacterial infection-mediated mucosal signalling induces local renal ischaemia as a defence against sepsis.* Cell Microbiol, 2008. **10**(10): p. 1987-98.

6. Schulz, A., et al., *Protective vascular coagulation in response to bacterial infection of the kidney is regulated by bacterial lipid A and host CD147.* Pathog Dis, 2018. **76**(8).
7. Wiles, T.J., et al., *Use of zebrafish to probe the divergent virulence potentials and toxin requirements of extraintestinal pathogenic Escherichia coli.* PLoS Pathog, 2009. **5**(12): p. e1000697.
8. Vo, A.H., et al., *Loss of fibrinogen in zebrafish results in symptoms consistent with human hypofibrinogenemia.* PLoS One, 2013. **8**(9): p. e74682.
9. Lin, H.F., et al., *Analysis of thrombocyte development in CD41-GFP transgenic zebrafish.* Blood, 2005. **106**(12): p. 3803-10.
10. Wright, K., et al., *Mycobacterial infection-induced miR-206 inhibits protective neutrophil recruitment via the CXCL12/CXCR4 signalling axis.* PLoS Pathog, 2021: p. 2020.12.14.422665.
11. Matty, M.A., S.H. Oehlers, and D.M. Tobin, *Live Imaging of Host-Pathogen Interactions in Zebrafish Larvae.* Methods Mol Biol, 2016. **1451**: p. 207-23.
12. Bennett, N.J., et al., *Infections in Australian Aged-Care Facilities: Evaluating the Impact of Revised McGeer Criteria for Surveillance of Urinary Tract Infections.* Infect Control Hosp Epidemiol, 2016. **37**(5): p. 610-2.
13. Umemura, Y., et al., *Efficacy and safety of anticoagulant therapy in three specific populations with sepsis: a meta-analysis of randomized controlled trials.* J Thromb Haemost, 2016. **14**(3): p. 518-30.
14. Scarlatescu, E., D. Tomescu, and S.S. Arama, *Anticoagulant Therapy in Sepsis. The Importance of Timing.* J Crit Care Med (Targu Mures), 2017. **3**(2): p. 63-69.
15. Iba, T., et al., *Efficacy and bleeding risk of antithrombin supplementation in septic disseminated intravascular coagulation: a secondary survey.* Crit Care, 2014. **18**(5): p. 497.
16. Deppermann, C. and P. Kubes, *Platelets and infection.* Semin Immunol, 2016. **28**(6): p. 536-545.
17. Davis, R.P., S. Miller-Dorey, and C.N. Jenne, *Platelets and coagulation in infection.* Clin Transl Immunology, 2016. **5**(7): p. e89.
18. Zhang, F., et al., *Antibiotic toxicity and absorption in zebrafish using liquid chromatography-tandem mass spectrometry.* PLoS One, 2015. **10**(5): p. e0124805.
19. Kirla, K.T., et al., *Importance of Toxicokinetics to Assess the Utility of Zebrafish Larvae as Model for Psychoactive Drug Screening Using Meta-Chlorophenylpiperazine (mCPP) as Example.* Front Pharmacol, 2018. **9**: p. 414.
20. Benedek, I.H., et al., *Variability in the pharmacokinetics and pharmacodynamics of low dose aspirin in healthy male volunteers.* J Clin Pharmacol, 1995. **35**(12): p. 1181-6.
21. Rosenkranz, B. and J.C. Frolich, *Plasma concentrations and anti-platelet effects after low dose acetylsalicylic acid.* Prostaglandins Leukot Med, 1985. **19**(3): p. 289-300.
22. Dobesh, P.P. and J.H. Oestreich, *Ticagrelor: pharmacokinetics, pharmacodynamics, clinical efficacy, and safety.* Pharmacotherapy, 2014. **34**(10): p. 1077-90.
23. Sun, S., et al., *Study on warfarin plasma concentration and its correlation with international normalized ratio.* J Pharm Biomed Anal, 2006. **42**(2): p. 218-22.
24. Routledge, P.A., et al., *Pharmacokinetics and pharmacodynamics of warfarin at steady state.* Br J Clin Pharmacol, 1979. **8**(3): p. 243-7.

Figure Legends

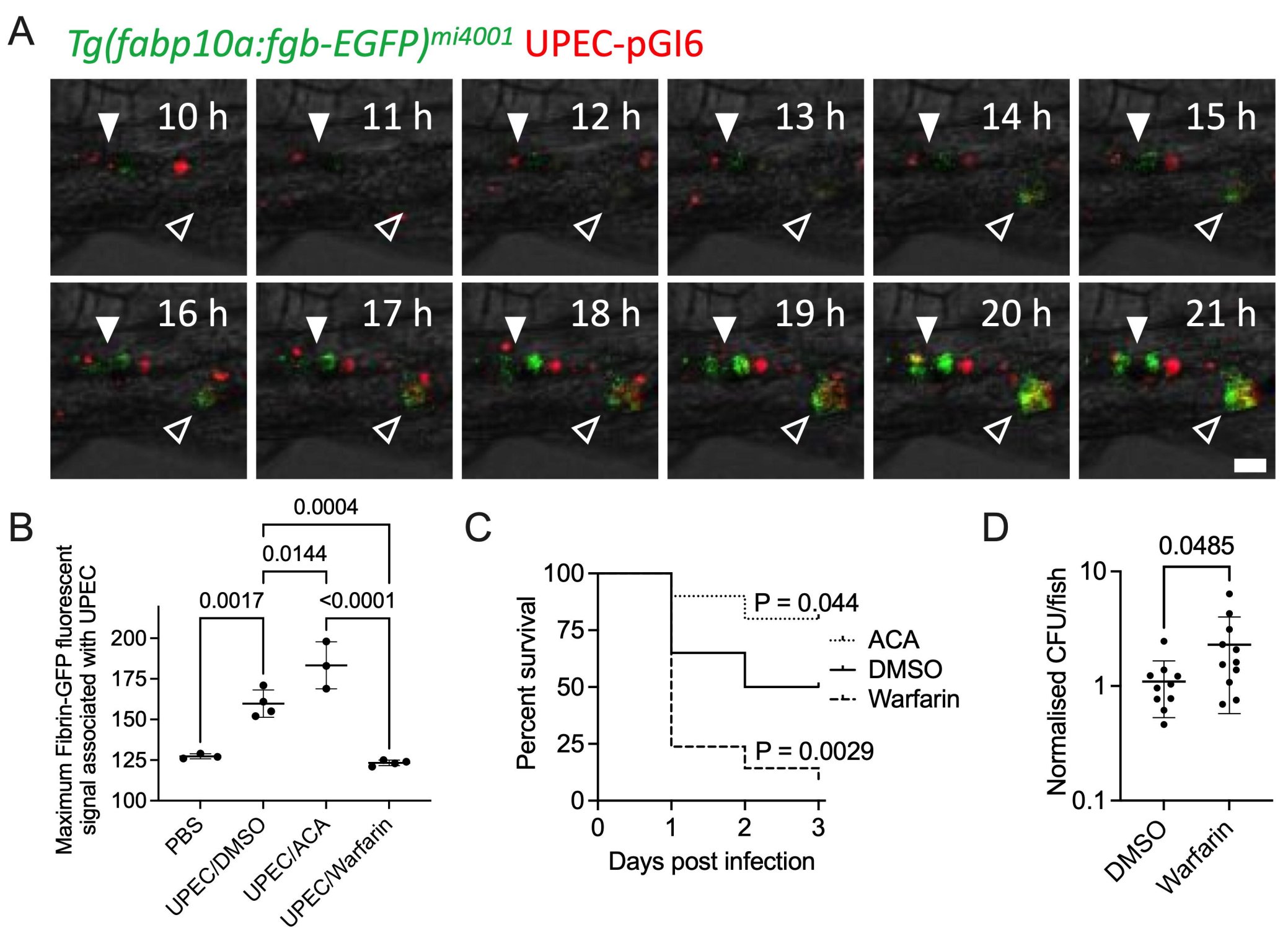
Figure 1: Clotting is host-protective against systemic UPEC infection.

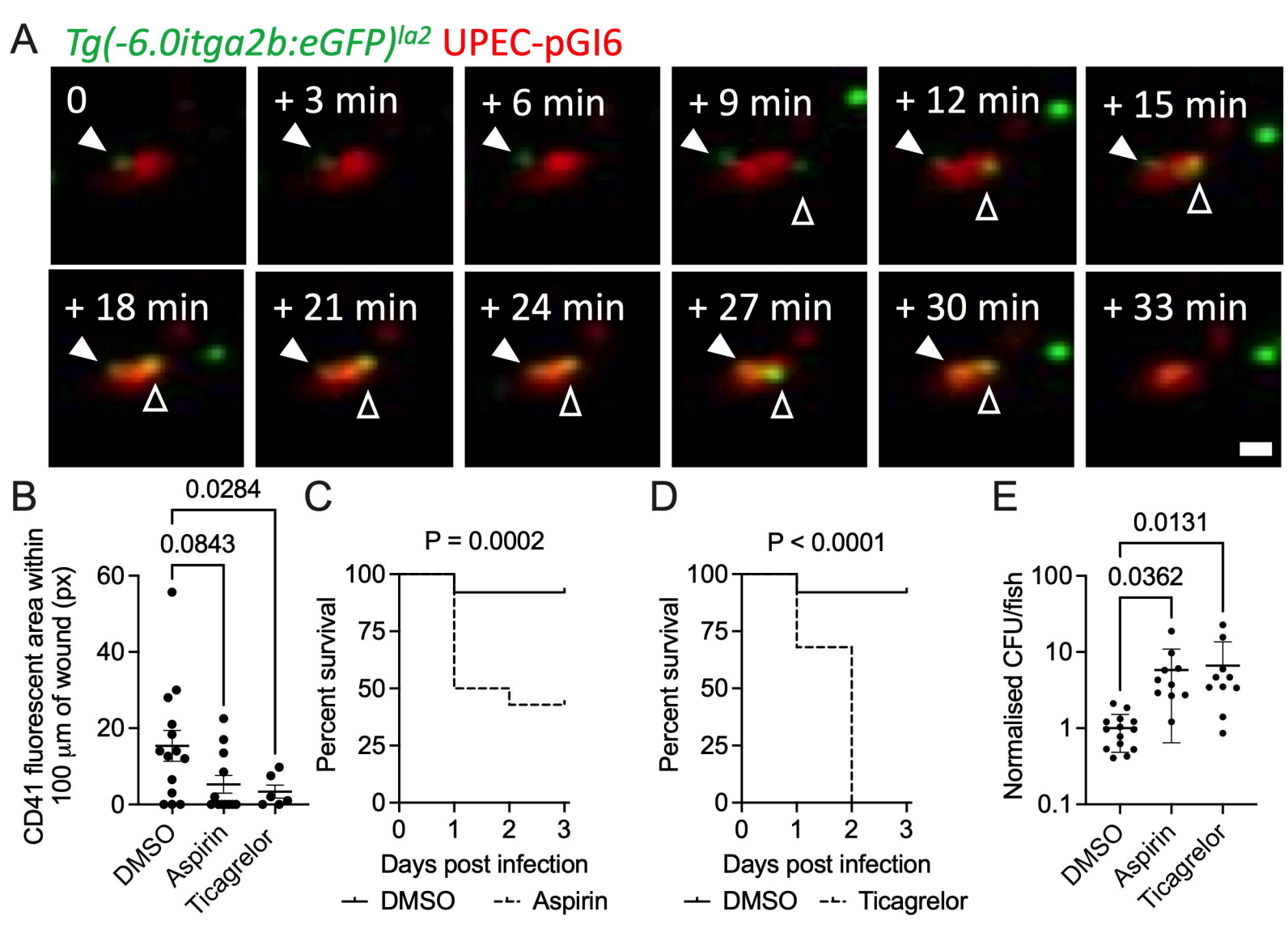
(A) Intravital microscopy of Fgb-GFP clot formation around UPEC pGI6 (red) in a *Tg(fabp10a:fgb-EGFP)*^{mi4001} embryo. Filled arrowhead indicates site of arterial clot formation, hollow arrowhead indicates site of venous clot formation. Scale bar indicates 10 µm. (B) Maximal Fgb-GFP fluorescent intensity 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. Scale bar indicates 10 µm. (B) CD41 (thrombocyte) fluorescent area at 2 hours post wounding. (C) Survival of UPEC-infected embryos treated with aspirin. n>=25 per group, data shown is from one experiment that is representative of two biological replicates. (D) Survival of UPEC-infected embryos treated with ticagrelor. n=25 per group, data shown is from one experiment that is representative of two biological replicates. (E) CFU recovery from 6 hpi embryos, data shown is pooled from two independent experiments.


A
Tg(fabp10a:fgb-EGFP)mi4001 UPEC-pGI6
10 h
11 h
12 h
13 h
14 h
15 h
16 h
17 h
18 h
19 h
20 h
21 h
B
0.0004
0.0144
0.0017
<0.0001
Maximum Fibrin-GFP fluorescent signal associated with UPEC
200
175
150
125
100
PBS
UPEC/DMSO
UPEC/ACA
UPEC/Warfarin
C
100
75
50
25
0
P = 0.044
ACA
DMSO
Warfarin
P = 0.0029
Percent survival
0
1
2
3
Days post infection
D
0.0485
10
1
0.1
Normalised CFU/fish
DMSO
Warfarin


A
Tg(-6.0itga2b:eGFP)la2 UPEC-pGI6
0
+ 3 min
+ 6 min
+ 9 min
+ 12 min
+ 15 min
+ 18 min
+ 21 min
+ 24 min
+ 27 min
+ 30 min
+ 33 min
B
0.0284
0.0843
CD41 fluorescent area within 100 µm of wound (px)
60
40
20
0
DMSO
Aspirin
Ticagrelor
C
P = 0.0002
Percent survival
100
75
50
25
0
0
1
2
3
Days post infection
DMSO
Aspirin
D
P < 0.0001
Percent survival
100
75
50
25
0
0
1
2
3
Days post infection
DMSO
Ticagrelor
E
0.0131
0.0362
Normalised CFU/fish
100
10
1
0.1
DMSO
Aspirin
Ticagrelor