

Evaluation of commonly used tests to measure the effect of single-dose aspirin on mouse hemostasis

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Abstract

Discrepancies in preclinical studies of aspirin (ASA) antiplatelet activity in mouse models of bleeding and arterial thrombosis led us to evaluate commonly reported methods in order to propose a procedure for reliably measuring the effects of single dose ASA on mouse hemostasis. FVB and C57Bl6 mice received 100 mg/kg of ASA or vehicle orally 30 min or 3 h prior to investigate either hemostasis using the tail bleeding assay or carotid thrombosis induced by FeCl₃, or to blood sampling for isolated platelet aggregation and TXB₂ generation.

Expected inhibition of COX1 by ASA was ascertained by a strong decrease in TXB₂ production, and its effect on platelet function and hemostasis, by decreased collagen-induced aggregation and increased bleeding time, respectively. Strikingly, we determined that anti-hemostatic effects of ASA were more predictable 30 min after administration than 3 h later. Conversely, ASA did not alter time to arterial occlusion of the carotid upon FeCl₃-induced thrombosis, suggesting ASA not to be used as reference inhibitor drug in this model of arterial thrombosis.

Key-words: aspirin, hemostasis, platelets, preclinical study, thrombosis

Background

Acetyl salicylic acid (ASA or aspirin) at low dose is broadly used for its antiplatelet effect in primary and secondary prevention of cardiovascular diseases [1]. ASA irreversibly acetylates the Ser529 residue of COX1, thereby leading to a steric hindrance of the COX channel that prevents access of the substrate to the catalytic site of the enzyme [2]. COX1 is responsible for the conversion of arachidonic acid into prostaglandins, that are precursors of thromboxane A₂ (TXA₂) in platelets [3]. By binding the thromboxane and prostanoid receptor (TP receptor), platelet-secreted TXA₂ enhances platelet activation initially triggered by agonists such as collagen. By limiting one major amplification pathway of platelet activation [4], ASA is therefore a relevant comparator for preclinical studies of antiplatelet agents under development.

Interestingly, whereas ASA pharmacology is well known in humans, its antiplatelet activity is inconsistently described in mouse models. The discrepancies between studies, such as inconsistent effects on mouse arterial thrombosis or bleeding time, could be the consequence of differences regarding the animal strain, the route of administration and ASA doses, as well as the hemostatic parameters evaluated *in vivo* and *ex vivo*. Doses most frequently found in the literature may vary from 1 to 100 mg/kg, given as single or repeated doses [5]–[10]. Various routes are also reported: intravenous, oral, intraperitoneal or subcutaneous. Whereas the most used bleeding model is the tail-tip transection, arterial thrombosis models in use are more diverse. Indeed, arterial thrombosis of carotid, cremaster or mesenteric artery may be induced by FeCl₃, laser or mechanical injury [11]. Finally, the delay of testing the effect of ASA after administration may vary from 10 min to 24 h after administration [7], [9], [12].

Objectives

Starting from commonly used experimental conditions concerning delivery of ASA and testing its antiplatelet effects in mice, our aim was to determine a procedure for reliably measuring the effects of single dose ASA on mouse hemostasis.

Methods

Animals

FVB and C57Bl6 (C57BL/6NRj) mice (males, 8-12 weeks old; weight $27.6 \text{ gr} \pm 2.3$, mean \pm SD) were from Janvier Labs (Le Genest-Saint-Isle, France). Anesthesia was induced by intraperitoneal injection of 80 mg/kg ketamine (Clorketam® 1000, Vetoquinol, Lure, France) and 10 mg/kg xylazine (Rompun® 2%, Bayer, La Garenne-Colombe, France), or with 2.5% inhaled isoflurane (Vetflurane®, Virbac, Carros, France) when performing tail bleeding time experiments. ASA (DL-lysine acetylsalicylate, Aspepic®, Sanofi-Aventis, Gentilly, France) dissolved in distilled water was administered orally by force-feeding or by intraperitoneal (i.p.) injection (100 mg/kg, 10 μ l/gr). *In vivo* experiments were done by an observer blinded to the treatment group. All animal studies were approved by the Ethics Committee on Animal Resources of Paris Descartes University (registration number: CEEA34.CBL.131.12).

Platelet preparation

Blood was collected by cardiac puncture into ACD-C solution (13 mM citric acid, 12.6 mM sodium citrate, 11 mM D-glucose) and diluted with wash buffer (36 mM citric acid, 5 mM D-glucose, 5 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium chloride, 103 mM

sodium chloride, pH 6.5) containing apyrase (0.1 U/ml; Sigma-Aldrich, St. Louis, MO, USA) and prostaglandin E₁ (PGE₁, 1 μM; Sigma-Aldrich). Diluted platelet-rich plasma (PRP) was obtained by 7 min centrifugation at 170 *g*. Platelets were washed twice with wash buffer containing apyrase and PGE₁, and then centrifuged for 10 min at 750 *g*. The pelleted platelets were resuspended in assay buffer (10 mM HEPES, 140 mM sodium chloride, 3 mM potassium chloride, 5 mM sodium bicarbonate, 0.5 mM magnesium chloride, 10 mM D-glucose, pH 7.35) to a concentration of 3.5 x 10⁸ /ml. Calcium chloride 2 mM was then added.

Platelet aggregation studies

Platelet aggregation was measured on a Discovery HT-R microplate reader (MWG Biotech AG, Ebersberg, Germany) coupled to the KC4 software for analysis of the data. FVB mouse washed platelets (3.5 x 10⁸/ml) were incubated for 2 min at 37°C under stirring in wells of a 96-well microplate (Greiner Bio-one, Frickenhausen, Germany) in a volume of 90 μl, then aggregation was induced by adding 5 μg/ml fibrillar type-I collagen from equine Achilles tendon (Horm, Nycomed, Linz, Austria) or 1 μM U46619, a TP synthetic agonist (Calbiochem, Merck, Darmstadt, Germany). Aggregation was monitored for 5 min and expressed as the percentage change in absorbance at 405 nm as previously described [13].

Bleeding assay

Tails of anaesthetized mice were pre-incubated in a 37°C saline solution during 5 min to homogenize vessel dilatation between animals. Then, bleeding time was measured following a 3-mm tail-tip transection, and immediate immersion of the tail in 10 ml of isotonic saline at

37°C. Bleeding time was set at cessation of blood leakage for at least 1 min. Blood loss was estimated by measuring the hemoglobin concentration in the saline, using the Drabkin method.

Thrombosis assay

Mice were anaesthetized and maintained at 37°C on a heating plate. The left carotid artery was exposed and dissected away from the vagus nerve and surrounding tissues. Carotid artery blood flow was monitored with a Doppler flow meter equipped with a Transonic flow probe (Model MA0.5PSB, Transonic System Inc, Ithaca, NY). Arterial thrombosis was induced by placing a 15% FeCl₃-saturated filter paper on the artery, 5 mm upstream the flow probe, for 2 or 4 min. Monitoring of blood flow was maintained for 5 min after the cessation of flow, and the time required for occlusion was recorded.

Thromboxane assay

Thromboxane B₂ (TXB₂) level was measured with the thromboxane assay kit from R&D system (Abingdon, UK). Assays were performed on washed platelet supernatant obtained after collagen-induced platelet aggregation. Ten min after the addition of collagen, 20 mM EDTA was added and the sample were centrifuged 2 min at 12000 g. The supernatant was kept frozen at -20°C until tested.

Data analysis

Data were expressed as medians [95% confidence interval (CI)] for non-normally distributed variables. Statistical analysis was performed with the Prism software package (GraphPad

Software, Inc., San Diego, CA, USA). The Mann-Whitney test was used to compare each parameter. Differences were considered significant when $P < 0.05$.

Results

The impact of ASA administration on mouse hemostasis was assessed by varying the time elapsed between ASA administration and testing (30 min *versus* 3 h), as well as the mouse strain (FVB *versus* C57Bl6).

Bleeding experiments

FVB mice. The impact of the period of time between oral ASA administration and bleeding time measurement was first tested in FVB mice. When ASA was given 30 min before measuring bleeding time, this hemostatic parameter was strongly increased compared to vehicle (88 sec [95% CI, 44-95] vs 343 sec [95% CI, 250-600] for vehicle and ASA respectively; $P < .0001$; Fig 1A). Conversely, when administered 3 hours before the bleeding assay, ASA inconsistently modified the bleeding time compared to vehicle (161 sec [95% CI, 45-600] vs 213 sec [95% CI, 99-600] for vehicle and ASA respectively; $P > .05$; Fig 1A), mostly because a high variability in values. However, no significant difference in bleeding time between the ASA groups was observed (30 min vs 3h, $p=0.16$).

Compared to bleeding time, measuring blood loss to evaluate the anti-hemostatic effect of ASA was not informative since it did not significantly differ from controls neither 30 min (14 μ l [95% CI, 6-29] vs 21 μ l [95% CI, 11-97] for control and ASA respectively; $P > .05$; Fig 1B) nor 3 h after

ASA administration (15 μ l [95% CI, 6-23] vs 36 μ l [95% CI, 10-73] for control and ASA respectively; $P > .05$; Fig 1B).

C57Bl6 mice. To evaluate the potential relevance of the animal genetic background on responsiveness to ASA, we tested whether the widely used C57Bl6 (C57BL/6NRj) strain could give comparable results than the FVB strain. When ASA was given to C57Bl6 mice 30 min before the assay, the bleeding time was also found strongly increased (44 sec [95% CI, 5-83] vs 320 sec [95% CI, 205-600] for control and ASA respectively; $P < .01$; Fig 1C). On another hand, blood loss was significantly, although slightly, increased by ASA (7.5 μ l [95% CI, 5.8-9.7] vs 10.5 μ l [95% CI, 9-18] for control and ASA respectively; $P < .05$; Fig 1D). However, such as for FVB mice, ASA administered 3 h before the tail cutting did not modify the bleeding time (53 sec [95% CI, 44-64] vs 128 sec [95% CI, 5-600] for control and ASA respectively; $P > .05$; Fig 1C) nor blood loss values (5.5 μ l [95% CI, 3.6-13.3] vs 10.4 μ l [95% CI, 5.5-83.2] for control and ASA respectively; $P > .05$; Fig 1D). No significant difference in bleeding time between the ASA groups was observed (30 min vs 3h, $p=0.18$, Fig 1C).

Carotid artery thrombosis

We next tested the impact of ASA on the most commonly used *in vivo* artery thrombosis model: the FeCl₃-induced injury of the carotid artery [11]. Surprisingly, ASA administered by oral route 30 min or 3 h before the experiment did not modify the time to the total occlusion whatever the mouse strain, FVB or C57Bl6 (Table 1).

Then, we tested a repeated administration of ASA for 4 days in C57Bl6 mice. In order to check if the absence of effect of aspirin on the thrombosis model was not due to a lack of sensitivity of our method, the FeCl₃ patch was left during only 2 min. Again, no difference in the time to occlusion was evidenced between aspirin and placebo groups (17 min [95% CI, 13.8-19.5] vs 16.3 min [95% CI, 15.1-17.3], respectively) (Supplemental Figure 1A).

Moreover, in order to verify that the route of administration was not responsible for the absence of anti-thrombotic activity of ASA in this model, we reproduced experiments using the i.p. route, which is largely used in preclinical pharmacology in mice, including ASA studies [6], [9], [14]. Similarly to the results obtained with the oral route, and whatever the time to assay after ASA administration (30 min or 3 h), we did not observe any effect of i.p.-administered ASA on carotid artery thrombosis, neither for FVB nor for C57Bl6 mice (Table 1).

Platelet aggregation and thromboxane generation

To ensure that the absence of anti-thrombotic effect was not due to a pharmacological inefficacy of a single oral ASA administration for COX-1 inhibition, platelet response to ASA exposure was also evaluated by testing platelet functions *ex vivo*. Aggregation assays were performed on washed platelets isolated from FVB mice, in response to 5 µg/ml collagen or 1 µM U46619, a specific agonist of the TP receptor.

When platelet isolation was done 30 min after ASA had been administered by the oral route,

and in line with the results obtained for bleeding time measurement, collagen-induced platelet aggregation was significantly reduced compared to control animals receiving vehicle alone (76% [95% CI, 62-88] vs 34% [95% CI, 20-83] for control and ASA respectively; $P < .01$; Fig 2A). This inhibition of platelet aggregation was associated with a high and significant reduction of about 85% TXB₂ generation by platelets of the ASA group (563 pg/10⁸ platelets [95% CI, 356-1430] vs 81 pg/10⁸ platelets [95% CI, 12-297] for control and ASA respectively; $P < .001$; Fig 2B).

When ASA was administered 3 h before platelet isolation, collagen-induced platelet aggregation was still significantly reduced compared to control platelets without ASA (59% [95% CI, 34-89] vs 13% [95% CI, 10-61] for control and ASA respectively; $P < .05$; Fig 2A). Of note, and whereas TXB₂ generation in platelet supernatant was also significantly decreased at 3 h (1058 pg/10⁸ platelets [95% CI, 545-1532] vs 210 pg/10⁸ platelets [95% CI, 82-270] for control and ASA respectively; $P < .05$; Fig 2B), the effect of ASA on platelet response to collagen was less significant compared to the 30 min time point (Fig 2A). Notably, there was no significant difference in collagen-induced aggregation between the ASA groups (30 min vs 3h; $p=0.25$).

The efficacy of aspirin after 4 days administration was also checked by a significant inhibition of collagen-induced washed platelet aggregation (82 % ± SEM 12% inhibition) and TXB₂ generation in the supernatant (97 % ± SEM 0.4% inhibition) (Supplemental Figure 1B-C). Overall, platelet inhibition measured ex vivo was more pronounced after ASA repeated administration compared to the single dose.

As a control for platelet responsiveness, aggregation induced by 1 μM U46619, which directly activates platelets *via* the TP receptor independently of TXA₂ synthesis, did not differ whatever the time of blood sampling after ASA delivery to animals (68% [95% CI, 30-88] vs 85% [95% CI,

49-92] and 64% [95% CI, 50-78] vs 66% [95% CI, 46-22] at 30 min and 3 h respectively, for control and ASA, respectively; $P > .05$; Fig 2C).

Discussion

Since aspirin remains the gold standard of antiplatelet treatment and an unavoidable reference for other antiplatelet molecules under development, this work was designed to settle appropriate in-house conditions to evidence its effects under a single dose regimen on mouse hemostasis. Indeed, there are major discrepancies found in the literature concerning *in vivo* effects of aspirin in mice. Our present results actually support other studies that failed to demonstrate the antiplatelet effect of ASA in various experimental setups. The two main parameters we chose to evaluate and to settle were (i) the period elapsed between ASA administration and bleeding or thrombosis assays, and (ii) the *in vivo* test to evaluate drug efficacy.

Regarding the ASA doses to be administered *per os* in such an evaluation, those that can be found in the literature are more frequently between 5 and 100 mg/kg [6], [7], [9], [14]–[17]. We show here that a 100 mg/kg ASA dose increased bleeding time as well as efficiently inhibited platelet activation *in vitro*, as evaluated by two recognized assays [18]: washed platelet aggregation and TXB₂ production. We also used a tenfold lower single dose of ASA (10 mg/kg) in some experiments, which turned to result in a high and unacceptable variability in read out data values (data not shown).

Importantly, we analyzed the influence of the period elapsed between ASA administration and

assays, and how it can affect evaluation of the ASA effect. In some previously reported studies, assays for ASA efficacy were performed between 16 and 24 h after drug administration [12], [19]. However Evangelista *et al.* have shown that a significant amount of newly released platelets with a fully active COX were present in the circulation 24 h after ASA administration [20]. Therefore, and as they are frequently used in published procedures, we focused on two short periods of time, 30 min and 3 h after ASA was administered orally. These time-points are in agreement with the rapid ASA effect reported for humans after an oral single dose [21]. Whatever the *in vivo* or *ex vivo* endpoint hemostatic test (bleeding time or platelet aggregation), our results show that the 30 min time point ensures more reproducible results as compared to 3 h. In these conditions, we also show that bleeding time is a better parameter than the blood loss to evidence an effect of ASA on hemostasis.

Considering the demonstration by Schiviz *et al.* of a variability for hemostatic parameters in mice in the absence of drug exposure, depending on the genetic background even between different strains of C57Bl6 [22], we compared the basal and ASA-modulated bleeding parameters in FVB and in C57Bl6 (C57BL/6NRj) mice, all males aged 8-12 weeks. We used these genetic backgrounds that correspond to the KO models currently used in our and many other labs [13][23]. Interestingly, in the absence of ASA treatment, bleeding time was significantly about twice shorter for control C57Bl6 as compared to FVB mice at 30 min, and thrice shorter at 3 h (Fig 1A vs 1C, black dots). In line, blood loss was also always higher in FVB as compared to C57Bl6 mice at either 30 min or 3 h (Fig 1B vs 1D, black dots). In the whole, bleeding experiments appeared to be more predictable-using the C57Bl6 (C57BL/6NRj) strain within the limits of the conditions tested.

Finally, we investigated the impact of ASA on arterial thrombus formation with a commonly used model, the carotid artery thrombosis induced by FeCl₃ [11]. Although we used a 15% FeCl₃ concentration for 4 min, we obtained similar results (see Table 1) than Li *et al.* who found a time to occlusion of 11.3 ± 3.16 min when using 7.5% FeCl₃ on C57Bl6 [24]. Whatever the delay after ASA administration and the mouse strain used, no anti-thrombotic effect of the drug was observed. Therefore, we wondered if this negative result could be due to the administration route. Huang *et al.* have shown that doses of ASA up to 150 mg/kg given intravenously were not sufficient to increase the time for occlusion of the mesenteric venule exposed to fluorescein sodium, and that 250 mg/kg was needed to observe an effect of ASA [15]. In the same study, however, the 150 mg/kg dose was, nevertheless, found to efficiently increase the tail bleeding time [15]. The same study showed, however, an effect of 40 mg/kg oral ASA on occlusion time. To note, the study used male ICR mice, a strain we did not use in the present work. Intraperitoneal administration being a commonly used route in pharmacological studies in murine models and ASA treatment [6], [9], [12], [14], [16], [19], [25], we also evaluated this type of administration. However, we did not either observe any effect of ASA on thrombus formation under this particular condition. On the whole, and given that an effective inhibitory activity of ASA on platelet functions was observed *ex vivo* at 30 min and at 3 h (see Fig 2A and 2B), we can conclude that the carotid artery thrombosis induced by FeCl₃ is not a suitable model for the evaluation of ASA anti-platelet effects, at least within the frame of our experimental conditions. Using the FeCl₃ injury model, some authors also failed to show any effect or a very moderate effect of ASA on thrombosis, if any [6], [9], [25], [26]. In a model of femoral artery thrombosis, Kondo *et al.* showed that ASA can increase the time to occlusion of the artery when thrombosis

is induced photochemically by using rose Bengal [7], while Nonne *et al.* failed to show any effect of intravenous ASA in laser-injured mesentery thrombosis in C57Bl6 strain [17]. More recently, Adili *et al.* showed that ASA induced a decreased platelet recruitment into the arterial wall thrombus in a model of laser-induced cremaster artery thrombosis, without affecting the increase in platelet surface P-selectin-expression within thrombi [27]. Thus, taken together, the already published and our current data show a wide variability in response to ASA treatment in arterial thrombosis models in mice. Data currently converge to a lesser contribution of platelets in the FeCl₃ injury model compared to mechanical injury; indeed, FeCl₃ injury induces significant damage of subendothelial proteins and attachment of platelets to bodies containing ferric ions and exposing large amounts of tissue factor [28].

Limitations of our study are (i) that models using laser to induce carotid injury were not considered, and (ii) that thrombus formation was not monitored using real-time intravital microscopy. Major reason for this is that we wanted to use the more commonly FeCl₃ injury model. On purpose, testing of a single ASA administration was firstly considered mostly because the objective of the present study was to evaluate this frequently used procedure, and after having verified that a strong TXA₂ generation inhibition was reached after a single dose. However, we cannot exclude that a daily administration of ASA could be more effective in limiting thrombosis. Indeed in a model of thrombus formation induced by in vivo injection of platelet agonist, Armstrong *et al.* observed that a chronic ASA dosing (300 mg/kg/day for 7 days) reduced thrombus formation [29]. However, more pathophysiologically relevant studies carried out in models of atherothrombosis did not observe an effect of daily administration of ASA on the lesion [5], [10]. To address this controversy, we tested the repeated administration of ASA

for 4 days in C57Bl6 mice. Interestingly, whereas global platelet inhibition measured ex vivo was more pronounced 4 days after ASA repeated administration compared to the single dose, again no difference in time to occlusion was evidenced between aspirin and placebo groups in the carotid thrombosis model (Supplemental Figure 1).

Moreover, it must be noted that, upon completion of our study, a recent publication suggested that the time to occlusion might not be the best parameter in order to analyze arterial thrombosis, and authors suggested including reflow events to maximize data interpretation [28].

Third limitation of our study is that the high dosage of aspirin used is not the one used in chronic treatment in patients with high cardiovascular risk.

We have thus demonstrated that specific experimental conditions are required in order to observe and adequately evaluate the effect of ASA on mouse hemostasis. Here, we show that the most relevant end-point is the tail bleeding time performed with a cut at 3 mm of the tip and at 30 min after oral 100 mg/kg ASA administration to C57Bl6 mice, as our optimal in-house experimental conditions. We do not recommend time to occlusion of FeCl₃-induced carotid arterial thrombosis as an index of ASA efficacy on platelets since it is inconsistently altered by ASA. A future consensus debate should define the more relevant method to explore the ASA antithrombotic effect.

Authorship Details

B. Decouture conceived the study, performed experiments, analyzed data and wrote the manuscript.

B. Dizier performed animal experiments and analyzed data.

T. Belleville-Rolland and A. Leuci performed in vitro aggregation assays and helped for in vivo experiments.

D Pidard critically read the manuscript.

C Bachelot-Loza and P. Gaussem conceived the study, analyzed data, and wrote the manuscript.

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Declaration of Interest

The authors have no conflicts of interest to disclose.

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Table 1. In vivo ASA effect on artery thrombosis

	30 min			3 hours		
	Control	ASA (100 mg/Kg)		Control	ASA (100 mg/Kg)	
Oral						
FVB	14.7 [10.7-21.2]	17.4 [10.3-25]	NS	12.8 [9.6-16.5]	14.0 [10.5-25]	NS
C57Bl6	11.7 [8.0-21.1]	14.2 [12.3-25]	NS	11.1 [9.4-25]	14.4 [10.5-16.1]	NS
I.P.						
FVB	11.9 [8.9-15.7]	12.5 [10.1-17]	NS	10.6 [10-11]	11.8 [9.6-14.9]	NS
C57Bl6	11.4 [7.3-14.6]	12.7 [7.9-16.2]	NS	10.4 [8.2-13.4]	12.5 [10.4-15]	NS

Vehicle or ASA were administered to FVB or C57Bl6 mice by the oral or intraperitoneal route (I.P.). Thirty min or 3h after ASA administration, carotid artery thrombosis was induced and the time to occlusion was recorded. Results are in minutes and expressed as median [95% CI].

Legends to figures

Figure 1. *In vivo* ASA effect on bleeding time and blood loss. Bleeding time was measured 30 min or 3 hours after vehicle (closed circles) or ASA at 100 mg/kg oral administration (open circles) for FVB (A; $n \geq 8$) or C57Bl6 (C; $n \geq 6$) mice. Hemoglobin concentration in the chamber effluent (containing 10 ml NaCl 0.9%) following bleeding time was measured for FVB (B; $n \geq 8$) and C57Bl6 (D; $n \geq 6$) mice.

Figure 2. *Ex vivo* ASA effect on platelet aggregation and TXB₂ synthesis. Blood from FVB mice was taken 30 min or 3 hours after vehicle (closed symbols) or ASA at 100 mg/kg oral administration (open symbols). Washed platelet aggregation was measured by monitoring OD (405 nm) variations of a platelet suspension at a concentration of 3.5×10^8 platelets per milliliter under stirring conditions in response to 5 $\mu\text{g/ml}$ collagen (A1; $n \geq 9$; A2: typical aggregation curves) or 1 μM U46619 (C; $n \geq 8$). Results are expressed as the percentage of aggregation (seen as a decrease in OD) 300 sec after agonist addition. After collagen-induced aggregation and a centrifugation step, TXB₂ was quantified in the supernatant (B; $n \geq 5$), and results are normalized relative to the vehicle condition (*Control, grey bars*) for each time point.

Figure 1

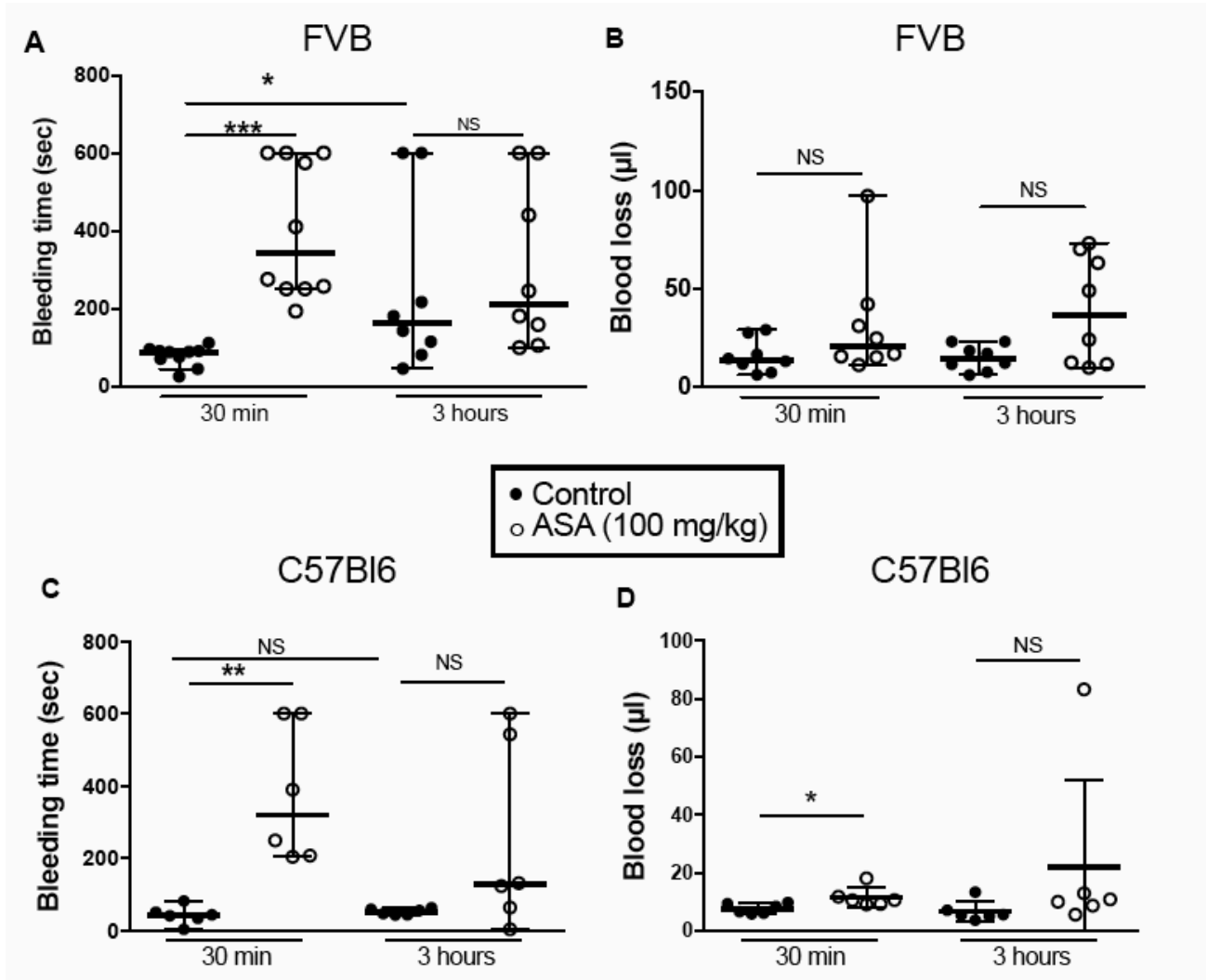


Figure 2

