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Letter to the Editors-in-Chief

Evaluation of a new generation platelet-derived hemostatic agent in a rabbit thrombocytopenic model

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1. Introduction

One of the leading causes of potentially survivable deaths (PSD) is trauma-induced hemorrhage, accounting for up to 90% and 26–40% of PSD in the military and civilian populations respectively [1,2]. A contributing factor to mortality following severe trauma/hemorrhage is trauma-induced coagulopathy (TIC), which is present at time of admission in 25% of patients, with low platelet counts and platelet dysfunction being principal components of TIC [3]. While fresh platelet transfusion has been used as a therapeutic strategy to correct TIC, fresh platelets have a limited shelf life of less than one week [4]. Thus, there is a need to develop strategies to increase the shelf life of platelets or provide platelet-like hemostatic function in the form of an alternative therapeutic to achieve hemostasis.

Platelet lyophilization increases shelf life and results in a therapeutic with platelet-like hemostatic capabilities which is called a platelet-derived hemostatic agent (PDHA). The manufacturing process involves the covalent cross-linking of surface membrane proteins and lipids that allows stabilization for lyophilization as well as results in a state of partial activation which theoretically improves the ability of the particles to home in on sites of endothelial disruption [5]. Prior to use, the PDHA is simply rehydrated with water.

A new generation of the PDHA Stasix® has been created using a proprietary process which provides a foundation for future product scale up. However, this new generation PDHA requires evaluation for efficacy, and determination of the dose-response curve for the therapeutic dosage range. The rabbit thrombocytopenic injury model is well-established for evaluating platelet function and hemostatic agents including human platelets [6,7]. In this study, we sought to determine the efficacy of the new generation of Stasix®, a PDHA, at increasing dosages in a rabbit thrombocytopenic model of injury. We hypothesized that this new generation PDHA would improve hemostasis in a dose-dependent manner.

2. Materials and methods

2.1. New Zealand White Rabbit Use and Institutional Animal Care and Use Committee (IACUC) compliance

The study protocol was approved by the IACUC at the 711th Human Performance Wing, JBSA-Fort Sam Houston and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011). New Zealand White rabbits (*Oryctolagus cuniculus*) were pair-housed at the Tri-Services Research Laboratory at Joint Base San Antonio (JBSA)-Fort Sam Houston, TX in accordance with the Secretary of the Navy Instruction (SECNAVINST) 3900.38C regulations.

2.2. PDHA formulation

The newest generation (proprietary formulation) of Stasix® (Entegrion, Research Triangle Park, NC) was reconstituted just prior to injury in sterile water per the manufacturer's instructions. The study tested three dosages of Stasix® particles/microliter (μL), which were based on animal weight and total blood volume: 1×10^4 particles/μL, 3×10^4 particles/μL, and 1×10^5 particles/μL. A thrombocytopenic control (saline) group as well as a healthy reference control group were also used for a total of 5 groups.

Abbreviations: PSD, potentially survivable death; TIC, trauma-induced coagulopathy; PDHA, platelet-derived hemostatic agent; MCF, maximum clot firmness; CBC, complete blood count; ROTEM, rotational thromboelastometry; SEM, standard error of the mean

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2.3. Surgical injury

Busulfan was prepared as described previously to induce thrombocytopenia with busulfan injections of 25 mg/kg on day 0 and 3 [6] and ear bleeding surgery performed as described on day 13 [7]. Animals were sedated with a combination of 20 mg/kg ketamine and 0.5 mg/kg acepromazine *via* intramuscular injection and anesthesia was maintained using inhalational isoflurane (2.0%). Vitals were monitored with a pulse oximeter/heart rate monitor. The femoral vein was cannulated utilizing an 18–23 g catheter while the ear vein used a 22–44 g catheter. Ear bleeding was initiated as previously described [7]. Animals were randomized to receive Stasix® or saline (vehicle control) injection into the femoral catheter immediately after injury ($n = 6$ per group). Investigators were blinded to treatment type and dosage. Healthy animals ($n = 3$) did not receive busulfan and were subjected to the same bleeding procedure with saline used as the treatment to produce non-thrombocytopenic controls.

2.4. Bleeding time

Two independent timekeepers recorded the time for visible bleeding to cease as described [7]. Laboratory technicians who were responsible for timekeeping were blinded to the treatment and doses.

2.5. Blood sample collection and laboratory assays

Whole blood was collected from either the ear catheter or the femoral catheter for laboratory assays. The laboratory assays included a complete blood count (CBC) using a ProCyte Dx® (IDEXX, Westbrook, ME) hematology analyzer to determine platelet count as well as rotational thromboelastometry (ROTEM® Delta system, TEM Systems Inc., Durham, NC, USA) to evaluate extrinsic coagulation pathway function (EXTEM) using maximum clot firmness (MCF). CBC blood was drawn before busulfan injection from the ear catheter (baseline), and then from the femoral catheter the day of surgery prior to injury (pre-treatment), and 1 min after injection of Stasix® or saline (post-treatment) in MiniCollect EDTA tubes (Greiner Bio-One, Kresmunster, Austria). ROTEM® blood was collected from the femoral catheter during pre-treatment and post-treatment of Stasix®-treated or saline-treated animals in BD Vacutainer® (Becton Dickson, Franklin Lakes, NJ) sodium citrate tubes.

2.6. Ex vivo analysis

ROTEM® was utilized for *ex vivo* evaluation of EXTEM MCF. Whole blood was collected from the femoral catheter at the time of exsanguination of thrombocytopenic animals. Saline or 1×10^5 Stasix® particles/μL was spiked into the thrombocytopenic blood and ROTEM® EXTEM MCF determined ($n = 3$ experimental replicates).

2.7. Statistical analysis

Statistical analysis was performed using one-way ANOVA (bleeding time) or two-way repeated measures ANOVA (for time-points) with *post hoc* Bonferroni analysis. Results are shown as mean ± the standard error of the mean (SEM) with significance at $p < 0.05$.

3. Results

Before busulfan injection (baseline), all animals were non-thrombocytopenic as demonstrated by the mean platelet count for each group and were similar to the healthy reference control animals. Busulfan treatment significantly reduced the platelet counts in all groups of the thrombocytopenic animals compared to the healthy reference controls at the pre- and post-treatment time-points ($p < 0.0001$ for all groups) (Fig. 1A). In the highest PDHA dosage group (1×10^5 particles/μL), the post-treatment platelet count was significantly increased compared to pre-treatment ($p < 0.0001$).

Healthy, non-thrombocytopenic animals displayed a rapid bleeding time (1.02 ± 0.31 min) while thrombocytopenic animals had a significantly increased bleeding time (12.46–14.3 min) ($p \leq 0.002$), regardless of treatment (Fig. 1B). No significant differences were observed between groups.

ROTEM analysis in healthy, non-thrombocytopenic animals had a significantly higher MCF compared to thrombocytopenic animals ($p < 0.05$) (Figs. 1C). In thrombocytopenic animals, MCF at the pre- and post-treatment time-points were not significantly influenced by PDHA injection between groups ($p > 0.99$ for all groups) or within groups ($p \geq 0.05$) (Fig. 1C). In *ex vivo* analysis, MCF was significantly lower ($p = 0.006$) with PDHA treatment compared to thrombocytopenic controls (Fig. 1D).

4. Discussion

In this study, the efficacy of a new generation of the PDHA Stasix® was evaluated in a rabbit model of thrombocytopenia. Using 3 different doses, no difference in ear bleeding time or MCF by ROTEM was observed between the controls and PDHA dosage groups. Further, ROTEM analysis *ex vivo*, did not demonstrate any significant improvement in MCF with the PDHA compared to controls, but instead had a slight but significant reduction, suggesting that the lack of efficacy is likely due to inactivity of the PDHA itself, rather than a short circulation time as circulation time has no role in *ex vivo* analysis.

Previous studies by our group and others have shown mixed results with PDHAs. A porcine hemorrhage model using an earlier generation of Stasix® found that a dosage of 2300 particles/μL achieved hemostasis in 60% of animals [8]. A follow-up study using an uncontrolled hemorrhage primate model with a later generation of Stasix® at 36,000 particles/μL observed no significant improvement with Stasix® [9]. Another report using lyophilized porcine and human platelets in a porcine model of liver injury at a dosage of 31,000 platelets/μL observed no significant reduction in blood loss with either type of PDHA [10]. Thus, the previous studies as well as the current report suggest that further refinement of PDHA's are indicated before transitioning into human trials.

5. Conclusions

The results of this study demonstrate that the new formulation of Stasix® does not significantly reduce ear bleeding time or improve MCF at