G\textsubscript{12/13} Signaling Pathways Substitute for Integrin αIIBβ3-Signaling for Thromboxane Generation in Platelets

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Abstract

**Background:** We have previously shown that ADP-induced TXA\textsubscript{2} generation requires signaling from αIIBβ3 integrin in platelets. Here we observed that, unlike ADP, protease-activated receptor (PAR)-mediated TXA\textsubscript{2} generation occurs independently of αIIBβ3. PAR agonists, but not ADP, activate G\textsubscript{12/13} signaling pathways. Hence, we evaluated the role of these pathways in TXA\textsubscript{2} generation.

**Principal Findings:** Inhibition of ADP-induced thromboxane generation by fibrinogen receptor antagonist SC57101 was rescued by co-stimulation of G\textsubscript{12/13} pathways with YFLLRNPy. This observation suggested an existence of a common signaling effector downstream of integrins and G\textsubscript{12/13} pathways. Hence, we evaluated role of three potential tyrosine kinases; c-Src, Syk and FAK (Focal Adhesion Kinase) that are known to be activated by integrins. c-Src and Syk kinase did not play a role in ADP-induced functional responses in platelets. Selective activation of G\textsubscript{12/13} pathways resulted in the activation of FAK, in the absence of integrin signaling. Interestingly, αIIBβ3-mediated FAK activation occurred in a Src family kinase (SKP)-independent manner whereas G\textsubscript{12/13} pathway caused FAK activation in a SKP and RhoA-dependent manner. A FAK selective inhibitor TAE-226, blocked TXA\textsubscript{2} generation. However, in comparison to WT mice, Pf4-Cre/Fak-Floxed mice did not show any difference in platelet TXA\textsubscript{2} generation.

**Conclusions:** Therefore, we conclude that differential activation of FAK occurs downstream of Integrins and G\textsubscript{12/13} pathways. However, the common effector molecule, possibly a tyrosine kinase downstream of integrins and G\textsubscript{12/13} pathways contributing to TXA\textsubscript{2} generation in platelets remains elusive.

Introduction

Platelet activation is an essential component of hemostasis and thrombosis, and involves engagement of complex signaling machinery. Injury to sub-endothelium results in platelet adhesion, and subsequent spreading on exposed collagen. Platelet activation also leads to reorganization of platelet cytoskeleton, release of granular contents from dense and alpha granules and finally culminates in integrin activation leading to platelet aggregation [1,2,3]. ADP released from the dense granules and the thromboxaneA\textsubscript{2} (TXA\textsubscript{2}) generated from activated platelets further act as positive feedback mediators and amplify the initial platelet responses and stabilize the hemostatic plug [4,5].

TXA\textsubscript{2} is generated from its precursor arachidonic acid through cyclooxygenase pathway [6]. TXA\textsubscript{2} acts on the TP (Prostanoid) receptors and recruits more platelets to the site of injury. Platelets can be activated by broad range of agonists, which can be further classified as strong or weak. ADP, serotonin, and epinephrine, are considered weak agonists [7] whereas thrombin, SFLLRNPy (PAR1 agonist), AYPGKF (PAR4 agonist) [8], and Convulxin (GP VI agonist) [9] are strong agonists. Not only these agonists activate platelets with varied potencies but they also induce distinct signaling pathways. Previous studies have shown that ADP-induced TXA\textsubscript{2} generation in platelets is integrin-dependent [10] however; it is not known whether stronger agonists such as thrombin depend on integrin-mediated signaling for TXA\textsubscript{2} generation.

ADP activates two G-protein coupled receptors P2Y\textsubscript{1} and P2Y\textsubscript{12}, activating G\textsubscript{q} and G\textsubscript{i} pathways, respectively [1,3]. In platelets, neither of the ADP receptors can couple to G\textsubscript{12/13} proteins whereas PAR receptors couple to G\textsubscript{12/13} proteins [11]. G\textsubscript{12/13} pathways have been shown to activate Rho kinase [12] and Src family kinases (SKFs) [13]. Earlier studies have shown that G\textsubscript{12/13} pathways mediate calcium-independent shape change [12,14], and play a potentiating role in Akt phosphorylation [13] and dense granule secretion [15]. Co-stimulation of platelets with G\textsubscript{12/13} and G\textsubscript{i} also leads to platelet aggregation [16].

Agonist binding to platelet receptors results in complex intracellular signaling events termed as inside-out signaling, which...
leads to the activation of integrins αIIbβ3 and α2β1. Activated integrins change conformation and bind multivalent ligands such as fibrinogen and von Willebrand Factor (vWF) [17]. Signaling events from ligand binding to fibrinogen receptor are termed as outside-in signaling which, in turn regulate platelet adhesion, spreading, and clot retraction [10]. Outside-in signaling also causes phosphorylation of β3 cytoplasmic tails [19,20], and activation of Phospholipase C (PLC-γ) [21], tyrosine kinases such as c-Src, Syk [21] and Focal adhesion kinase (FAK) [22]. FAK is a 125 kDa protein, expressed in both megakaryocytes and platelets [23]. FAK is tyrosine phosphorylated on six tyrosine residues viz., Y397, Y407, Y576/577, Y681 and Y925 [24].

Other signaling molecules, involved in outside-in signaling are Protein Tyrosine Phosphatase-1B (PTP-1B) [25], Protein Phosphatase 1C (PP1c) [26], Calcium and integrin binding protein (CIB) [27] and Protein Kinase C-β (PKC-β) [28,29].

In this study we show that, G_{12/13} pathways, cause TXA₂ generation even when signaling from integrin is blocked. We present an evidence that c-Src and Syk kinase do not play a role in ADP-induced functional responses and FAK can be activated downstream of integrin αIIbβ3 as well as G_{12/13} pathways. However, G_{12/13}-mediated activation of FAK occurs via Rho kinase and SFKs, whereas integrin-mediated FAK activation is independent of SFKs. Studies to evaluate the role of FAK in thromboxane generation showed that FAK does not contribute to thromboxane generation downstream of integrins. In conclusion, neither c-Src, Syk nor FAK contribute to integrin-mediated thromboxane generation in platelets. Therefore, the common signaling molecule downstream of integrins and G_{12/13} remains yet to be identified.

**Results**

**Regulation of thromboxane generation in platelets by G_{12/13} pathways**

ADP-induced TXA₂ generation is dependent on integrin activation and as pre-treatment of platelets with fibrinogen receptor antagonist (SC57101) abrogated ADP-induced TXA₂ generation [10]. We investigated the effect of a fibrinogen receptor antagonist (SC57101) on PAR-mediated TXA₂ generation. Searle Research developed SC57101 (Skokie, IL) based on the RGDS structure. SC57101 and its analogs are fibrinogen receptor antagonist and do not affect inside out signaling [10]. As shown in Fig. 1A, varying concentrations of AYPGKF, a PAR4 agonist, caused TXA₂ generation in the presence or absence of SC57101, indicating that PAR agonists cause thromboxane generation independently of integrin signaling. One of the main signaling differences between ADP and PAR agonists is that only PAR agonists can activate G_{12/13} pathways [11]. In order to evaluate the role of G_{12/13} pathways in platelet TXA₂ generation we stimulated platelets with 2MeSADP and/or YFLLRNP. 2MeSADP is a more potent agonist than ADP at the P2Y1 and P2Y12 receptors [30] and YFLLRNP is a weak agonist of PAR1 that selectively activates G_{12/13} pathways (at low doses) [16,31]. As shown in Fig. 1B, 2MeSADP-induced thromboxane generation was completely blocked in the presence of SC57101. YFLLRNP alone did not cause any significant TXA₂ generation in human platelets. However, co-stimulation of platelets with 2MeSADP and YFLLRNP in presence of SC57101 caused TXA₂ generation, indicating that the G_{12/13} pathways can rescue the inhibition rendered by fibrinogen receptor antagonist. Hence, G_{12/13} pathways can substitute for integrin-mediated signaling by probably activating similar effector molecules.

![Figure 1. Regulation of thromboxane generation by G_{12/13} pathways.](image)

Role of c-Src and Syk downstream of integrin αIIbβ3 in thromboxane generation in platelets

Previous studies have shown that c-Src and Syk are signaling effectors in platelets that are regulated by integrins [32]. In order to identify the common signaling molecules downstream of fibrinogen receptor and G_{12/13} pathways, we evaluated the role of these individual kinases in ADP-induced TXA₂ generation. We reasoned that as these tyrosine kinases are activated downstream of fibrinogen receptor and because ADP-induced thromboxane generation requires signaling events from fibrinogen receptor, at least one of these kinases could be crucial for ADP-induced thromboxane generation. 2MeSADP-induced phosphorylation of c-Src Y416 occurred in a time and concentration-dependent manner (Figs. 2A and B). However, 2MeSADP-induced TXA₂ generation in wild type mice was not different from the c-Src knockout mice (Fig. 2C), suggesting that either other Src family members may compensate for the absence of c-Src or that c-Src activation downstream of ADP receptors is not involved in TXA₂ generation. We then studied Syk kinase activation downstream of ADP receptors. Syk kinase was not activated downstream of ADP receptors (Fig. 2D), as determined by the phosphorylation of the Tyr525/526 residues [33]. Taken together, these results suggest,
that c-Src and Syk may not be not essential for ADP-induced thromboxane generation in platelets.

Focal Adhesion Kinase is activated downstream of integrins and G_{12/13} pathways

We next evaluated the activation of FAK by ADP receptors, using Y397 phosphorylation as an activation marker. FAK contains multiple tyrosine phosphorylation sites and the sequential tyrosine phosphorylations of these sites causes complete FAK activation beginning with autophosphorylation Y397 phosphorylation [34]. As shown in Figs. 3A & B, FAK is activated downstream of ADP receptors and this activation is blocked by a fibrinogen receptor antagonist but not by a pan SFK inhibitor PP2. These results indicate FAK activation by ADP occurs in an integrin-clustering-dependent manner, independent of SFKs.

We further investigated FAK activation downstream of G_{12/13} pathways. PAR agonists activate both G_{q} and G_{12/13} pathways in platelets [11]. The G_{q} pathways can be inhibited by YM254890 [35] without affecting the G_{i} or G_{12/13} pathways [13]. Since YFLLRNP is a weak, agonist for G_{12/13} pathways at low concentrations and at higher concentrations can also activate G_{q} pathways [16], we chose to stimulate platelets with AYPGKF (500 μM) in the presence or absence of YM254890 (150 nM) (C). The lysates were then subjected to western blotting analysis and probed with anti- phospho- FAK (Y-397) and total FAK antibodies as lane loading control. The data represents the mean ± S.E.M (n = 3). The data was analyzed by ANOVA and * P<0.05 was considered significant (B). Aspirin-treated washed platelets were stimulated with AYPGKF (500 μM) in presence or absence of YM254890 (150 nM) (C). The lysates were then subjected to western blotting analysis and probed with anti- phospho- FAK (Y-397) and total FAK antibodies as lane loading control.

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YM254890 abrogates AYPGKF-induced platelet aggregation and secretion [13] and hence autophosphorylation of FAK on Y397 is through G_{12/13} pathways but not by outside-in signaling.

**Signaling pathways regulating FAK activation downstream of G_{12/13} pathways**

We have shown that G_{12/13} pathways activated by AYPGKF in the presence of YM254890 can activate FAK (Fig. 3A). Under these conditions we used pharmacological inhibitors to evaluate the signaling molecules that could regulate FAK phosphorylation. As shown in Figs. 4A&B, PP2, a pan SFK inhibitor abolished FAK phosphorylation mediated by G_{12/13} pathways. Our results from Figs. 4C&D show that Rho kinase inhibitors H1152 and Y27632 also markedly inhibited G_{12/13}-Mediated FAK phosphorylation. These results indicate that both SFKs and Rho kinase play an important role in FAK activation downstream of G_{12/13} pathways in platelets. In order to further delineate the signaling pathways downstream of G_{12/13}, we also studied Src Y416 phosphorylation in presence of Rho kinase inhibitors. As shown in Figs. 4E&F, G_{12/13}-Mediated Src Y416 phosphorylation was dramatically inhibited in presence of Rho kinase inhibitors H1152 and Y27632, thus suggesting that SFKs are activated downstream of Rho kinase in the G_{12/13} signaling cascade.

**Evaluation of FAK as a common signaling effector molecule regulating thromboxane generation downstream of integrins and G_{12/13} pathways**

We next evaluated whether activated FAK played a role in ADP-induced TXA_2 generation. TAE-226 has recently been identified as a selective inhibitor of FAK with an IC_{50} of 5.5 nM [36]. To determine the effect of FAK inhibition on ADP-induced thromboxane generation platelets were treated with varying concentrations of TAE-226. As demonstrated in Fig. 5A, TXA_2 generation was significantly inhibited by TAE-226 at a higher concentration of 2 μM. Pharmacological inhibitors are often known to have off target and broad-spectrum effects. The specificity of TAE-226 was never evaluated in platelets and reports suggest that TAE-226 inhibits Pyk2 (a Focal Adhesion kinase family member) with an IC_{50} of 5 nM [37]. Hence, we studied thromboxane generation in WT and Pf4-Cre/FAK-floxed mice platelets. Murine platelets from WT and Pf4-Cre/FAK-floxed were stimulated with 100 nM of 2MeSADP and thromboxane levels were measured from WT and Pf4-Cre/FAK-floxed mice samples. As shown in Fig. 5B there was no significant difference observed thromboxane levels and aggregation tracings (Fig. 5C) between WT and Pf4-Cre/FAK-floxed. These results suggest that TAE-226 might exhibit some non-specific effects, and FAK is not the common signaling molecule regulating thromboxane generation downstream of integrins and G_{12/13} pathways.

**Discussion**

Activated platelets release positive feedback mediators such as ADP and TXA_2. The molecular mechanisms involved in thromboxane generation by different platelet agonists are not clearly understood. Weaker agonists such as ADP require outside-in signaling through activated fibrinogen receptor to cause thromboxane generation in platelets. Patients with Glanzmann’s

![Figure 4. Signaling pathways regulating FAK activation downstream of G_{12/13} Pathways.](https://www.plosone.org/figure/4.4)

Aspirin-treated, washed human platelets were pre-treated with different inhibitors (as indicated) for 5 minutes at 37°C followed by stimulation with AYPGKF (500 μM) for 90 seconds under stirring conditions at 37°C in an aggregometer. The lysates were then subjected to western blotting analysis and probed with anti-phospho-FAK (Y-397) and total FAK antibodies as lane loading control (A &C) or anti-phospho- Src (Y416) and total c-Src antibodies as lane loading control (E). Quantitative data, normalized to the lane loading control, are representative of mean ± S.E.M (n = 3). The data was analyzed by ANOVA and * P<0.05 was considered significant (B, D, and F).

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Figure 5. Evaluation of FAK as a common signaling effector molecule regulating thromboxane generation downstream of integrins and G_{12/13} pathways. Non-aspirin-treated, washed human platelets were pre-treated with varying concentrations of TAE-226 for 5 minutes at 37 °C (A) and murine platelets from WT and Pf4-Cre/Fak-Floxed mice (B) were stimulated with 2MeSADP (100 nM) for 3.5 minutes and TXB\textsubscript{2} levels were analyzed as described for Figure 1. Aggregation tracings were measured from WT and Pf4-Cre/Fak-Floxed mice and representative tracings are shown (C). The values are representative of 3 independent experiments mean ± S.E.M (n = 3). The data were analyzed by ANOVA and student t-test, *P<0.05 was considered significant.
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thrombasthenia show defective thromboxane generation with ADP but not with thrombin [30]. Our results show that PAR agonists mediate TXA2 generation independent of the integrin signaling. Previous studies by Stefanini et al [39] showed that stimulation of platelets with high and low doses of GPVI agonist convulxin in the presence of integrin blockers showed a marginal decrease in thromboxane generation, indicating that thromboxane generation downstream of GPVI receptors is not solely dependent on integrins. Thus, these results indicate that platelets can generate thromboxane in an integrin-dependent and-independent manner.

Since PAR receptors can activate G12/13 pathways we reasoned that there might be a common signaling molecule, which could be activated by both G12/13 pathways (which are not activated by ADP) and integrins. This common signaling molecule might regulate TXA2 generation downstream of G12/13 pathways independent of integrins.

It has been known that several platelet agonists cause activation of G12/13, but not much is known about the intracellular signaling pathways downstream of this activation. Studies from our group have shown that G12/13 pathways activate Src family kinases in platelets [13]. Klages et al [12] showed that G12/13 proteins can activate tyrosine kinases such as Syk and c-Src. Interestingly these kinases are also known to be activated downstream of outside-in signaling [32]. Although integrins and G12/13 pathways activate c-Src, it is clear that this kinase has no significant role in thromboxane generation downstream of ADP receptors (Fig. 2C). Interestingly, thromboxane A2, an agonist that couples to Gq and signaling [32], shows that G13 binds to integrin signaling. Gong et al claim that Rho activation downstream of G12/13 pathways are independent of each other. Upstream of FAK, SFKs regulate FAK activation downstream of integrins and G12/13 pathways [13]. SFKs activate RhoA/Rho kinase pathways leading to calcium-independent shape change [12,14]. These pathways are also known to regulate dense granule release through RhoA/Rho kinase pathways [13]. Our studies show that SFKs regulate Rho kinase pathways regulate FAK activation through SFKs (Figs. 4E&F). This is a novel observation and indicates that Rho kinase pathways are the nodal point of SFK activation, ppIMβ phosphatase, and other pathways regulating dense granule release. We postulate that these pathways are independent of each other as; inhibition of SFKs (with PP2) has no significant effect on PAR-mediated shape change or dense granule release in aspirin-treated platelets [41]. Hence, SFK activation occurs downstream, rather than upstream, of RhoA/Rho kinase pathways upon G12/13 stimulation as outlined in Fig. 6. If SFK activation were to occur upstream of Rho kinase pathways, then SFK inhibition would have affected platelet shape change as well as dense granule release reaction in aspirin-treated platelets.

Furthermore, complementary approaches e.g. pharmacological FAK inhibitor TAE-226 and Pf4-Cre/Fak-Floxed mice were employed to evaluate role of FAK in thromboxane generation. Pre-treatment of platelets with various doses of TAE-226 did not lead to significant decrease in thromboxane levels. However, inhibition of thromboxane generation was observed at 2 μM concentration only (Fig. 5A). Similarly thromboxane generation was not affected in Pf4-Cre/Fak-Floxed mice, when compared to WT littermates (Fig. 5B). These results indicate that FAK does not contribute to thromboxane generation downstream of integrins and TAE-226 might have some non-specific effects on platelets.

Since our data indicates that loss of FAK does not translate into diminished thromboxane generation, it is possible that some other tyrosine kinases might be compensating for the loss of FAK in platelets, however it might be unlikely since previous studies in Pf4-Cre/Fak-Floxed mice showed differences in tail bleeding times and platelet spreading and no upregulation of Pyk2 expression was observed in FAK-Floxed mice [23]. Recently, another FAK inhibitor PF-573,228 was shown to inhibit platelet aggregation [42]. However, our platelet aggregation studies comparing WT and Pf4-Cre/Fak-Floxed mice did not show any differences in aggregation (Fig. 5C). Furthermore, as our previous studies have shown that ERK can be activated even in the presence of integrin antagonists [43] therefore, ERK cannot be the common effector downstream of integrins and G12/13 pathways.

Thus, we conclude that G12/13 pathways through a Rho kinase/SFK dependent manner activate FAK. However, FAK activation downstream of integrins occurs independently of SFKs. Finally none of the three-tyrosine kinases c-Src, Syk or FAK seems to play a role in thromboxane generation downstream of integrins. Thus, the common signaling effector, possibly a tyrosine kinase, contributing to thromboxane downstream of G12/13 pathways and integrins remains yet to be identified.

Ethical Statement

Approval for this study was obtained from the Institutional Review Board of Temple University (Philadelphia, PA), and mice were used for physiological measurements using the protocol ID number 3364, approved by the Institutional Animal Care and Use Committee (IACUC).

Materials and Methods

Materials

2MeSADP, Apyrase grade VII, human fibrinogen, acetylsalicylic acid, were obtained from Sigma (St. Louis, MO). SC57101 was gift from Searle Research and Development (Skokie, IL). Hexapeptide AYPGKF was custom synthesized at Invitrogen (Carlsbad, CA). Convulxin was purchased from Centerchem Inc. (Norwalk, CT). The heptapeptide, YFLLRNP was synthesized by Research Genetics (Norwalk, CT). The heptapeptide, YFLLRNP was synthesized by Research Genetics (Norwalk, CT). The heptapeptide, YFLLRNP was synthesized by Research Genetics (Norwalk, CT). The heptapeptide, YFLLRNP was synthesized by Research Genetics (Norwalk, CT).

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The Rho kinase inhibitor H1152 was obtained from Toronto Research Chemicals (North York, ON). Y-27632 was obtained from Calbiochem (San Diego, CA). YM-254890 was a gift from Yamanouchi Pharmaceuticals Co., Ltd (Ibaraki, Japan). TAE-226 was generously provided by Novartis Pharma AG, Switzerland. The other reagents were of reagent grade, and de-ionized water was used throughout.

Animals
8–12 weeks old Pf4-Cre, and Pf4-Cre/FAK-floxed mice were generated in accordance with previously described protocol [44]. The generation of Src KO mice was described previously [45] and these mice along with wild type littermates in C57BL/6 background were used in the experiments.

Preparation of washed human and murine platelets
All experiments with human volunteers were performed in accordance with Declaration of Helsinki. Whole blood was drawn from healthy, human volunteers selected from students, staff or workers at the Temple University with written informed consent. Donated blood was collected in tubes containing one-sixth volume of acid citrate dextrose (ACD) (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of de-ionized water). Citrated blood was centrifuged and platelets were isolated with previously established protocol [46].

Isolation of mouse platelets
Blood was collected from the vena cava of anaesthetized mice into syringes containing one-tenth blood volume of 3.8% sodium citrate as anticoagulant. Red blood cells were removed by centrifugation at 100,000 g for 10 min. PRP was recovered, and platelets were pelleted at 400,000 g for 10 min. The platelet pellet was resuspended in Tyrode’s buffer (pH 7.4) containing 0.01 unit/ml apyrase. The isolated platelets were subsequently used for experiments.

Western blot analysis
Aliquots of aspirin-treated, washed human platelets were lysed using Laemmli buffer in presence of dithiothreitol (DTT) (100 mM) and boiled for 10 min. The platelet lysates were loaded onto a 10% -Tris-glycine gel, subjected to SDS-PAGE (Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis), and transferred to PVDF membrane. Nonspecific binding sites were blocked by incubating the membrane in Tris–buffered saline-Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% [vol/vol] Tween 20) containing 3% [wt/vol] bovine serum albumin (BSA) and 5% [vol/vol] Irish cream for 30 min at RT, followed by incubating it overnight at 4°C with gentle agitation in the primary antibody (1:1000 dilution for anti-FAK Y397, anti-Src Y416, anti-Syk Y525/526, anti-Syk, Anti-FAK and anti-Src in TBST with
3% BSA). After washing with TBST, the membranes were probed with an alkaline phosphatase-labeled secondary antibody (1:5000 dilutions in TBST with 3% BSA) for 1 hour at RT. After additional washing steps, membranes were incubated with chloroformazepoxide (CDP, Staar® chemiluminescent substrate (Tropix, Bedford, MA) for 10 min at RT, and immune-reactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-3000 CH, Tokyo, Japan).

Measurement of TXA₂ generation in human and mice platelets

Washed human platelets (300 µl brought to a concentration of 2 x 10⁸ platelets/mL) were stimulated with 2µM S-nitrosoglutathione (100 nM) in a lumi-aggregometer at 37°C with stirring at 900 rpm, in the presence or absence of TAE-226. Similarly murine platelets from WT and Pf4-Cre/FAK-floxed mice (250 µl brought to a concentration of 2 x 10⁸ platelets/mL) were stimulated with 2µM S-nitrosoglutathione (100 nM). After 3.5 min of stimulation, the reaction was stopped by quickly freezing the sample in a dry ice-methanol bath. The level of TXB₂, the stable metabolite of TXA₂ was measured using a previously established protocol [47].

Statistical Analysis

The results were quantified, expressed as mean ± S.E.M. The data was statistically analyzed using Student’s t-test and ANOVA. *P<0.05; **P<0.01 were considered significant.

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Author Contributions

Conceived and designed the experiments: KB ISH AS SPK. Performed the experiments: KB PRI RTD JJ. Analyzed the data: KB JJ SPK. Contributed reagents/materials/analysis tools: ISH AS. Wrote the paper: KB SPK.
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