European Journal of Pharmacology 883 (2020) 173385

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Demonstration of an action pathway in mouse platelets leading to prolongation of bleeding time by fluoxetine

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ARTICLE INFO

Keywords:
α-granule release
Bleeding time
Fluoxetine
Platelet aggregation
Serotonin

ABSTRACT

Fluoxetine is one of SSRIs commonly used as first-line antidepressants. It also induces adverse effects, including bleeding events. This study clarified the bleeding effect of fluoxetine and explored the action cascade of this drug leading to a longer bleeding time. A total of 48 male adult mice were evenly distributed into four groups and given fluoxetine in saline at 0, 4, 8, or 16 mg/kg, for 14 days. On day 15, tail bleeding time of 6 mice/group was measured, and their blood samples were collected for analyses of relevant platelet functions. The remained mice were allowed to survive for another 14 days without fluoxetine, and subjected to the same analyses on day 29. A significant effect of fluoxetine was reveled on bleeding time ($F_{(3,20)} = 16.842$, $P < 0.01$) and intraplatelet serotonin ($F_{(3,20)} = 90.967$, $P < 0.01$). Moreover, fluoxetine effectively inhibited platelet aggregation ($F_{(3,20)} = 30.247$, $P < 0.01$), decreased amount of GPIbα ($F_{(3,20)} = 23.855$, $P < 0.01$), suppressed GPIIb/IIIa activation ($F_{(3,20)} = 89.441$, $P < 0.01$), and lowered P-selectin ($F_{(3,20)} = 7.960$, $P < 0.01$) on platelet surface. Negative correlations existed between bleeding time and the aforementioned four indices, whereas correlations between intraplatelet serotonin and the same indices were positive. All changes returned to same levels as Control group after fluoxetine withdrawal. These data suggest an action pathway of fluoxetine starting at binding to serotonin transporter, followed by decreased intraplatelet serotonin, increased GPIbα shedding, suppressed GPIIb/IIIa activation, and inhibited α-granule release, and concluding with prolonged bleeding time in mice.

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are a class of antidepressants that have been used as first-line drugs for the treatment of depressive disorders. These drugs share a common action of binding to serotonin transporter (SERT) and inhibiting the reuptake of serotonin from extracellular space into presynaptic cells by SERT (Artigas et al., 2002). Via this action, SSRIs are able to increase the level of neurotransmitter serotonin in the synaptic cleft available to binding to the postsynaptic receptors. This approach to increasing the effectiveness of a neurotransmitter (monoamine) has been thought to be the mechanism of action of most of antidepressants including SSRIs and serotonin-norepinephrine reuptake inhibitors. In addition to depressive disorders, SSRIs are also frequently prescribed for anxiety disorders including social anxiety disorder, panic disorder, obsessive-compulsive disorder, eating disorders, social phobia, and premenstrual dysphoric disorder (Masand and Gupta, 1999).

With the extensive application of SSRIs in clinical practice, there is increasing clinical reports associating SSRIs with a number of potential drug-drug interactions and adverse effects including gastrointestinal disturbances, headache, sedation, insomnia, activation, weight gain, impaired memory, excessive perspiration, paresthesia, and sexual dysfunction (Joshi, 2018; Masand and Gupta, 1999). Examples of drug-drug interaction are those between SSRIs and warfarin or clopidogrel, which increased bleeding risk, such as minor bruising, even bleeding (Hemeryck and Belpaire, 2002; Yuet et al., 2019). Moreover, SSRIs were reported to increase risk of gastrointestinal tract (GI) bleeding and intracranial bleeding independent of other bleeding risk factors (Dalton et al., 2003). The SSRIs-induced GI bleeding has been associated with a drug's potency of inhibiting serotonin reuptake or its receptor affinity as suggested by a case-control study (Lewis et al., 2008), in which increased odds of GI bleeding were attributed to moderate or high-affinity SSRIs among 359 case participants and 1889 control participants. Similar findings were noted in a cohort study with

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https://doi.org/10.1016/j.ejphar.2020.173385
Received 11 April 2020; Received in revised form 13 July 2020; Accepted 20 July 2020
Available online 23 July 2020

36,389 antidepressant users registered in an electronic medical record database in Massachusetts (Castro et al., 2012). 333 cases of GI bleeding were observed in the low-affinity group (n = 14,927) while 601 GI bleeding cases were found in the high-affinity group (n = 21,462).

In exploring the underlying mechanism for the increased odds of bleeding in human patients treated with SSRIs, investigators have paid attention to effects of SSRIs on platelets, which play central roles in hemostasis process comprising successive four steps of adhesion, aggregation, secretion, and procoagulant activity (Halperin and Reber, 2007). As early as 1992, abnormal platelet aggregation was associated with fluoxetine therapy (Alderman et al., 1992). Paroxetine, another SSRI, was shown to inhibit platelet plug formation while decreasing intraplatelet serotonin in human beings. Moreover, paroxetine was reported to lower expression of CD63, a platelet activation marker, in response to two concentrations of thrombin receptor-activating peptide (Hergovich et al., 2000). In patients with congestive heart failure treated with antecedent aspirin, SSRIs decreased platelet activity as manifested by significant reductions in adenosine diphosphate (ADP) and collagen-induced aggregation, as well as lower expression of the platelet surface glycoprotein (GP) receptors GPIb and GPIIb/IIIa. SSRIs also resulted in a reduced formation of platelet-leukocyte microparticles (Serebruany et al., 2003). In a recent study, citalopram inhibited platelet aggregation, adhesion and thromboxane production in human volunteers (Roweth et al., 2018).

Although some clinical studies suggest that SSRIs may affect haemostasis and thrombosis as reviewed above, these effects are not well understood mechanistically and useful animal study is scarce. The present study aimed to clarify effect of fluoxetine, a high-affinity SSRI, on bleeding time in mice, and to explore the mechanism for the effect. On the basis of previous clinical studies, we expected fluoxetine would increase bleeding time in mice. The main work of this study was to explore the mechanism for the effect of fluoxetine on bleeding time via examining effects of the drug on the main steps of hemostasis process, i.e., platelet adhesion, aggregation, and secretion.

2. Materials and methods

2.1. Materials

Fluoxetine, bovine serum albumin (BSA), HEPES and apyrase were purchased from Sigma Aldrich (St. Louis, MO, USA). Prostaglandin E1 (PGE1) was purchased from Meilun Biotech (Dalian, China). Bovine plasma thrombin was purchased from Solarbio Life Science (Beijing, China). Antibodies used for flow cytometry including anti-GPIbα (CD42b-Dylight 649), anti-GPIIb/IIIa (Leo.F2-Dylight 649), anti-JON/A-PE, and anti-CD62P-FITC were purchased from EMFRET ANALYTICS (Wórzburg, Germany). Serotonin ELISA kits were purchased from Elabscience (Wuhan, China).

2.2. Animals

Adult male ICR mice (8-weeks old, 30–35 g) were obtained from Vital River Laboratory (Beijing, China). The mice were housed in groups of 2 in standard polypropylene cages under a temperature-controlled condition (23 ± 1 °*C*, 50–60% humidity) with a 12-h light/dark cycle (lights on at 08:00) and food and water available ad libitum. All experiments involving animals were performed in strict accordance with the Guidelines established by Chinese National Institutes of Health. The protocol was approved by the Ethics Committee of Animal Experiments of Shantou University Medical College (SYXK, 2017–0079).

2.3. Experimental design and fluoxetine administration

A total of 48 mice were randomly assigned into four groups (12 mice/group) and given fluoxetine in saline by gavage at 0, 4, 8, or 16 mg/kg, for 14 days (referred to as Control, F4, F8, and F16 groups, respectively). Fluoxetine was dissolved in sterilized saline at the concentrations of 0.5, 1.0, and 2.0 mg/ml for the F4, F8, and F16 groups, respectively. The volume of saline and fluoxetine solution administered each time was 0.24 ml. These doses of fluoxetine approximately equate to 20, 40, and 80 mg/d for a person with the body weight of 60 kg according to a formula of dose translation (Reagan-Shaw et al., 2008).

Two hours after the last gavage, half number of mice in each group were subjected to bleeding time measurement under anesthesia with 1% sodium pentobarbital (40 mg/kg). Blood samples of mice were collected, and platelet-rich plasma was prepared for next analyses including platelet aggregation test, enzyme-linked immunosorbent assay (ELISA) to measure intraplatelet serotonin, and flow cytometry analysis with specific antibodies to label corresponding targets on platelets. The remained mice (6 mice/group) were allowed to survive for an additional 2 weeks in the absence of fluoxetine. At the end, these mice were subjected to the same experimental analyses mentioned above.

2.4. Measurement of tail bleeding time

Mouse was anaesthetized intraperitoneally with 1% sodium pentobarbital (0.04 ml/10 g). Then his tail was incised transversely at 5 mm from the tip with a sterilized knife and immediately immersed in a tube filled with sterilized warm saline (37 °C). The time from incision to complete bleeding cessation was recorded. Bleeding time exceeding the cut-off time (12 min) was noted as 720 s.

2.5. Sample preparations for flow cytometry, platelet aggregation test and ELISA

Under the anesthesia condition as described before, blood of mouse was drawn from cardiac ventricle using an anticoagulant-coated syringe and used for subsequent analyses. For flow cytometry, 40 μl blood sample from each mouse was pipetted into a centrifuge tube containing 10 μl of 3.2% sodium citrate solution (1: 4) and further diluted (1: 20) in 1 ml modified Tyrode's buffer. The diluted blood sample then was centrifuged at 900×*g* for 5 min at room temperature (RT). The supernatant was removed, and pellet was resuspended with 1 ml modified Tyrode's buffer.

For platelet aggregation test, blood sample was centrifuged at 100×*g* for 10 min at RT, then the platelet-rich plasma (PRP) was pipetted into a new tube. After the addition of 5 nM PGE1, the PRP was centrifuged at 1200×*g* for 5 min at RT. Then the pellet was resuspended in modified Tyrode's buffer supplemented with 0.02 U/ml apyrase and 5 nM PGE1. After repeating the same step, the platelet pellet was resuspended in 600 μl Tyrode's buffer containing 0.02 U/ml apyrase.

For ELISA, 480 μl blood sample collected from each mouse was pipetted into a tube containing 120 μl 3.2% sodium citrate solution and centrifuged at 100×*g* for 10 min at RT. The supernatant was transferred into a new tube and Tyrode's buffer was added to 1 ml. After sufficient mixing, 200 μl of this diluted PRP was centrifuged at 4500×*g* for 10 min at 4 °C. The supernatant was discarded, and 200 μl double distilled water was added to resuspend platelet pellets. The isolated platelets were then stored under −80 °C until use for ELISA.

2.6. Platelet aggregation test

The platelet aggregation test was performed. Briefly, 295 μl of platelet suspension from each mouse was used and stirred at 37 °C with a small magnet in an aggregometer (PRECIL, China), which detects platelet aggregation in PRP according to the changes in light transmission (wavelength: 740 nm, 37 °*C*). To activate platelets response, thrombin was added at the final concentration of 0.1 U/ml. The transmittance alterations were expressed as a curve and monitored for 5 min, which resulted in the maximum aggregation rate (MAR) of platelets. Double measurements were performed with the sample from each mouse. Of the various platelet agonists, thrombin was chosen in this

study given that it is the most potent activator of platelets (Kahn et al., 1998). The final concentration of thrombin used in this study was 0.1 U/ml, which is much lower than those required for the other platelet agonists (2, 5, 10, 20 μM for ADP; 1, 5, 10, 20 μg/ml for collagen) (Zhou et al., 2020). As for VWF, the binding of it to its platelet receptor occurs under high shear rate flow conditions present in arteries and arterioles thus discouraging the ex vivo application of it (Savage et al., 1996). Serotonin per se is a weak agonist of platelet, but it regulates numerous biological processes including cardiovascular function, bowel motility, bladder control, and so on (Berger et al., 2009). In addition, serotonin dose-dependently enhances platelet activation induced by ADP and, in particular, thrombin in whole blood (Li et al., 1997).

2.7. Flow cytometry analysis

For platelet surface receptors, after preparation of washed whole blood sample, single-labelling analysis was performed. In brief, 26 μl washed whole blood sample was pipetted into each of three FACS (fluorescence-activated cell sorting) tubes (tube 1, 2, & 3) containing 9 μl PBS, then 5 μl of PBS, Dylight 649-conjugated anti-CD42b or anti-GPIIb/IIIa was added into tube 1, 2, & 3, respectively. Tube 1 was used as control. After 3-sec vortex mixing, the mixed solutions were incubated at RT in the dark for 15 min. The incubation was stopped by addition of 400 μl PBS. Then the samples were analyzed by flow cytometry after gentle mixing. The platelets in tube 1 were identified by a FACSCalibur (Becton Dickinson, USA) based on logarithmic amplification in forward scatter (FSC) and side scatter (SCC). The data were analyzed using FlowJo 7.6 (Tree Star, Ashland, USA) after gating on the platelet population according to FSC/SCC. Theoretically, this approach may exclude bigger and smaller platelets from counting if they are beyond the size range determined by FSC and SCC. Logically, bigger platelets may result from platelet aggregation while smaller ones may be due to damage or split of platelets. However, the sample preparation procedure employed in this study unlikely produced platelet aggregation in absence of thrombin and any other platelet agonist. Nor was the procedure able to split platelets as it involved only vortex mixing of washed whole blood sample diluted in PBS.

For investigation of platelet activation, 26 μl of washed blood sample in a FACS tube containing 4 μl of PBS or thrombin (0.1 U/ml) was labeled simultaneously by 5 μl of Leo.F2-Dylight 649 and of FITC-conjugated anti-P-selectin (CD62P), or by 5 μl of anti-CD42b-Dylight 649 and of PE-conjugated anti-activated GPIIb/IIIa (JON/A). The mixed solutions were vortex mixed for 3 s followed by incubation in the dark at RT for 15 min. Then the reaction was ceased by addition of 400 μl PBS just before the measurement. All the tests were completed within 1 h. The proportion of positive labelling and mean fluorescence intensity (MFI) were measured by the Calibur flow cytometer and the data were analyzed using FlowJo 7.6.

2.8. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to measure intraplatelet serotonin following the instruction provided by the manufacturer (Elabscience, Wuhan, China). Briefly, the standard and sample solutions in duplicate (50 μl/well) were added into the ELISA plate wells. Then, 50 μl biotinylated detection antibody (anti-5-HT) working solution was added immediately to each well, followed by the incubation for 45 min at 37 °C. After rinsed with PBS three times, 100 μl HRP-conjugated working solution was added to each well, followed by the incubation for 30 min at 37 °C. After rinsed with PBS three times, 90 μl of substrate reagent was added to each well, followed by the incubation for 15 min at 37 °C. Finally, 50 μl of stop solution was added and immediately the optical density of each well was readout under a microplate reader (Tecan, Switzerland) set to 450 nm. The readouts were analyzed with ELISA Calc software (Customized Applications, Inc., Chicago, USA).

2.9. Statistical analysis

Data were presented as mean ± S.D. One-way ANOVA was performed followed by Student- Newman-Keuls (SNK) test for post-hoc comparisons. The Pearson correlation coefficients between bleeding time and the functional parameters of platelets were calculated. Bootstrap method based on 1000 bootstrap samples was applied to determine 95% confidence interval (CI) of the correlation coefficient. $P < 0.05$ was regarded statistically significant.

3. Results

3.1. Subchronic administration of fluoxetine prolonged tail bleeding time in mice while decreased intraplatelet serotonin in a dose dependent manner

SSRIs-related hemorrhage usually occurs as post-surgery and post-partum events (Perrotta et al., 2019; Roose and Rutherford, 2016; van Haelst et al., 2010), suggesting a prolonged bleeding time subsequent to SSRIs administration. On the other hand, it has been reported that SSRIs decrease intraplatelet serotonin (Blardi et al., 2002; Knorr et al., 2019; Zhuang et al., 2018). Therefore, the first task of this study was to check if intraplatelet serotonin decrease is concomitant with a prolonged bleeding time in fluoxetine-treated mice. Fluoxetine at four dosages (0, 4, 8, 16 mg/kg) in sterilized saline was given to mice once a day by gavage for consecutive 14 days. At the end, tail bleeding time and intraplatelet serotonin of mice were evaluated. The data from the four groups were analyzed by one-way ANOVA followed by SNK post-hoc comparisons. The results indicated a significant effect of drug treatment on bleeding time in mice ($F_{4,20} = 16.842$, $P < 0.01$). Post-hoc comparisons indicated: 1) F4 group was comparable to Control group; 2) compared to the Control group, F8 and F16 groups had significantly longer bleeding time; 3) the difference between F8 and F16 groups was also significant (Fig. 1A). As for intraplatelet serotonin, one-way ANOVA showed a significant effect of fluoxetine too ($F_{4,20} = 90.967$, $P < 0.01$). Post-hoc comparisons indicated: 1) all fluoxetine-treated mice presented significantly lower intraplatelet serotonin compared to Control group; 2) the higher the dose of fluoxetine, the lower the intraplatelet serotonin (Fig. 1B). Pearson correlation coefficient between intraplatelet serotonin and bleeding time was $r = -0.64$ (95% CI -0.83, −0.32; $P < 0.01$).

3.2. Subchronic administration of fluoxetine inhibited platelet aggregation

The prolonged bleeding time in fluoxetine-treated mice may be due to decreased platelet number (numeral density) and/or suppressed platelet aggregation, which is crucial in thrombus formation (Bismuth-Evenzal et al., 2012). With this consideration, we counted platelet number by flow cytometry after gating on the platelet population according to FSC/SCC (Fig. 2A), in addition to measuring the platelet aggregation in mice. The former was expressed as platelet number/-sample (3×10^4 cells), and the latter was recorded as MAR (Fig. 2B). The data of the two experiments were analyzed by one-way ANOVA followed by SNK post-hoc comparisons. One-way ANOVA showed no effect of fluoxetine ($F_{4,20} = 0.640$, $P = 0.598$) on platelet numbers in mice (Fig. 2C). However, fluoxetine showed a significant effect on MAR ($F_{4,20} = 30.247$, $P < 0.01$). Post-hoc comparisons indicated: 1) F4 group was comparable to Control group; 2) compared to the Control group, F8 and F16 groups had significantly lower MARs; 3) the difference between F8 and F16 groups was also significant (Fig. 2D).

3.3. Subchronic administration of fluoxetine decreased platelet surface GPIbα in mice

Of the four steps in hemostasis process, the first one is the adhesion of platelets to subendothelial matrix. This step is mediated by the binding of von Willebrand factor (vWF) to platelet surface receptor GPIb/IX/V complex. The major ligand-binding subunit of the complex is GPIbα. As

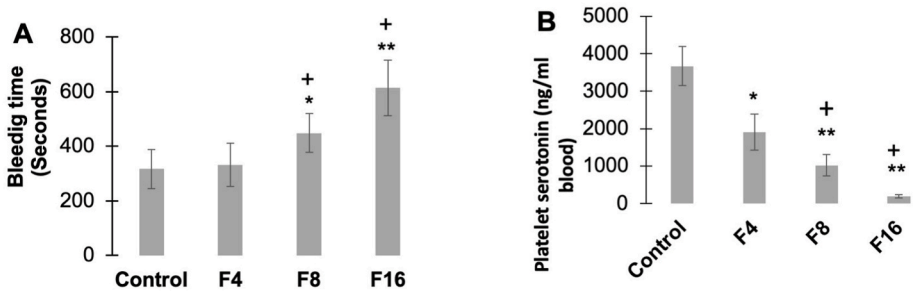


Fig. 1. Fluoxetine prolonged tail bleeding time while decreased intraplatelet serotonin in a dose-dependent manner. All fluoxetine-treated mice, except for those in F4 group, showed longer tail bleeding time compared to controls. The difference between F8 and F16 groups was also significant (A). Fluoxetine decreased platelet serotonin content in a dose-dependent manner (B). Data were expressed as means ± S.D. (n = 6/group). *P < 0.05, **P < 0.01, compared to Control group. +P < 0.05, comparisons were made between F8 and F4, or between F16 and F8 groups.

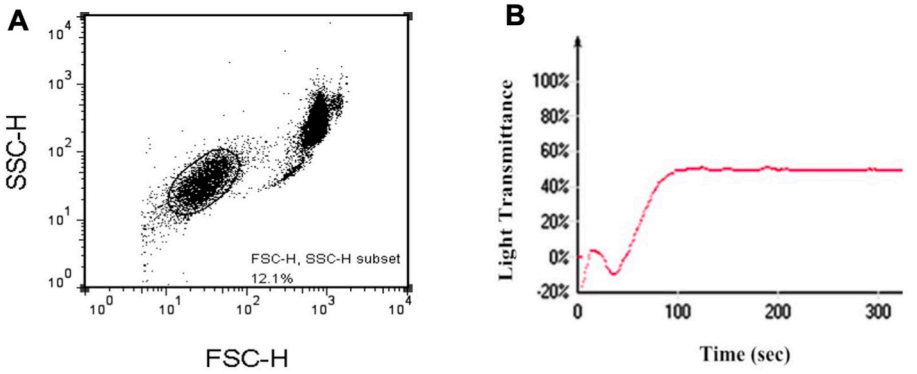
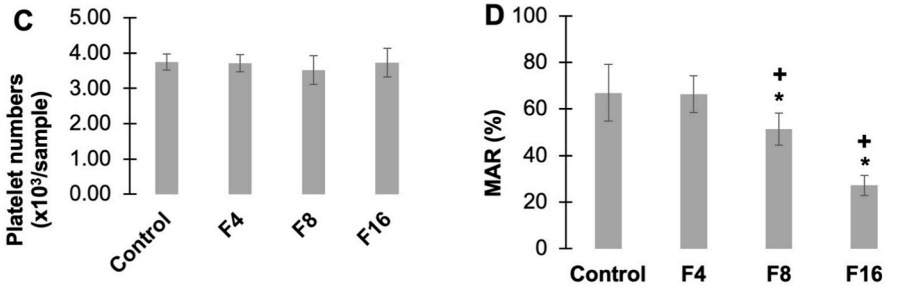


Fig. 2. Fluoxetine decreased platelet aggregation ratio but had no effect on platelet number in the mouse. Select platelets in every 30,000 blood cells via FSC/SSC gating strategy (A). Platelet aggregation rate curve in the presence of thrombin (0.1 U/ml) (B). All fluoxetine-treated mice were comparable to Control group in terms of platelet number (C). All fluoxetine-treated mice, except for those in F4 group, showed lower platelet aggregation rates compared to Controls (D). Data were expressed as means ± S.D. (n = 6/group). *P < 0.05, compared to Control group. + P < 0.05, comparisons were made between F8 and F4, or between F16 and F8 groups.



such, we identified the presence of GPIbα and quantified the amount of it on platelet surface using anti-CD42b-Dylight 649 by flow cytometry. In absence of thrombin, all four groups were comparable in terms of ratios of CD42b-Dylight 649 labeled platelets in total platelets ($F_{4,20} = 0.987$, $P = 0.419$) (Fig. 3A). However, differences did exist among the four groups in terms of the MFI of CD42b-Dylight 649 labelling ($F_{4,20} = 23.855$, $P < 0.01$), suggesting that fluoxetine treatment deceased GPIbα amount on platelet plasma membrane. Post-hoc comparisons indicated: 1) F4 group was comparable to Control group; 2) compared to the Control group, F8 and F16 groups had significantly lower MFIs; 3) the difference between F8 and F16 groups was also significant (Fig. 3B).

3.4. Subchronic administration of fluoxetine suppressed the thrombin-induced activation of GPIIb/IIIa

The platelet aggregation depends upon the most prominent platelet membrane protein GPIIb/IIIa, which is also known as integrin (αIIbβ3), and its ability to serve as a receptor for extracellular ligands–fibrinogen and vWF (Koltai et al., 2017). In resting platelets GPIIb/IIIa presents in its inactive form. Once activated by ADP, thrombin, or other agonists,

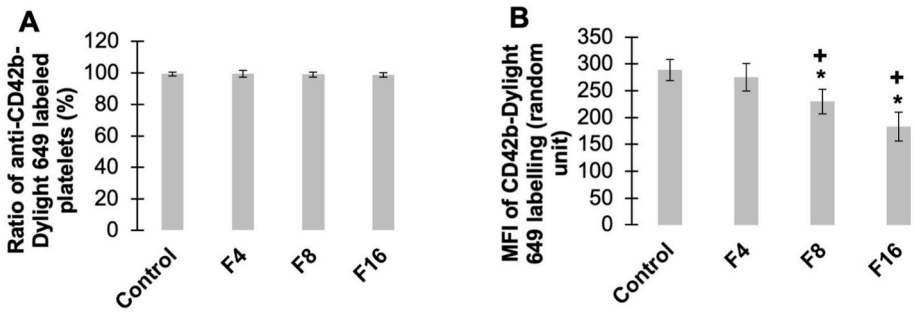


Fig. 3. Fluoxetine decreased GPIbα amount on platelet surface although had no effect on intraplatelet GPIbα in the mouse. All fluoxetine-treated mice were comparable to Control group in terms of intraplatelet GPIbα (A). All fluoxetine-treated mice, except for those in F4 group, showed lower levels of GPIbα on platelet surface compared to Controls (B). Data were expressed as means ± S.D. (n = 6/group). *P < 0.05, compared to Control group. +P < 0.05, comparisons were made between F8 and F4, or between F16 and F8 groups.

the conformation of it changes into its fibrinogen binding form (active form). And the receptor-bound fibrinogen connects two GPIIb/IIIa molecules on nearby platelets (Bennett, 2015; Kauskot and Hoylaerts, 2012). With this rationale in mind, we identified and quantified the inactive and active forms of GPIIb/IIIa by means of double-labeled flow cytometry.

The active form of GPIIb/IIIa was identified by PE-conjugated anti-activated GPIIb/IIIa (JON/A) while the inactive GPIIb/IIIa was identified by Leo.F2-Dylight 649. The washed whole blood sample was incubated with the above antibodies in PBS or 0.1 U/ml thrombin in PBS. The ratio of JON/A-PE labeled platelets/total platelets and ratio of Leo.F2-Dylight 649 positive platelets/total platelets were calculated; in addition, the MFI of JON/A-PE and MFI of Leo.F2-Dylight 649 were quantified. The former two indices indicate the presence of active and inactive GPIIb/IIIa on platelet surface while the latter two relate to the amount of active and inactive forms of GPIIb/IIIa. In absence of thrombin, all four groups were comparable in terms of ratios of Leo.F2-Dylight 649 labeled platelets ($F_{4,20} = 0.765$, $P = 0.527$) and MFI of Leo.F2-Dylight 649 labelling ($F_{4,20} = 0.875$, $P = 0.470$), suggesting that fluoxetine had no effect on the resting platelets (Fig. 4A & B). In presence of thrombin, all four groups presented comparable ratios of JON/A-PE positive platelets ($F_{4,20} = 0.135$, $P = 0.938$), suggesting that fluoxetine had no effect on number of platelets carrying activated GPIIb/IIIa (Fig. 4C). However, differences did exist among the four groups in terms of the MFI of JON/A-PE ($F_{4,20} = 89.441$, $P < 0.01$), suggesting that fluoxetine treatment inhibited the thrombin-induced activation of GPIIb/IIIa. Post-hoc comparisons indicated: 1) F4 group was comparable to Control group; 2) compared to the Control group, F8 and F16 groups had significantly lower MFIs; 3) the difference between F8 and F16 groups was also significant (Fig. 4D).

3.5. Subchronic administration of fluoxetine reduced the thrombin-induced α-granule release

The third step of the hemostasis process is platelet granule release. Of the three types of platelet granules, α-granules store adhesive molecules such as vWF and fibrinogen which are important for platelet adhesive properties and building of a stable thrombus (Maynard et al., 2007; McNicol and Israels, 1999). During the degranulation process of α-granules, the membrane of them fuses with the plasma membrane of platelets. As such, P-selectin (CD62P), one of the most abundant glycoproteins on the membrane of α-granule, can translocate to platelet membrane. Through the interactions with its ligands, the P-selectin on platelet surface determines size and stability of platelet aggregates (Merten and Thiagarajan, 2000).

Here, we examined possible effects of subchronic fluoxetine medication on α-granule release in platelets by means of flow cytometric analysis. Washed whole blood sample was incubated with anti-CD62P-FITC in absence or presence of 0.1 U/ml thrombin. Platelets were identified by expression of GPIIb/IIIa. The ratios of CD62P-FITC positive platelets/total platelets and MFI of CD62P-FITC were quantified. In absence of thrombin, all four groups showed comparable ratios of CD62P-FITC positive platelets (Fig. 5A), indicating that subchronic administration of fluoxetine had no effect on the number of platelets containing α-granules ($F_{4,20} = 1.792$, $P = 0.181$). In presence of thrombin, however, all four groups were different in terms of MFI of CD62P-FITC in platelets ($F_{4,20} = 7.960$, $P < 0.01$), suggesting that the treatment did influence the extent of α-granule release in platelets. Post-hoc comparisons indicated: 1) F4 group was comparable to Control group; 2) compared to the Control group, F8 and F16 groups had significantly lower MFIs (Fig. 5B).

3.6. Correlations between fluoxetine-related changes in platelet function

To analyze the correlations between the fluoxetine-induced changes in platelet function measured in this study, the Pearson correlation coefficients between bleeding time and each of the others (except for intraplatelet serotonin) and correlation coefficients between intraplatelet serotonin and each of the others (except for bleeding time) were calculated. The results are shown in Table 1. Moderate negative correlations were found in measurements of bleeding time/amount of GPIbα [r = −0.63 (95% CI, −0.83, −0.31)] and in bleeding time/α-granule release (P-selectin) [r = −0.58 (95% CI, −0.80, −0.23)]; Strong negative correlations were revealed in bleeding time/GPIIb/IIIa activation [r = −0.81 (95% CI, −0.91, −0.60)] and in bleeding time/platelet aggregation [r = −0.83 (95% CI, −0.92, −0.64)]; Strong positive correlations were found in intraplatelet serotonin/amount of GPIbα [r = 0.85 (95% CI, 0.67, 0.93)] and in intraplatelet serotonin/GPIIb/IIIa activation [r = 0.77 (95% CI, 0.51, 0.89)]; Moderate positive correlations were seen in intraplatelet serotonin/platelet aggregation [r = 0.68 (95% CI, 0.39,

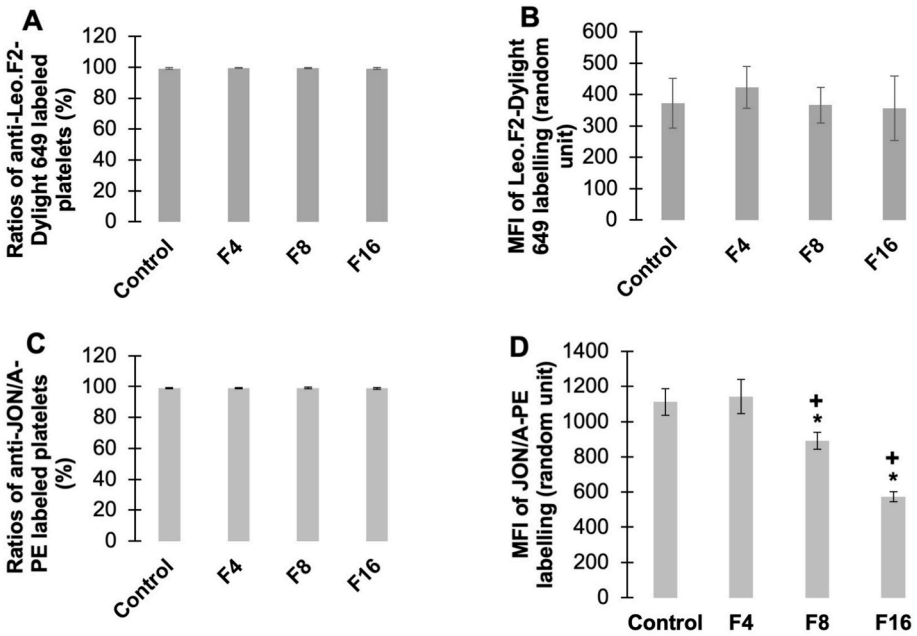


Fig. 4. Fluoxetine inhibited the thrombin-induced activation of GPIIb/IIIa on platelet surface. In absence of thrombin, all four groups were comparable in terms of ratios of Leo.F2-Dylight 649 labeled platelets and MFI of Leo.F2-Dylight 649 labelling, suggesting that fluoxetine had no effect on the expression of (inactive) GPIIb/IIIa in platelets (A & B). In presence of thrombin, all four groups presented comparable ratios of JON/A-PE positive platelets, suggesting that fluoxetine had no effect on number of platelets carrying activated GPIIb/IIIa (C). In presence of thrombin, platelets from F8 and F16 groups showed lower MFI of JON/A-PE, suggesting that fluoxetine treatment inhibited the thrombin-induced activation of GPIIb/IIIa (D). Data were expressed as means ± S.D. (n = 6/group). *P < 0.05, compared to Control group. +P < 0.05, comparisons were made between F8 and F4, or between F16 and F8 groups.

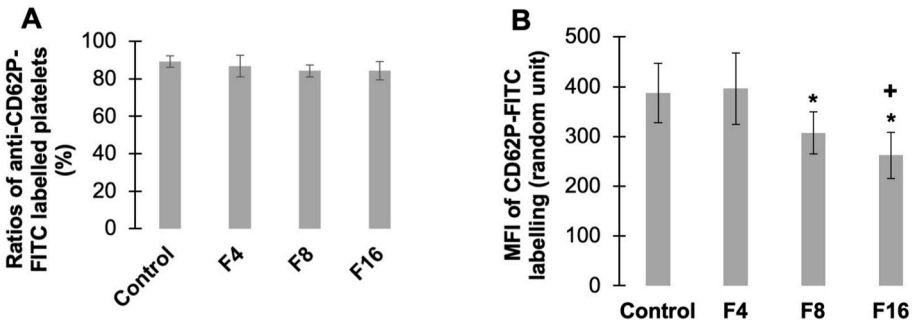


Fig. 5. Fluoxetine reduced the thrombin-induced α-granule release in the mouse. In absence of thrombin, all four groups showed comparable ratios of CD62P-FITC positive platelets, indicating that fluoxetine had no effect on the number of platelets containing α-granules (A). In presence of thrombin, platelets from F8 and F16 groups showed lower MFI of CD62P-FITC in platelets, suggesting that the treatment did influence the extent of α-granule release in platelets (B). Data were expressed as means ± S.D. (n = 6/group). *P < 0.05, compared to Control group. +P < 0.05, comparison was made between F16 and F8 groups.

Table 1
Correlations between outcomes resulted from fluoxetine administration.

Outcomes	MAR	MFI of CD42b-Dylight 649 labelling	MFI of JON/A-PE labelling	MFI of CD62p-FITC labelling
Bleeding time	−0.83 (−0.92, −0.64)	−0.63 (−0.83, −0.31)	−0.81 (−0.91, −0.60)	−0.58 (−0.80, −0.23)
Intraplatelet serotonin	0.68 (0.39, 0.85)	0.85 (0.67, 0.93)	0.77 (0.51, 0.89)	0.70 (0.42, 0.86)

Note: Data are expressed as r (95% CI). N = 24. r > 0.7 indicates a strong correlation; 0.4 < r < 0.7 indicates a moderate correlation. MAR, maximum aggregation rate.

0.85)] and in intraplatelet serotonin/α-granule release [r = 0.70 (95% CI, 0.42, 0.86)].

3.7. Complete restoration of fluoxetine-induced changes after the drug withdrawal

In order to provide further evidence that fluoxetine prolongs bleeding time via effecting on platelets, we checked if the fluoxetine-induced changes can recover to normal levels after a recovery period. For this purpose, we did the same analyses (except for ELISA) as described before using blood/platelet samples from the second batch of animals (n = 6/group) that continued to survive for two weeks after withdrawal of fluoxetine. As shown in Table 2, all fluoxetine-induced changes in F8 and F16 groups returned to normal levels in Control group. These data suggest that the fluoxetine-induced changes in platelet-relevant indices are reversible.

4. Discussion

As early as 1990, Hergovich et al., 2000; Humphries and Wheby, 1990; Joshi, 2018 described a patient who developed petechiae and

Table 2
Complete restoration of fluoxetine-induced changes following the drug withdrawal.

	Control	F8	F16	F	P
Bleeding time (S)	303.33 (79.92)	296.67 (65.01)	326.67 (54.28)	0.329	0.724
MAR (%)	64.83 (18.80)	71.68 (13.44)	59.81 (21.22)	0.650	0.536
MFI of JON/A-PE	1042.00 (123.34)	1048.67 (122.81)	1154.50 (124.22)	1.568	0.241
MFI of CD42b-Dylight 649	306.00 (71.67)	336.17 (25.69)	320.00 (24.58)	0.641	0.541
MFI of CD62p-FITC	356.33 (44.72)	355.00 (68.60)	422.33 (53.20)	2.797	0.093

Note: Data are expressed as mean (SD). N = 6/group. MAR, maximum aggregation rate.

prolongation of the bleeding time while received fluoxetine therapy. Since then, this adverse effect of fluoxetine has obtained special attention as evidenced by intermittent case reports describing fluoxetine-induced bleeding (Ottervanger et al., 1993; Vandel et al., 2001), fluoxetine-associated bruising (Pai and Kelly, 1996) and ecchymosis (Mirsal et al., 2002). However, cohort studies reported inconsistent results. For example, Berk et al. (1995) failed to demonstrate any compromised hemostatic function (international normalized ratio, partial thromboplastin time, factors II, V, VII, VIII:C, IX, X, XI, XII, fibrinogen, thrombin time, bleeding time, and so on) associated with fluoxetine therapy given to ten patients with major depression. In another study (Lainé-Cessac et al., 1998), the hemostatic function (prothrombin time, partial thromboplastin time, thrombin time, plasma fibrinogen, platelet counts, bleeding time, platelet aggregation induced by ADP, epinephrine, ristocetin, collagen, and arachidonic acid) of depressive patients was studied before and after fluoxetine treatment (20 or 40 mg daily) for one month. Fluoxetine treatment did not increase bleeding time although it decreased platelet aggregation. In a recent study, patients receiving fluoxetine (20 mg per day) were followed up for 3 months to examine the effect of the drug on coagulation parameters encompassing bleeding time, clotting time, platelet count, prothrombin time and partial thromboplastin kaolin time. At the end of 3rd month, fluoxetine-treated patients showed a significantly prolonged bleeding time, but within the normal range (Siddiqui et al., 2011). In summary, these data from clinical studies suggest that effects of fluoxetine on bleeding time and hemostatic function of depressive patients may be influenced by multiple factors such as dose and duration of the medication. In attesting this inference animal experiments possess advantages over clinical studies as a variety of doses of a drug can be tested in one animal study. As an example, mice in the present study were given fluoxetine at three doses of 4, 8, and 16 mg/kg/d, which approximately equate to 20, 40, and 80 mg/d for adult persons (Reagan-Shaw et al., 2008). The two higher doses, but not the lowest dose of fluoxetine, significantly prolonged bleeding time in mice (Fig. 1A). This result is coherent with the aforementioned human studies in which 20 mg/d fluoxetine had no effect or only mild effect on bleeding time (Lainé-Cessac et al., 1998; Siddiqui et al., 2011). Taken together, all these data confirm that 20 mg/d fluoxetine used for adult persons is generally safety while it actions as an antidepressant.

Fluoxetine significantly decreased intraplatelet serotonin in mice relative to controls in a dose-dependent manner (Fig. 1B). This is consistent with the results of human studies showing that fluoxetine (20 mg/d for 4 weeks or 30 days) decreased intraplatelet serotonin while improved the clinical presentations of depressive patients (Blardi et al., 2002; Zhuang et al., 2018). This decrease in intraplatelet serotonin is due to the inhibition of serotonin uptake into platelets by fluoxetine (Lemberger et al., 1985), specifically the inhibition of platelet SERT activity by fluoxetine as demonstrated in our recent human study (Zhuang et al., 2018). Similarly, citalopram-treated patients had significantly lower intraplatelet serotonin compared to control subjects (Li et al., 2015). Furthermore, the present study showed that bleeding time prolongation was concomitant with intraplatelet serotonin

decrease in fluoxetine-treated mice. Furthermore, a significant negative correlation existed between intraplatelet serotonin and bleeding time in fluoxetine-treated mice. In line with our findings, serotonin decrease in platelets isolated from citalopram-treated mice paralleled bleeding phenotype in a recent study (Oliver et al., 2016). In humans, paroxetine decreased intraplatelet serotonin concentrations while inhibited platelet plug formation (Hergovich et al., 2000). This causal relationship between platelet serotonin decrease and bleeding phenotype encouraged further investigations on effects of fluoxetine on the platelet functions central in hemostasis process.

Following the aforementioned rationale, we counted platelet number and measured the platelet aggregation in mice as the first task. As expected, subchronic administration of fluoxetine did not change platelet numbers in mice (Fig. 2A), but significantly decreased MAR of platelets in a dose-dependent manner (Fig. 2B). This result is in accordance with previous human studies showing abnormal platelet aggregation associated with fluoxetine therapy (Alderman et al., 1992; Lainé-Cessac et al., 1998). Pertinently, citalopram was shown to inhibit platelet aggregation (Roweth et al., 2018), specifically inhibit ADP- and collagen-induced platelet aggregation (Atar et al., 2007; Tseng et al., 2010). Moreover, the MAR of fluoxetine-treated mice was positively correlated to intraplatelet serotonin (0.68; 95% CI: 0.39, 0.85), but negatively correlated to bleeding time (−0.83, 95% CI: 0.92, −0.64) (Table 1), suggesting that fluoxetine prolonged bleeding time via decreasing intraplatelet serotonin which in turn inhibited platelet aggregation. A question is, however, what did occur between intraplatelet serotonin decrease and platelet aggregation inhibition?

To answer the above question, we examined effect of fluoxetine on GPIbα, which is the major ligand-binding subunit of the GPIb/IX/V complex on platelet surface and a major player in adhesion step of the hemostasis process. A previous in vitro study reported that platelet surface expression of GPIb decreased in the escitalopram-pretreated samples (Atar et al., 2007). In a clinical study, the augmented expression of GPIb in major depression patients was normalized following escitalopram treatment for 24 weeks (Lopez-Vilchez et al., 2014). In accordance with these previous studies, subchronic administration of fluoxetine in mice deceased platelet surface GPIba in the presence of thrombin in a dose-dependent manner but the treatment did not change the ratio of anti-CD42b-Dylight 649 labeled platelets to the total platelets in absence of thrombin (Fig. 3). This result suggests that GPIba decrease on platelet surface of fluoxetine-treated mice was likely due to increased shedding of it in the presence of thrombin, which is known to induce GPIbα shedding (Bergmeier et al., 2004). Why did the GPIbα shedding happen at different extents in the Control and fluoxetine-treated mice? Our interpretation is, via inhibiting the uptake of serotonin from plasma into platelets, fluoxetine decreased intraplatelet serotonin (Fig. 1B) but increased plasma serotonin, which in turn led to more activation of 5-HT2AR on platelet surface thus increased GPIba shedding. In support of this interpretation, plasma fluoxetine and serotonin levels increased concurrently after drug administration and reached the highest levels on the 30th day of fluoxetine (20 mg); whereas intraplatelet serotonin decreased following the drug administration and the lowest values were observed on the same day (Blardi et al., 2002). Further supporting evidence came from one of our recent studies showing that fluoxetine administration (started at 20 mg/d and titrated to 40 mg/d) for 4 weeks significantly decreased the higher levels of intraplatelet serotonin in depression and anxiety disorder patients while it effectively improved scores of HAMD and HAMA of the patients (Zhuang et al., 2018). Furthermore, in a very recent study of our team, patients withdrawn from SSRIs for more than 4 weeks showed the same higher levels of intraplatelet serotonin as the patients who never took the drugs before participated in the study (unpublished data). In line with these human studies, an animal study elegantly demonstrated: 1) serotonin induced shedding of GPIbα in isolated platelets, and this effect was increased to 60% in the presence of fluoxetine; 2) ketanserin (a 5-HT2AR antagonist) reversed the effect of fluoxetine plus serotonin whereas DOI (a selective 5-HT2AR agonist) elicited a similar response as serotonin; 3) $Tph1^{-/-}$ mice (TPH1 is the rate-limiting enzyme for the synthesis of peripheral serotonin; Walther et al., 2003) contained significantly less glycocalicin in the plasma relative to wild-type mice (Duerschmied et al., 2009). Although the present study did not measure plasma glycocalicin in mice, GPIba decrease on platelet surface of fluoxetine-treated mice reflected the same event of increased shedding of GPIbα given glycocalicin is a product of enzymatic cleavage of GPIbα.

Different from GPIbα, GPIIb/IIIa is a major player in platelet aggregation. Once activated by its agonists, GPIIb/IIIa changes its conformation into active form able to bind to fibrinogen (Bennett, 2015; Kauskot and Hoylaerts, 2012). In the present study, fluoxetine showed no effect on the expression of GPIIb/IIIa in platelets, but it inhibited the thrombin-induced activation of GPIIb/IIIa at the two higher doses (Fig. 4). This finding is coherent with the data of a previous study indicating that citalopram inhibited GPIIb/IIIa activation while suppressed convulxin-induced platelet aggregation (Tseng et al., 2010). However, this consistence does not necessarily mean that citalopram and fluoxetine share a same mechanism for the inhibition of GPIIb/IIIa activation. Indeed, lower (nanomolar) concentrations of citalopram inhibited serotonin uptake into platelets but had no effect on other platelet functions, which were inhibited by higher (micromolar) concentrations. These data indicate that citalopram-induced inhibition of platelets in vitro is not mediated by blockade of SERT (Roweth et al., 2018). On the other hand, platelets isolated from $SERT^{(-/-)}$ or citalopram-treated mice showed reduced activation of G-proteins coupled to 5-HT2ARs and receptor surface expression, indicating that sustained SERT function loss reduces 5-HT2AR surface expression critical for the synergistic activation of GPIIb/IIIa by serotonin and ADP (Oliver et al., 2016). The data from the present study suggest that both serotonin and functional SERT are necessary for the activation of GPIIb/IIIa, although we did not examine effect of fluoxetine on the expression/function of 5-HT2AR on platelet surface.

The third step of hemostasis process is platelet secretion manifested as platelet granule release. We examined effect of subchronic fluoxetine medication on α-granule release and found the inhibition of thrombin-induced α-granule release by fluoxetine (Fig. 5B). In line with this result, citalopram was shown to inhibite α-granule secretion from platelets in response to collagen as evidenced by a reduced expression of P-selectin (Tseng et al., 2010). Pertinently, patients treated with paroxetine for one week showed significantly lower plasma levels of platelet α-granule release products beta-thrombo-globulin (BTG) and platelet factor 4 (PF4) relative to baseline values. The BTG and PF4 levels remained low at 3- and 6-week measurements (Pollock et al., 2000). Although none of these studies investigated the mechanism underlying the inhibition of platelet α-granule release by SSRIs, it is plausible that inhibiting GP IIb/IIIa activation may adversely influence α-granule release in platelets given that activation of GP IIb/IIIa occurred within the first 10 s, whereas P-selectin was expressed progressively over a period of $\geq$ 10 min (Merten and Thiagarajan, 2000). In support of this speculation, the initial bridging of GP IIb/IIIa-fibrinogen was considered to be necessary to approximate P-selectin with its binding site (Boukerche et al., 1996). Therefore, we may suggest that the both events (inhibition of GP IIb/IIIa activation and α-granule release) may be due to platelet serotonin decrease resulted from subchronic administration of fluoxetine.

It is important to note that fluoxetine-treated mice presented negative correlations between bleeding time and levels of the aforementioned three major players (GPIba, GPIIb/IIIa, and P-selectin) in the platelet adhesion, aggregation and α-granule release steps, respectively (Table 1). This finding is in agreement with the distinct roles of these three members in the successive three steps of hemostasis process as discussed above. On the other hand, they all positively correlate with intraplatelet serotonin (Table 1), confirming that they are distinct members in a series or an action pathway of the hemostasis process.

Taken together, the action pathway started from platelet serotonin decrease, which resulted from subchronic administration of fluoxetine, and concluded with bleeding time prolongation.

Last but not least, all the fluoxetine-related changes returned to the same levels as those in Control group on day 14 following withdrawal of the medication (Table 2). The outcomes include bleeding time, MFI of CD42b-Dylight 649, MAR, MFI of JON/A-PE, MFI of CD62p-FITC. The first two derived from resting platelets while the last three were obtained following ex vivo thrombin stimulation. These results not only consolidate the conclusions drawn from the first data set, but also indicate the reversibility of the fluoxetine-induced bleeding complications. Again, these data are in favor of the application of fluoxetine in clinical practice as one of first-line antidepressants.

Although the fluoxetine-induced suboptimal platelet responses described above were observed in the presence of thrombin in this study, the drug may exert the same effects on basal platelet activation in circulation given that these effects are correlated to decreased intraplatelet serotonin due to blockade of SERT by fluoxetine. In support of this inference, paroxetine decreased intraplatelet serotonin by 83% while prolonged closure time by 31% and lowered platelet activation in healthy male volunteers (Hergovich et al., 2000). On the other hand, the observed prolongation of bleeding time in fluoxetine-treated mice may involve the other factors such as those in the coagulation cascade, in addition to the platelet functions examined in this study. Indeed, paroxetine was shown to increase prothrombin ratio while it effectively improved depressive symptoms in patients with depression (Lederbogen et al., 2001).

5. Conclusions

In conclusion, the data of this animal study clarified the inhibiting actions of fluoxetine on platelet function. Specifically, via inhibiting SERT function, fluoxetine decreased intraplatelet serotonin but increased extracellular plasma serotonin level, which in turn promoted GPIbα shedding in the presence of thrombin. The inhibition of SERT function by fluoxetine also inhibited αIIbβ3 activation. In addition, platelet α-granule release subsequent to the initial platelet aggregation was suppressed due to decreased granule serotonin and/or loss of serotonin signaling in platelets. All the events happened in an action pathway and ultimately prolonged bleeding time in mice. Two weeks after withdrawal of fluoxetine, all the drug-induced changes completely returned to the same levels as those in the Control group.

Authorship contribution

Ru Li: Designed and carried out experiments, analyzed data and wrote original draft. Jingsi Qu, Cauru Wu, and Zeman Fang: Carried out some of experiments. Xiaohong Hong: Designed and supervised experiments. Haiyun Xu: Designed experiments, interpreted results, wrote the second draft, reviewed, and edited the final versions of the manuscript.

Declaration of competing interest

The authors declared no conflict of interest.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 81573510).

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