The Role of Glutamine in the Prevention of Ultraviolet-C-Induced Platelet Activation

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Background and Objectives. The primary function of platelets is to prevent bleeding. The use of UV-C light in the treatment of platelets has become a valuable method for preserving the efficacy of platelet concentrates in blood banks. However, its deleterious effect remains, such as the activation of platelets, thus causing the platelets to lose their physiological function. In this study, we intended to demonstrate the impact of UV-C on platelets and how the use of glutamine could mitigate the loss of physiological function of the platelets caused by UV-C.

Materials and Methods. This study was conducted using mouse platelets. We assessed calcium signaling using Fura-2 AM incubation and dense granule secretion of the platelets using luminescence assay by measuring ATP. At the molecular level, the activation of integrin using PAC-1 antibody was analyzed. Phosphorylation of immune-precipitated cPLA2 was assessed using a specific antibody. All the experiments were carried out with or without glutamine in the presence of UV-C. Positive and negative controls were used in all experiments to validate the findings.

Results. We have demonstrated that physiological and biochemical damage arises as a result of the exposure of platelet concentrate to UV-C and that the use of glutamine could alleviate this damage. Various experiments, thrombus formation, integrin activation, and phosphorylation of cPLA2 were preserved using 50mM of glutamine in the presence of UV-C, which reduces 50% of platelet viability.

Conclusions. Our study demonstrates that the storage of platelet concentrates under the UV-C activates their physiological process and renders them to the thrombus formation, hence decreasing their viability. The presence of a moderate amount of glutamine can alleviate the toxic effect of UV-C, and platelet concentrates could be kept viable for a long time.

1. Introduction

Platelets are tiny cellular fragments of large bone marrow cells (megakaryocytes). They make up only a minute fraction of the total blood volume. The primary function of platelets is to prevent bleeding.

Platelet storage in blood banks could lead to bacterial contamination and activation upon agitation, causing the platelets to lose their physiological function. Various measures have been adopted to minimize the loss of physiological function of stored platelet concentrates. The use of broad-spectrum wavelength MirasolTM Pathogen Reduction Technology System of ultraviolet (UV) light (UV-A (60%), UV-B (100%), and UV-C (20%)), in the presence of a photosensitizer riboflavin, has been widely adopted all over the world as the standard procedure for the prevention of bacterial and viral contamination [1]. Furthermore, this technology has advanced to include the narrow band shortwave, “theraflexTM” with a specific wavelength (254 nm) of UV-C light. It has become a valuable tool for preserving the safety and efficacy of platelet concentrates in blood banks [2, 3]. In some of the developed countries, this system has been adopted such that the platelets are subjected to a “one-shot” treatment and do not require any additives. However, it is the prolonged exposure to high energy UV, especially UV-B and UV-C, which can lead to severe malfunctioning of the platelets. In the developing or underdeveloped countries, MirasolTM system still prevails [4–9].
Alternatively, the INTERCEPT™ system is being employed in the developed countries which uses amotosalen, a photoactive compound that specifically targets DNA and RNA, coupled with UV-A illumination, and has shown encouraging results, [10] and above all, it is cost-effective [11, 12].

The beneficial effects of UV-C on platelet concentrates have been well documented [13, 14]. However, its pitfalls remain, such as its ability to decrease the physiological function of platelets [15] and generate ROS [16]. Other biomolecular processes that are transformed during the prolonged exposure of platelet concentrates to UV-C are lactic acid production, P-selection exposure, and phosphatidyl-serine exposure [16].

Despite the mentioned drawbacks of UV-C, some studies state to the contrary that UV-C light has an insignificant impact on platelet concentrates, and the pathogen inactivation increases the quality of platelets [14, 17]. As documented in the literature, UV-C irradiation of platelets effectively prevents the growth of pathogens [18, 19], but at the same time, it induces platelet aggregation [18] and somewhat decreases the physiological function of platelets. Such a decrease in physiological function inevitably leaves the platelets useless.

In a very insightful study conducted by Murphy et al., they estimated the level of 17 amino acids in the plasma during the storage of platelet concentrates. They concluded that the concentration of 16 amino acids in plasma remained unchanged, whereas the concentration of glutamine fell to zero by day four [20]. In our study, we used 50 mM concentration of L-glutamine in freshly prepared human or mouse platelets exposed to UV-C light for short intervals.

2. Materials and Methods

2.1. Reagents and Antibodies. Thrombin (T6884), L-glutamine (glutamine) (G3126), and ATP assay kit (FLAA) from Sigma, Fura-2 AM (F1221) from Invitrogen, CCK-8 (CK04) from Dijindo Laboratories, anti-human PAC-1 (340507) monoclonal antibody (mAb) from BD Biosciences, cPLA2 from Dijindo Laboratories, anti-human PAC-1 (340507) antibody (2832), and phospho-cPLA2 (Ser505) antibody (2831) from Cell Signaling Technology were obtained. Various other reagents for buffers were obtained from Sigma-Aldrich.

2.2. Platelet Preparation. Male mice, C57BL/6, 4 to 6 weeks, were inbred and maintained at the animal house of the university. All animal studies were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Chonbuk National University Medical School. Blood was drawn by cardiac puncture into acid citrate dextrose (ACD) (20 mM citric acid, 110 mM sodium citrate, and 5 mM glucose) 1:10 ratio v/v. Platelets were prepared as previously described [21]. Platelets in tyrode buffer (TB) or platelet-rich plasma (PRP) were then allowed to settle for 60 minutes at 37°C before their use for the experiment. For the calcium study, 0.02 U of apyrase was added during the resting period to minimize the effect of ADP and ATP. Calcium was added if stated, or else calcium-free buffer was used.

2.3. Platelet Aggregation. Aggregation assays, as previously described [21], were performed with Thermomax microplate reader (Molecular Devices SpectraMax plus) and connected to a computer using SOFTmax plus 4.0. The microplate reader was turned on at least 30 minutes earlier and prewarmed to 37°C. In brief, in clear flat-bottomed 96-well plates, an appropriate volume of TB-suspended platelets was added and stimulated with the agonist, thrombin 0.5 U/ml. The platelets were stirred for 5 seconds in a microplate reader, where the turbidity change at 650 nm was measured in units of % of light transmission (%LT) every 15 seconds, with the agitation of 3 seconds between readings for 10 minutes.

2.4. UV-C Irradiation. The appropriate volume and concentration of platelets with or without glutamine were irradiated from the above in 96- or 24-well plates, using Vilber Lourmat BLX-254 emitting at 254 nm. According to the company’s specification, platelets were irradiated with a constant intensity of 0.5 joules for 100 seconds at room temperature, with continuous shaking in an uncovered Petri dish of 60 × 15 mm.

Since Gravemann et al. had demonstrated that the use of 0.2 joules of UV-C in Theraflex™ causes minimal damage to αIIbβ3 and 0.6 joules of UV-C causes significant damage to integrin [22], we used 0.5 joules of UV-C to demonstrate that maximum exposure of platelets to UV-C, which can cause significant changes in integrin, could be ameliorated by the use of glutamine.

2.5. Measurement of Intracellular Calcium. TB-washed platelets were incubated by adding 1% bovine serum albumin (BSA) and 5 μM Fura-2 AM for 40 minutes. After the required time of incubation and after one wash, 1 ml of Fura-2-loaded platelets were subjected to a monochromator, Photon Technology International (PTI) in magnetically stirred cuvette at 37°C, with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in [Ca²⁺]i were monitored using Fura-2 340 nm/380 nm fluorescence ratio.

2.6. Immunoprecipitation and Western Blotting. Washed platelets were stimulated with 0.5 U/ml of thrombin or exposed to UV-C with or without 50 mM glutamine. The reaction was stopped with ice-cold cell lysis buffer containing 20 mM of HEPES (pH 7.2), 1% (v/v) Triton X-100, 10% glycerol, 100 mM of NaCl, 1 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride, 50 mM of NaF, 1 mM of Na₂VO₄, 10 g/ml of leupeptin, 10 μg/ml of pepstatin, and 10 μg/ml of aprotinin. Samples were left on ice for 30 minutes, and supernatants were obtained after centrifugation at 15,870 g for 10 minutes. For immunoprecipitation, cell lysates were precleared, and proteins were subjected to SDS-PAGE on 10% gel. After transferring to
the nitrocellulose membranes, the blots were incubated in blocking buffer and then incubated with primary Ab, cPLA2 (1:2000), or P-cPLA2 (1:2000) dilution in 1% BSA of blocking buffer overnight at 4°C. The blots were rinsed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad STAR121P) (1:5000 dilution) in the blocking buffer at room temperature for 60 minutes. The immune-reactive proteins, with the respective secondary antibody, were determined using an enhanced chemiluminescence kit (Amersham Biosciences AB) and exposed to a LAS-1000 ImageReader Lite (Fujifilm, Japan).

2.7. ATP Assay. TB-suspended platelets were stimulated with thrombin for the desired time at 37°C with or without glutamine in the EP tubes for 2 minutes. After the desired time period, the tubes were then spun at 15870 g for 1 minute. Appropriate volumes of the supernatant were transferred into other tubes and were measured, with Lumat LB 9507, by adding the appropriate volume of a luciferase luciferin mixture to the sample. Values obtained were plotted against the standard value of ATP.

2.8. Flow Cytometry. Washed platelets were stimulated with thrombin or exposed to UV-C, as described above, over a specified period, after which the reactions were stopped by adding 500 µl of ice-cold PBS. After 15 minutes in ice, the appropriate volume of the antibody was added and left on the rotor in a cold room for 5–10 minutes and then was subjected to BD FACS Calibur, along with positive and negative control. A total of 10,000 events per sample were collected and plotted as an one-parameter overlaid histograms of fluorescein fluorescence.

2.9. Viability Assay. TB-suspended platelets in a 96-well plate, with a final volume of 100 µl/well, were incubated in 50 mM of glutamine and exposed to UV-C or in a lytic buffer and left nontreated for the desired time. The viability assay was assessed using 10 µl of methylthiazoletetrazolium (MTT, Sigma). After 4 hours of incubation at 37°C, the medium was replaced with 100 µl of dimethylsulfoxide (DMSO, Sigma). The optical density was measured at 570 nm using a microplate spectrophotometer.

2.10. Statistics. Statistical analysis was carried out on raw data in SigmaPlot 9 using unpaired Student’s t-test; a probability value, $p < 0.05$, was considered statistically significant. Values are expressed as means with standard deviation, and "n" indicates the number of experiments.

3. Results

3.1. Glutamine Is Nontoxic and Does Not Prevent the Release Reaction of Dense Granules of Platelets. In order to assess whether glutamine could be toxic to the platelets, we used the viability assay (Figure 1(a)) depicting the nontoxic effect of glutamine on platelets. However, UV-C has decreased the viability of platelets to 50%. Furthermore, a release reaction of dense granules, which evoke secondary aggregation, was also not impaired when the platelets were stimulated with thrombin in the presence of glutamine (Figure 1(b)).

3.2. Glutamine Did Not Impair the Physiological Functions of Platelets. Having demonstrated the nontoxic effect of glutamine on the platelets, it was imperative to show if this dose of glutamine would impair the physiological functions of platelets. Intact calcium signal is cardinal to the functions of platelets. The presence of glutamine did not impair their physiological function when the platelets were stimulated with thrombin (Figure 2(a)). Statistical analysis of Figure 2(a) is shown in Figure 2(a).

Aggregation of platelets, upon stimulation with thrombin in the presence or absence of glutamine, is demonstrated in Figure 2(b). No significant difference was observed between the presence of 25 mM (data not shown so that graph is not congested with lines) and 50 mM concentration of glutamine. However, 100 mM of glutamine did show some oscillations after 5 minutes, but eventually, complete aggregation was achieved at the 10th minute. Statistical analysis of Figure 2(b) is shown in Figure 2(b).

3.3. Glutamine Impeded UV-C-Induced Integrin Activation. It was earlier demonstrated that UV-C-induced platelet activation by affecting the function of integrin, and this activation did not depend on intracellular pathways. However, the presence of glutamine impaired the UV-C-induced integrin activation. PAC-1 binding was assessed in the presence or absence of glutamine after exposing the platelets to UV-C (Figure 3). A significant left shift of the peak was seen with glutamine incubation, suggesting a decrease in the activation of integrin.

3.4. Glutamine Abolished the UV-C-Induced Phosphorylation of cPLA2. To show the effect of glutamine at the molecular level, we assessed the phosphorylation of cPLA2. In our experiment, we observed that when the platelets were exposed to UV-C, the P-cPLA2 ratio increased as of the control; this phenomenon was prevented when platelets were incubated with glutamine before exposure to UV-C (Figure 4). Stimulation with thrombin served the purpose of positive control in our experiment.

4. Discussion

Transfusion of viable platelets is critical. Different platelet concentrate storage solutions have been investigated; the use of a diluted autologous plasma [23], ViaCyte™, and preservative solution was all shown to preserve the ability of platelets to aggregate and secrete granule contents [24]. Theraflex™ has been widely employed for this purpose. A number of studies have been published showing its toxic effect, such as decreased thrombus formation property [15] and decreased collagen-induced aggregation [2] with platelet exposure to UV-C. In our study, we used glutamine to nullify the toxic effect of Theraflex™.
FIGURE 1: The glutamine toxicity effect was studied using CCK-8 kit (mouse platelets-A (a); \( n \geq 4 \)). The presence of glutamine was not toxic to platelets; however, viability was reduced to 50% with UV-C exposure. **\( P < 0.05 \). For control purposes, lysis buffer (LB) was used. The result expressed is means with standard deviation. The release reaction of dense granule was assessed by measuring ATP secretion using the luciferase assay. Mouse platelets-B (b). The histogram represents mean ± SD from five experiments. *\( P < 0.05 \), measured by the t-test.

FIGURE 2: Glutamine does not impair the physiological functions of platelets: washed platelets stimulated with 0.5 U/ml of thrombin for [Ca^{2+}]i measurement (mouse platelets-A) (a). The presence of 50 mM glutamine showed an intact calcium signal. Histogram (A1) represents mean ± SD from four experiments. Aggregation (mouse platelets-B) (b) was also unaffected in the presence of a high dose of glutamine. Histogram B1 represents mean ± SD from four experiments (color scheme of Figure B1 is depicted in Figure B).
Glutamine is a simple amino acid and is actively metabolized in human platelets, representing a preferential mitochondrial oxidative substrate in these cells [25]. Furthermore, exogenous glutamine is metabolized by platelets to glutamate, aspartate, and CO2; however, no lactate is formed [26]. This makes the platelets more viable, as they are less exposed to acidic environments. In our study, 50 mM of glutamine mitigates the thrombus formation induced by UV-C.

Calcium signaling and the release of dense granules [14], both of which are crucial biochemical pathways for the physiological function (aggregation) of platelets, were used to illustrate if 50 mM of glutamine is toxic to platelets. This hallmark function of platelets remained intact when glutamine-incubated platelets were exposed to UV-C. Conformational change in platelet integrin [15, 27] results in the disruption of disulfide bonds of integrin, thus pushing them to open confirmation for the attachment of the integrin to fibrinogen present in the plasma [28–30]. The use of glutamine in our study successfully reversed these phenomena, making the platelets viable.

It has been well documented that cPLA2 and integrin potentiate each other’s function upon stimulation of the platelets. The prominent effect at molecular levels includes phosphorylation of cPLA2 [31]. Various reports have confirmed that stress [32] or UV-C induces [33] nonreceptor activation of this intracellular protein. Furthermore, cPLA2 and integrin cross-talk each other and enhance the physiological function in signaling platelets [34]. As observed in our study, this phosphorylation was eradicated when the washed platelets were exposed to UV-C light in the presence of glutamine.

5. Conclusion

There is not much focus on this topic in the literature. In this study, we demonstrated that the physiological and biochemical damages done during the exposure of platelet concentrates to UV-C could be alleviated by glutamine. We have delineated different experiments, showing that glutamine preserves the physiological functions of platelets (intact calcium signal and dense granule secretion reaction) when exposed to UV-C. This study raises some questions. How and where does glutamine work in the anucleated cell to alleviate the toxic effect of UV-C? Does the presence of glutamine in the platelets have any direct impact on the growth of pathogens?

Furthermore, could the 50 mM concentration of glutamine be sufficient for an extended period? If so, for how long? Above all, what concentration of glutamine would be consumed daily by the platelets when stored as a concentrate in the storage bag? These speculations leave this study...
subject to further metabolic studies on oxidative phosphorylation and glycolysis in the presence of UV-C and glutamine.

Data Availability

Data and other required materials can be submitted if requested.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Mazhar Mushtaq designed the research work, carried out the research work, made an analysis of the results, and writes the manuscript. Uh-Hyun Kim encourages, supports, advises, and supervises all the work done.

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Supplementary Materials

The immune-precipitated cPLA2 was analyzed by immuno blotting the same blot with anti-cPLA2 or anti-P-cPLA2. Full-length blots are shown in the supplementary data. (Supplementary Materials)

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