Biomedical Sciences
2021; 7(1): 36-40
http://www.sciencepublishinggroup.com/j/bs
doi: 10.11648/j.bs.20210701.17
ISSN: 2575-3924 (Print); ISSN: 2575-3932 (Online)

Addition of Glutamine in the Human Platelets Could Prevent Toxic Effect of Ultraviolet-C Induced Platelets Activation

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To cite this article:
Mazhar Mushtaq. Addition of Glutamine in the Human Platelets Could Prevent Toxic Effect of Ultraviolet-C Induced Platelets Activation. *Biomedical Sciences*. Vol. 7, No. 1, 2021, pp. 36-40. doi: 10.11648/j.bs.20210701.17

Received: March 1, 2021; **Accepted**: March 17, 2021; **Published**: March 26, 2021

Abstract: The primary function of platelets is to prevent bleeding. Transfusion of platelets can be prophylactic or therapeutic. From the storage perspective of blood and blood components, standard protocols are followed around the world to ensure the safe operation of blood banks. The Use of UV-C light in treating platelets has become a valuable method for storage and efficacy of platelets concentration in the blood bank. However, its deleterious effect remains, such as activation of platelets, thus losing their physiological function. In this study we intend to demonstrate that addition of glutamine in the platelets concentrate could prevent the toxic effect of UV-C. This study was conducted using human or mouse platelets. Use of positive and negative control in all experiment were ensured to validate the findings. In vitro thrombus formations was assessed before and after exposure of platelets to appropriate dose of UV-C. To assess the cytotoxic effect of 50 mM of glutamine on the platelets, methylthiazoletetrazolium (MTT) was used to validate the viability effect. We have successfully demonstrated that physiological damages done during the exposure of platelet concentrate to UV-C could be alleviated by the use of glutamine. Our study demonstrated that the toxic effect of UV-C on the platelets could be preserved by adding appropriate concentration of glutamine.

Keywords: Platelets, Glutamine, UV-C, Thrombus Formation

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.

Various pathological disorders are attributed to their numbers and functions. Many factors have been documented in literature regarding the root cause of the decrease in number or functions of platelets. Frequently, such individuals are transfused with platelets to maintain the hemostasis.

The use of broad-spectrum wavelength Mirasol™ Pathogen Reduction Technology System of ultraviolet (UV) light (UV-A (60%), UV-B (100%), and UV-C (20%)) and beneficial effect of UV-C on the platelet concentrate has been well documented [1, 2]. However, its pitfall remains, such as decrease in the physiological function, [3] and generation of ROS [4]. Other biomolecules that are altered/produced during the exposure of platelets concentrate are; lactic acid production, P-selection exposure and phosphatidylserine exposure [3]. In an interesting study, it was demonstrated that UV-C pathogen inactivation treatment does not influence the activation of integrin's of the platelets; however, it mitigates thrombus formation property of platelets, when subjected to micro fluidic flow chamber [5]. Such a decrease of physiological function leaves the platelets, inevitably of no-use, if they are to be transfused, especially for those who are suffering from severe platelet disorders.

In a very interesting study conducted by Murphy *et al*., they estimated the level of 17 plasmas' amino acid during the storage of platelet concentrate and concluded that the concentration of 16 amino acid remained unchanged, whereas the concentration of glutamine fell to zero by day four [6].

In this study, we intend to demonstrate that the addition of glutamine in the human platelets concentrate could prolong

the physiological function. We, used 50 mM concentration of L-glutamine in freshly prepared human or mouse platelets and they were exposed to UV-C light for short intervals.

2. Materials and Methods

2.1. Reagents

Thrombin, L-Glutamine (Glutamine). CCK8 Kit from Dijindo Laboratories and various other reagents 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 of Chonbuk National University Medical School. Blood was drawn by cardiac puncture into Acid Citrate Dextrose (ACD) (20mM citric acid, 110 mM sodium citrate, and 5mM glucose) 1:10 ratio V/V. Platelets were prepared as previously described [7]. Platelets in Tyrode Buffer (TB) or Platelets Rich Plasma (PRP) were then allowed to settle for 60 minutes at 37°C, before their use for the experiment. For the calcium study, 0.02U of apyrase was added during the resting period to minimize the effect of ADP and ATP. Calcium was added if stated, else calcium-free buffer was used.

Human platelets were prepared using the same protocol with phlebotomy after informed consent was obtained from the participants, as per guideline of the Biochemistry department. Participants were explained the purpose of it. The participants were also screened before for the potential risk assessment, and essential safety procedures were followed approved by hospital laboratory.

2.3. 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 254nm. 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 60x15mm.

Since Gravemann et al. had demonstrated that the use of 0.2 joules of UV-C in Theraflex^{TM} causes minimal damage to αIIbβ3 and 0.6 joules of UV-C causes significant damage to integrin [8], we used 0.5 joules of UV-C to demonstrate that maximum exposure of platelets to UV-C, which can cause significant changes to integrin, could be ameliorated by the use of glutamine.

2.4. In vitro Thrombus Formation

Platelet were stimulated with thrombin 0.5U/ml or exposed to UV-C with or without glutamine under continuous shaking. After the desired time, extra platelets were aspirated and the thrombus formed at the bottom was fixed with 1% formalin for 15 min. After one wash, Phase contrast images from 6 random microscopic fields were captured using Nikon Eclipse TE2000-S. Images acquired were captured using Focus Lite Version 2.88 software. Covered area of the thrombi seen in the images were calculated as described previously [7].

2.5. Viability Assay

TB-suspended platelets in a 96-well plate, with a final volume of 100 µl/well, were incubated in 50mM of glutamine and exposed to UV-C, or in a lytic buffer and left non-treated 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 100µl of dimethylsulfoxide (DMSO, Sigma). The optical density was measured at 570 nm using a microplate spectrophotometer.

2.6. 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. Result and Discussion

Glutamine prevent the UV-C induced activation of platelet. Washed human platelets were re-suspended in TB, supplemented with 1mm Ca2+ (Right panel, Figure 1. A.), PRP; not supplemented with calcium (Left panel, Figure 1. A.) were expose to UV-C in the presence or absence of glutamine. Addition of thrombin (positive control) produces multiple foci of thrombus formation in both panels. Similarly, platelets in their natural habitat i.e. plasma or TB washed platelets showed similar pattern of activation when exposed to UV-C, large mesh formation of induced platelets is noticeable in both, panels. Glutamine addition, however, abolishes this phenomenon in both the panels. Figure 1. A1, represents the statistical analysis of Figure 1. A, which shows the percentage of the covered area in the petri dish. Thrombin induced (positive control) and VU-C exposure lead to 50% to 65% respectively, of the area covered area. Addition of glutamine, however, reversed the UV-C induced activation of platelets. These results demonstrated that glutamine could prevent the UV-C induced activation of platelets. Another intriguing answer we got from this experiment was the use of PRP or the TB washed platelets produces the same result.

In order to assess whether 50mM of glutamine could be toxic to cells, we used the viability assay (Figure 2) depicting the non-toxic effect of glutamine on platelets. In order to validate the data of our earlier publication [9] where we have demonstrated at length the effect of glutamine on mouse platelets, the viability assay of both (Human and Mouse) did not give much difference between the platelets under the same conditions.

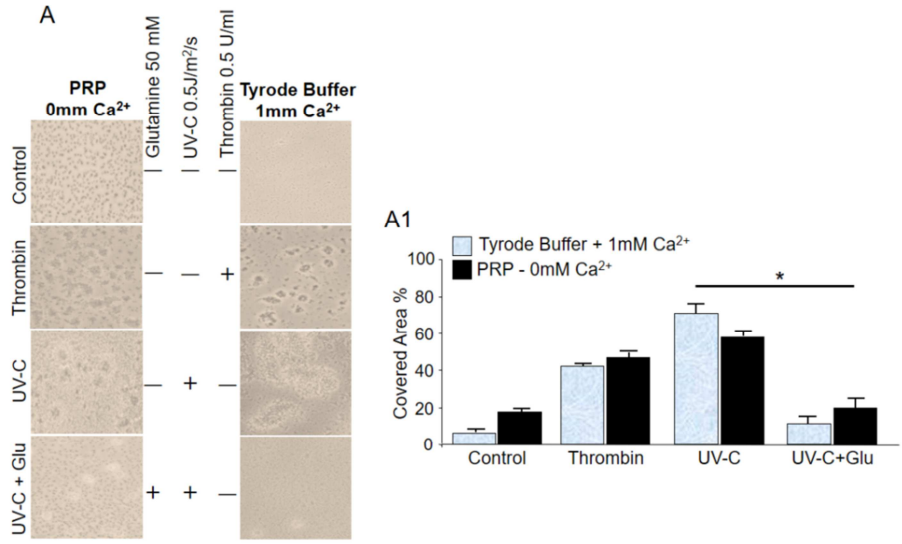

Figure 1. *Glutamine can prevent the UV-C induced activation of human platelet: In vitro thrombus formation was assessed after exposing the human platelets to UV-C with or without 50mM glutamine; thrombin 0.5U was used as positive control. (A; n=6). TB washed human platelets, supplemented with 1mM Ca2+ were exposed to UV-C with or without 50mM Glutamine (right panel). Platelet Rich Plasma, (left panel) was similarly exposed. Significantly decreased, platelet covered area was observed with glutamine (A1), *P < 0.05, measured by t test.*

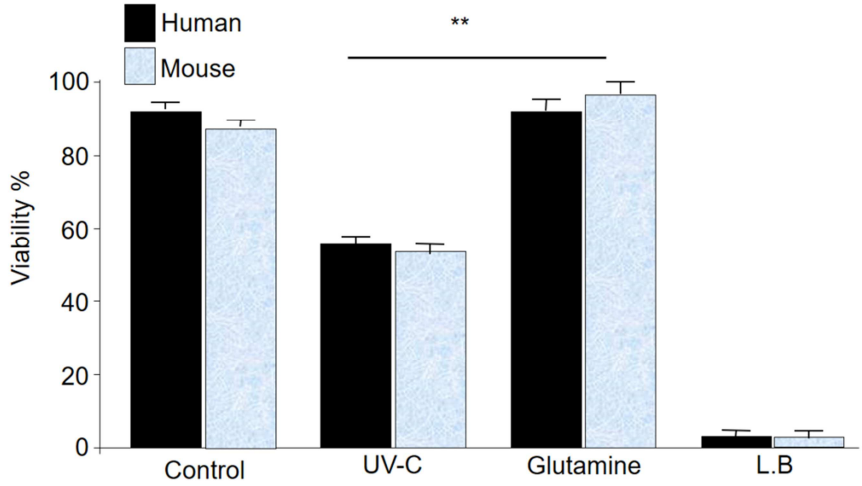

Figure 2. *Glutamine toxicity effect was studied using CCK-8 kit (B; n=4) of mouse and human platelets. The presence of glutamine was not toxic to platelets; however, viability was reduced to 50% with UV-C exposure, **P < 0.05. For control purpose, lysis buffer (LB) was used.*

Platelet storage in the blood bank has been a critical issue. Frequent agitation, bacterial contamination and activation upon agitation result to the loss of their physiological function. Various measures have been employed to minimize the loss of physiological function of stored platelets concentrates. The use of broad spectrum wavelength, in the presence of a photosensitizer Riboflavin [10]; Mirasol Pathogen Reduction Technology System of ultraviolet (UV) light; UV-A (60%), UV-B (100%), and UV-C (20%) was widely adopted all over the world as a standard procedure to prevent bacterial and viral contamination [11]. Furthermore, this technology has advanced to narrow band shortwave; "Theraflex", with specific wavelength (254 nm) of UV-C light and has become a valuable tool for safety and efficacy of platelets concentrate in the blood bank [12, 13]. In some of the developed country this system has been adopted which expose the platelets as "one-shot" treatment and it does not require any additives, however, it's prolong exposure could severely produce malfunctioning of the platelets. In the developing or underdeveloped countries, Mirasol system still prevails [14-16]. Figure 3 sums up the differential properties of the different UV lights. Transfusion of viable platelets is critical. Different platelet concentrates storage solutions have been investigated; the use of a diluted autologous plasma, ViaCyte™, and preservative solution, were all shown to preserve the ability of platelets to aggregate and secrete granule contents [17-19]. 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 [20] and decreased collagen-induced aggregation [12] with platelet exposure to UV-C. In our study, we used glutamine to nullify the toxic effect of Theraflex™.

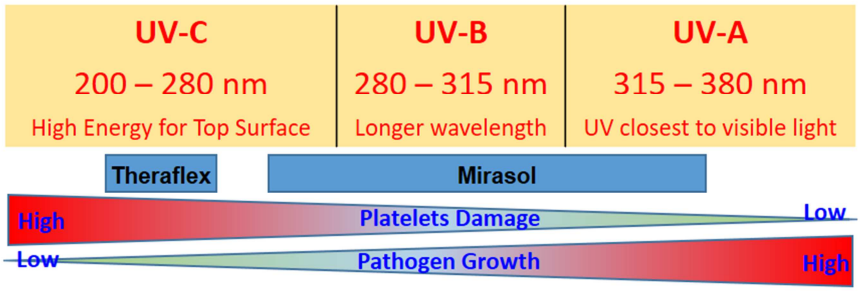

Figure 3. *Differential properties of the different UV lights. UV-C narrow wavelength, high energy producing potential damage to platelets, however effective germicidal.*

Glutamine after its transport [21] is actively metabolized in human platelets, representing a preferential mitochondrial oxidative substrate in these cells [22]. Furthermore, exogenous glutamine is metabolized by platelets to glutamate, aspartate, and CO_2; with no lactate formation [12]. This make the platelets more viable, as they are less exposed to acidic environment.

4. Conclusion

There is not much focus on this topic in the literature. In this study, we demonstrated that the occurrence of physiological changes due to the exposure of platelet concentrates to UV-C could be alleviated by glutamine. We have delineated the different experiments in the mouse model, showing that glutamine preserves the physiological functions of platelets. In this study we have use the human platelets and have shown well preserve function of the platelets under the UV-C. However, a more in-depth study is required at the molecular level to assess the detailed pathway and how glutamine maintains the physiological functions of platelets. 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? These speculations leave this study subject to further metabolic studies on oxidative phosphorylation and glycolysis in the presence of UV-C and glutamine.

Acknowledgements

I am appreciative to Prof. Uh-Hyun Kim, head of Biochemistry Department in the medical section of Chonbuk National University, South Korea, who's endless efforts in the supervision and guidance of this work.

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