Artigo / Article

Evaluation of platelet aggregation in platelet concentrates: storage implications

Efeito das catequinas (catequina e epicatequina) na agregação plaquetária

Teresinha J.C. Neiva[1]
Marcos J. Machado[1]
Monica Hoehn[2]
Elizabeth M. Hermes[3]
Cidônia L. Vituri[1]
Jovino S. Ferreira[4]
Elbio A. D'amico[5]

*The use of hemo-derivatives is nowadays a fundamentally important therapeutic modality in the exercise of medicine. Among the various hemo-components employed, we have the platelet concentrate (PC), indicated in cases of hemorrhagic disturbances. We previously showed that platelet function in blood donors is reduced in their screening phase and after the separation process of PCs. Currently, we are providing evidence for the existence of biochemical and functional changes in PC preparations stored for three days at temperatures of 20 ± 2 ^{0}C. Platelet concentrates from 40 healthy donors, collected in CPD anticoagulant and PL-146 polyvinylchloride containers, were examined in order to determine the pH value, pCO_2, pO_2 and lactate concentrations. In addition, the aggregation of platelets with thrombin and collagen were examined to evaluate platelet function. A pH increase from 7.07 ± 0.04 to 7.36 ± 0.07 ($p < 0.01$) was observed. The pCO_2 concentration decreased progressively from 69.2 ± 7.7 mmHg to 28.8 ± 6.2 mmHg ($p < 0.001$) during the storage period. In contrast, pO_2 value increase from 103.4 ± 30.6 to 152.3 ± 24.6 mmHg ($p < 0.001$) was evidenced during the 48 hours of storage. The lactate concentration increased from 17.97 ± 5.2 to 57.21 ± 5.7 mg/dl ($p < 0.001$). Platelet aggregation using 0.25 U/ml-thrombin and 2.0 µg/ml-collagen showed significant hypofunction from 61.8 ± 2.7% to 24.8 ± 9.8% and 62.7±5.0 to 33.4± 6.2 ($p < 0.001$), respectively. We concluded that the evaluated biochemical parameters and the platelet function changed significantly when the platelets were kept under routine storage conditions. Rev. bras. hematol. hemoter. 2003; **25**(4): 207-212.*

Key words: *Platelet aggregation; platelet count; platelet concentrates, storage implications.*

[1] *Departamento de Análises Clínicas, Universidade Federal de Santa Catarina-SC – Brasil.*
[2] *Curso de Graduação em Farmácia – Análises Clínicas, Universidade Federal de Santa Catarina-SC – Brasil.*
[3] *Laboratório de Análises Clínicas – HU, Universidade Federal de Santa Catarina-SC – Brasil.*
[4] *Serviço de Hemoterapia – HU, Universidade Federal de Santa Catarina-SC – Brasil.*
[5] *Laboratório de Coagulação – Fundação Pró-Sangue Hemocentro de São Paulo-SP – Brasil.*

Correspondência para*: T. J. C. Neiva*
Departamento de Análises Clínicas – CCS,UFSC
88010-970 –Florianópolis-SC – Brasil
e-mail: neiva@ccs.ufsc.br

Introduction

Platelet transfusions (random donor platelet concentrates and single donor apheresis platelets) are effective for the prevention and treatment of bleeding in patients who have quantitative and/or functional disorders.[1,2,3] Transfusion efficacy in clinical practice for clinically stable thrombocytopenic patients is mainly based on the quantitative increase of platelets, the functional aspect of transfused platelets not being considered. In clinically stable patients, both fresh and stored platelets had comparable increments and survival rates; however, in clinically unstable patients, PCs stored for two days or less had significantly better results than those stored for longer intervals.[4]

Studies conducted with PCs showed these cells lose their viability very quickly during the storage period, implying the need for a constant renewal of stock.[5,6,7] In a previous report we established that platelet aggregation reduced significantly ADP and adrenaline in PC.[8] Thus, the question is whether some of the variability in post transfusion platelet responses seen in clinically ill patients is related to the metabolic modification during platelet storage or to the effects of the patient's underlying disease and medications, or both or on platelet viability. Thus, the aim of the present study was to investigate the biochemical changes affecting PCs during the storage period and their implications on platelet function.

Material and Methods

Reagents

Thrombin and collagen were purchased from Chronolog Corporation, Havertown, PA, USA. All other chemicals were of the highest quality available and were obtained from commercial sources.

Subjects

The study included 40 healthy volunteers of both sexes, who after informed consent underwent clinical and laboratory screening at the UFSC Hemotherapy Service.

PC separation

Platelets, collected in CPDA anticoagulant and PL-146 polyvinylchloride containers, were isolated by means of centrifugation of total blood at 1600 x g for 8 minutes at a temperature of 22°C to obtain platelet-rich plasma (PRP). The obtained PRP was once more centrifuged at 2400 x g for 8 minutes under the same experimental conditions. After the final centrifugation the supernatant (PPP) was separated, and the residual pellet with the platelets was re-suspended in a mean volume of 50 ± 0.9 ml of the respective plasma. The bag with the PC was left to rest for one hour, and then placed in an agitator under constant stirring until the moment of its use. The PCs were evaluated for periods of up to 48 hours of storage.

Platelet aggregation studies

Platelet aggregation was determined by the turbidimetric method,[12] using a Net Lab aggregometer. Aliquots of 400 µl of platelets were put into a small cuvette using a pipette and stirred at a constant speed of 1,000 rpm at 37 °C before the addition of 0.25 U/ml-thrombin and 2.0 µg/ml-Collagen. The PCs were adjusted to 3 x 10^8 cells/ml with their respective plasma (PPP) obtained by centrifugation at 2400 x g for 8 minutes, to be stimulated with collagen. When thrombin was used, aliquots of PCs were submitted to a washing procedure (platelet isolation).

Platelet isolation

The pH of PCs (5 ml) was adjusted to pH 6.2 by addition of 1M citric acid. PCs aliquots were than centrifuged at 400 x g for 10 minutes. The obtained "pellet" was re-suspended in a buffer containing 3.8 mM Hepes, 140 mM NaCl, 2.1 mM KCl, 5 mM EGTA, 0.1% glucose, and 1 µM prostacyclin at pH 6.2. After the last centrifugation, platelets were re-suspended in 3.8 mM Hepes, 140 mM NaCl, 2.1 mM KCl, 0.1% glucose, 1.5 mM $MgCl_2$ and 1 mM $CaCl_2$, at pH 7.4 and adjusted to a concentration of 3 x 10^8 cells/ml.

Platelet, leukocyte, and erythrocyte quantification

The study was conducted on PC samples counted in a Neubauer chamber.

pH, pO_2, and pCO_2 monitoring

Aliquots (2 ml) were collected, under sterile conditions using a syringe, from the PC and analyzed on a AUL-993 Model.

Lactate Dosage

Determination was conducted on PC samples; PC portions of 0.5 ml were centrifuged at 900 x g for 10 minutes. The supernatant (plasma) was used to quantify the lactate by means of a Dade-Dimension-AR model analyzer.

Statistic Analysis

Data are reported as means ± standard deviations (SD). The Student *t*-test was employed to estimate differences between groups, and Pearson's Linear Correlation coefficient was determined. Differences were considered to be significant when the probability, $p < 0.05$. The statistical program Instat-2 was used for analysis.

Results

The functional study of platelets was performed by platelet aggregation, using as agonists 0.25 U/ml-thrombin and 2.0 µg/ml-collagen at different storage periods (Table I). Results demonstrated a significant platelet hypofunction following a 24-hour storage period for both agonists ($p<0.001$). The study also showed, after fractionating, that the concentration of platelets in PCs was significantly reduced after 24 hours (Table II). The concentrations of leukocytes and erythrocytes, on the other hand, did not present significant alterations.

To take into consideration the possibility of platelet hypofunction occurring due to biochemical changes caused by alterations of the post-storage levels of oxygenation and/or lesions to the platelet membrane, we evaluated the pH and amounts of pO_2, pCO_2, and lactate production. Obtained results indicated significant alterations in the levels of oxygenation and lactate production after 24 hours of storage, as well as a significant correlation between these alterations and the platelet function ($p <0.01$) (Tables III and IV).

Discussion

This work aimed at evaluating biochemical alterations of PCs over 48 hours of storage and the implications of this on platelet function. Results showed a significant reduction of platelet aggregation after 24 hours of storage (Table 1). Similarly, a reduction was observed in the number of platelets existing in the PCs, as well as alterations

Table 1
Platelet aggregation

Storage (hours)	Aggregation(%) 0.25 U/ml Thrombin	Aggregation(%) 2.0 µg/ml Collagen
0	62.0 ± 1.2	62.7 ± 5.0
24	27.0 ± 2.7*	48.2 ± 2.7*
48	24.2 ± 4.5*	33.4 ± 6.2*

Platelets were stimulated with 0.25 U/ml-thrombin and 2.0 µg/ml-collagen. Data are reported as means ± standard deviation; n=40; p < 0.001 (Student t-test)*

Table 2
Quantification of platelets, leukocytes and erythrocytes

Storage (hours)	Platelets x 10^6/mm^3	Leukocytes x 10^3/mm^3	Erythrocytes x 10^5/mm^3
0	1.84 ± 0.9	0.28 ± 0.1	3.1 ± 2.4
24	1.65 ± 0.7	0.27 ± 0.2	5.6 ± 2.1
48	0.93 ± 0.2*	0.23 ± 0.1	3.6 ± 2.0

Data are reported as means ± standard deviation; n = 40; p < 0.01 (Student t-test)*

in pH, pCO_2 and lactate concentration. On the other hand, the pO_2 concentration of this medium presented several significant alterations only after the bags had been stored for 48 hours (Table 3). Such results show that during PC storage, cellular lesions may happen caused by alterations of the extracellular medium, implying not only a quantitative reduction in platelets but also biochemical changes (variations or changes in their metabolism), with a resulting loss or decrease in function. Nevertheless, obtained results of aggregation do not reflect changes introduced by the quantitative reduction of platelets, as the evaluation of platelet function was made with various amounts of readjusted cells. In this way, we suggest the observed platelet hypofunction is apparently caused by biochemical alterations during storage, with consequent changes in the intracellular metabolism. In fact, obtained results show pH, pO_2, and pCO_2 values significantly changed following the storage period (Table 3). An increase in pH and a reduction in pCO_2 were seen after a 24-hour storage period. Levels of pO_2, on the other hand, had a significant increase only after 48 hours. The observed rise in pH appears to be a consequence of a greater permeability of the plastic bag (used to store the PC) to gases,

Table 3
Effect of post-storage pH value, pO_2, pCO_2 and lactate concentrations. Platelet concentrates (PCs) were obtained from normal volunteers and storage in container for 48 hours at 20 to 22 ^{0}C

Storage (hours)	pH	pO_2 mmHg	pCO_2 mmHg	Lactate mg/dL
0	7.07 ± 0.04	103.4 ± 30.6	69.2 ± 7.7	17.9 ± 5.2
24	7.23 ± 0.08*	119.5 ± 44.9	40.6 ± 10.6*	37.2 ± 5.8*
48	7.36 ± 0.07*	152.3 ± 24.6*	28.8 ± 6.2*	57.2 ± 5.7*

Data are reported as means ± standard deviation; n = 40; p < 0.01 (Student t-test)*

Table 4
Pearson correlation coefficients between aggregation and pH value, PO_2, PCO_2 and lactate concentrations

	Aggregation (r)	p
pH	-0.55	0.0008*
pO_2	-0.37	0.0320*
pCO_2	0.50	0.0031*
lactate	-0.87	0.0001*

Significant correlation was found (n=40)

particularly the CO_2, already evidenced during the first 24 hours. This would introduce an imbalance in H_2CO_3/HCO_3 concentrations of the medium, with a resulting inefficient buffering of the system and the variation of pH. Susceptibility to greater gas exchange between plasma and the atmosphere, when conditioned in plastic bags and without CO_2 supplementation in the storage place, has been demonstrated by several authors being greater at temperatures of 22 °C. Regarding pO_2, its delayed rise is apparently related to its greater concentration in the plasma (in relation to pCO_2).

The greater lactate contents found in extracellular medium confirm the increased lysis of platelets in a more alkaline pH, probably resulting from a greater fragility of its membranes. This content is apparently related to a greater activation of the glycolytic via (oxidation of glucose to an intracellular lactate) in the stored platelets. Baker et al[9] have already observed metabolization of glucose into lactate in human platelets is strongly influenced by the pH of the extracellular medium, attaining greater speeds when the pH is more alkaline. Considering the low contamination by leukocytes and erythrocytes of the assessed PCs, and also the absence of significant variations of their quantities, the verified contents of lactate come apparently from the existing platelets.

Studies correlating the biochemical variables of the extracellular medium and platelet aggregation seemingly point to functional alterations originated from medium alterations. A good correlation was seen to exist between pH and aggregation (Table 4). Similarly, a good correlation was found between pCO_2 and aggregation; however, this latter was directly dependent.

On the other hand, the correlation between pO_2 and aggregation was weak and inversely dependent. Such studies confirm the existing interrelation between the reduction in functionality of platelets and the alterations seen in the extracellular medium. Modification of platelet function was already observed after 24 hours in storage, whereas the total number of platelets present in the PCs only had a significant variation after a 48-hour storage period. Thus the participation of biochemical variables in the medium (pH and pCO_2) is corroborated in inducing platelet hypofunction. A possible explanation might be the variation in the composition of the platelet membrane, as well as other factors involved in aggregation (preferential anaerobic metabolism, due to the high contents of L-lactate), and alterations caused by a increased extracellular pH.

Various authors[5,10,13,14] have suggested platelet-conserving solutions should be used, instead of plasma, for PC preparation. The best conservation of platelets, particularly of their function, is apparently observed with these solutions, in which there is a reduction in glucose metabolization by the glycolytic via, and also a greater retention of pH of the extracellular medium. However, other factors such as temperature, volume, agitation and the kind of plastic used for conservation of PCs, are possible determinants of biochemical changes in the medium.[11,15] Rock & col[15] demonstrated the survival duration of transfused platelets varies according to the kind of bag used. The above results, when analyzed as a whole, lead us to the conclusion that the observed gas exchanges are capable of causing platelet lesions, altering their metabolism. As an indicative of cell lesion, we analyzed the production of lactate in the medium

and results showed a significant increase after 24 hours of storage (Table 3).

In addition, a correlation was observed between extracellular L-Lactate and aggregation, which it proved to be very important and inversely dependent (Table 4).

In spite of a few authors[5,14] admitting the lactate levels in PC are caused by a quantitative increase of platelets, we observed in our study that a significant reduction in platelets happened during storage. In this way, we inferred the observed increase of lactate would be a consequence of greater platelet lesion with a resulting liberation of lactate. The observed production of lactate comes predominantly from platelets, considering that in PC the presence of other cell elements such as erythrocytes and leukocytes is not augmented (Table 2).

According to Noral et al,[16] only 66% of all the transfused platelets circulate freely, and various factors are capable of affecting the function of these cells, such as infections, the use of antibiotics and anti-inflammatory drugs, as well as previous lesions caused by the separation and storage processes. Results of this study make it possible to conclude the processes of separation and storage are capable of introducing significant platelet activation, reducing its functional capacity. In this way it is concluded that, to offer greater clinical benefit to patients under this kind of treatment, PCs must be transfused after a storage time that is as short as possible.

Acknowledgments

The authors would like to thank the staff of the UFSC Hemotherapy Service in Santa Catarina for their valuable help in the screening of donors and separation of platelet concentrates.

Resumo

*Os flavonóides representam um dos grupos fenólicos mais importantes e diversificados entre os produtos de origem natural. Dentre os compostos fenólicos temos os derivados flavânicos (flavan-3-óis e flavan-3,4-dióis) que podem se polimerizar originando taninos. O representante mais importante do grupo dos flavan-3-óis é a catequina. Estes compostos exibem inúmeros efeitos biológicos incluindo ação antiviral, antioxidante e antitrombótica. No presente trabalho foi avaliado o efeito das catequinas (catequina e epicatequina) sobre a função plaquetária. Foram estudados vinte indivíduos adultos, clinicamente saudáveis. A agregação plaquetária foi avaliada em plasma rico em plaquetas (PRP) e plaquetas lavadas utilizando-se agonistas colágeno (2,0 µg/ml) e trombina (0,25U/ml), respectivamente. Como controle utilizou-se o veículo da droga (DMSO 0.2%). O pré-tratamento das plaquetas com epicatequina (200 µg/ml) causou inibição significativa da agregação para o colágeno (9.0 ± 7.8%) e para a trombina (10.0 ± 2.2) em relação aos respectivos controles (70.0 ± 7.8) e (68.0 ± 5.0), (p<0.001; Student t-test). Por outro lado, a catequina não promoveu inibição da resposta de agregação. Conclui-se que a epicatequina apresenta potencial antiagregante. Rev. bras. hematol. hemoter. 2003; **25** (4): 207-212.*

Palavras-chave: *Agregação plaquetária; contagem de plaquetas; concentrado de plaquetas; conservação.*

References

1. Fabris F, Soini B, Sartori R, Randi ML, Luzzatto G, Girolami A. Clinical and laboratory factors that affect the post-transfusion platelet increment. Transfusion Science 2000;23:63-68.
2. Slichter SJ. Platelet transfusions a constantly evolving therapy. Thrombosis Haemostasis 1991;66:178-188.
3. Aubuchon JP. Platelet transfusion therapy. Clinical. Laboratory Medicine 1996;16:797-816
4. Peter-Salonen K, Bucher U, Nydegger UE. Comparison of post-transfusion recoveries achieved with either fresh or stored platelets concentrates. Blut 1986;54:207- 209.
5. Wildt-Eggen J, Schrijver JG, Bins M, Gullikson H. Storage of platelets in additive solutions: effects of magnesium and/or potassium. Transfusion 2002; 42:76-80.
6. Gulliksson H. Platelet storage. Transfusion and Apheresis Science 2001;24:241-244.
7. Holme S, Sawyer S, Heaton, A. Studies on platelets exposed to or stored at temperature below 20 degrees C or above 24 degrees C. Transfusion 1997;37:5-11.
8. Neiva TJC, Brocardo G, Ferreira JS, Ferreira VL, Castro CES. Avaliação da função plaquetária de doadores de sangue. RBAC 2000;32:249-252
9. Backer JM, Candy DJ, Haweker RJ. Influences of pH on human platelet metabolism. Platelets 2001; 12: 333-342.
10. Gulliksson H, Aubuchon JP, Vesterinen M, Sandgren P, Larsson S, Pickard CA, Herschel I, Roger J, Tracy J, Langweiler M. Storage of platelets in additive solutions: a pilot in vitro study of effects of potassium and magnesium. Vox Sanguinis 2002;82:131-136.

11. Holme S, Vaidja K, Murphy S. Platelet storage at 22 ^{0}C: Effect of type of agitation on platelet morphology, viability and function in vivo. Blood 1978;52:425-427.

12. Neiva TJC, Benedetti AL, Tanaka SMCN, Santos JI, D'Amico EA. Determination of serum aluminum, platelet aggregation and lipid peroxidation in hemodialysed patients Brazilian Journal of Medical and Biological Research 2002;35:345-350.

13. Van Der Meer P, Pietersz R, Reesink H. Leukoreduced platelet concentrates in additive solution: an evaluation of filters and storage containers. Vox Sanguinis 2001; 81:102-107.

14. Glaser A, Friedlein H, Zingsem J, Zimmermann R, Weisbach V, Ruf A, Eckstein R. Storage of single donor platelet concentrates: Paired comparison of storage as single or double concentrates. Journal of Clinical Apheresis 2001;6:148-154.

15. Rock G, Scherring VA Tittley P. Five-day storage of platelet concentrates. Transfusion 1984;24:147-152.

16. Norol F, Kuentz M, Cordonnier C, Beaujean F, Haioun C, Vernant JP. Influence of clinical status on the efficiency of stored platelet transfusion. British Journal of Haematology 1984;86:125-129.

Avaliação:
Editor e dois revisores externos
Conflito de interesse: não declarado

Recebido: 14/02/2003
Aceito após modificações: 06/10/2003