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RESEARCH ARTICLE

RABBIT PYROGEN TEST STUDY OF INFUSIBLE PLATELET MEMBRANE AS A PLATELET SUBSTITUTE FOR BLOOD TRANSFUSION

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ABSTRACT

Blood transfusion centers are under considerable pressure to produce platelet concentrates with a shelf life limited to 3-5 days. Many approaches have been investigated experimentally to produce new hemostatically active platelet products that are capable of long term storage. In this article infusible platelet membrane (IPM) as a platelet substitute was investigated with regard to rabbit pyrogen test as a requirement for parenteral drugs according to the EU Pharmacopoeia monograph to demonstrate its safety. Lyophilized IPM was prepared from fresh and outdated platelet concentrates. Platelet concentrates were pooled, disrupted by freeze-thaw procedure and pasteurized for 20 hours to inactivate possible viral or bacterial contaminants. Sterility test, size particle test and rabbit pyrogen test were then performed. Administration of IPM at various dosage forms were carried out on a group of three rabbits with a total of five groups. The summed response of body temperature of each group was calculated. At dosage forms of 1.0, 2.0, 4.0, 6.0 and 9.0 mg per kg of body weight, the summed responses of each group were 0.1, 0.2 , 0.5, 0.7 and 1.0 degree centigrade, respectively. As might be expected, there was shown a strong direct correlation between dose and response with r=0.996. Preparations being examined passed the test according to the EU Pharmacopoeia monograph. It was concluded that IPM as a platelet substitute is sterile and safe without endotoxins and non-endotoxin pyrogens that may be originated from bacteria, intracellular and extracellular proinflamatory cytokines and other biologic response modifiers in conventional platelet concentrates.

Keywords: Infusible platelet membrane, rabbit pyrogen test, platelet substitute

INTRODUCTION

Platelet transfusion is an effective therapy to control bleeding in thrombocytopenic patients. Unfortunately, blood platelet units are generally stored in blood banks for 3-5 days; thereafter they are discarded[1, 2]. For preserving platelets for a long period, a number of attempts have been taken to develop substitutes for platelets, as possible alternatives to currently available platelet concentrates. A number of studies have shown that platelet preparations with impaired metabolic or functional integrity still retain a certain degree of hemostatic property[3-8]. Infusible platelet membrane (IPM) prepared from fresh or outdated human platelets have been developed as an alternative to standard platelet concentrates, with the additional advantage of long shelf life and increased pathogen safety and have confirmed useful in shortening bleeding time in rabbits with experimentally induced thrombocytopenia[9-13]. The preliminary clinical study of lyophilized platelet material in patients with secondary thrombocytopenia showed no toxicity or thromboembolic sequelae[14]. Evaluation of the thrombogenicity of IPM, using the method of Wessler *et al.*[15], indicated that IPM is not thrombogenic[10]. In normal human volunteers, infusions of IPM were well tolerated and had no effect on biochemical or coagulation parameters and no evidence of immunogenicity was reported[16]. On the other hand, the earliest trials with IPM preparations were not successful *in vivo* and produced considerable distress in experimental animals[17,18]. This line of research was postponed for nearly three decades until experiments in thrombocytopenic rabbits provided preclinical evidence of the preparations haemostatic efficacy without significant morbidity[9].

The crucial step in the quality control insurance of different pharmaceutical products requires absence of fever-inducing contaminants (pyrogens), especially in drugs intended for the parenteral application. Given the seriousness of this issue, rabbit pyrogen test has been taken into consideration of European Pharmacopoeia as indicated general chapter on Pyrogens; 2.6.8. Since 1942, the rabbit pyrogen test (RPT) has been established as a golden standard for testing various pyrogens (Gram-negative endotoxins, non-endotoxin pyrogens, fungal pyrogens). Optionally, the Limulus-Amebocyte-Lysate test (LAL) is used as a highly specific test for detection of Gram-negative bacteria endotoxin, but it fails to detect non-endotoxin pyrogens and therefore can not be used solely, instead of RPT. The principle of proposed tests is measurement of proinflammatory cytokines such as tumor necrosis factor (TNF), interlukin (IL-1, IL-6) released from human blood cells in response to the presence of exogenous pyrogens[19]. Bacterial products and toxins

exemplify exogenous mediators. Endogenous mediators are classified based on source or origin as plasma derived and cell or tissue derived.

In this paper we want to show that our lyophilized IPM which is prepared from fresh and outdated human platelets is safe and has no adverse effects of pyrogenicity or fever induction due to bacterial contamination, proinflamatory cytokines and membrane fragments of leukocytes and platelets, at the various injection doses during rabbit pyrogen test study.

MATERIALS AND METHODS

Preparation of IPM

IPM is prepared from 58 fresh and outdated platelet units of Tehran Blood Transfusion Center. The units were pooled and centrifuged for 15 min at 1000 RPM in order to remove contaminating red and white cells. The supernatant was centrifuged for 30 min at 2500 RPM to remove plasma. The precipitate was suspended in 150 ml of physiological saline solution (0.9 g%). For lysis and disruption of platelets, freeze-thaw procedure was repeated three times at -50°C and room temperature for 24 and 3 hours, respectively. The solution was then washed twice with physiological saline solution to remove intracellular components by centrifugation (30 min at 2500 RPM). The precipitate was then suspended again in a 94 ml of the same solution.

Pasteurization

The IPM suspension with 0.4 M sodium caprylate concentration was prepared and heated at 60°C for 20 hours to inactivate possible viral or bacterial contaminants and was then formulated with sucrose 1 M and human serum albumin 0.1% [12].

Lyophilization

After filling and capping, the vials were lyophilized by Freeze-Dryer (Usifroid Model SMH 150) in an aseptic condition for three days.

Sterility Test

The test for sterility was carried out under aseptic conditions according to the EU Pharmacopoeia monograph (general chapter on sterility 2.6.1).

Particle size assay

We used a particle-sizing instrument (Malvern Zetasizer Nano ZS, UK) to measure the angular distribution of light scattered from a sample illuminated by a laser. The corresponding size distribution was calculated by using the software supplied with the instrument.

Rabbit pyrogen test

Healthy, adult rabbits of either sex, weighing not less than 1.5 kg were purchased from Tehran Institute Pasteur and the test for pyrogens was performed according to the EU Pharmacopoeia monograph (Ph. Eur. general chapter on Pyrogens; 2.6.8). The lyophilized IPM was reconstituted with a pyrogen-free distilled water and was injected slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 minutes. The temperature degrees of the animals were recorded, beginning before injection and continuing for 3 hours after injection of the IPM being examined. The initial and maximum temperatures for each rabbit were determined before and after the injection of IPM respectively by pyrogen testing instrument (PD 85 instrument, Ellab, Copenhagen, Denmark). The amount of the samples to be injected on five groups (three rabbits each) were1.0, 2.0, 4.0, 6.0 and 9.0 mg per kg of body weight. The volume of the injection was less than 10 ml per kg of body weight.

RESULTS

In our previous investigations the effectiveness of IPM was demonstrated by the reduction in the bleeding time in thrombocytopenic rabbits [11] with its dose-dependent response property [13]. In this study we investigated that our IPM product pyrogen levels in sterile are within established limits according to sterility and pyrogenecity requirements of the European Pharmacopoeia for injectable drug products.

The results of rabbit pyrogen test of infusible platelet membrane are summarized in Table I.

The principal of the test

The test consists of measuring the rise in body temperature inducing in rabbits by the intravenous injection of a sterile solution of the IPM being examined.

Determination of the initial and maximum temperatures

The initial temperature of each rabbit is the mean of two temperature readings recorded for that rabbit at an interval of 30 minutes in the 40 minutes immediately preceding the injection of the material being examined. The maximum temperature of each rabbit is the highest temperature recorded for that rabbit in the 3 hours after injection. The

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difference between the initial temperature and the maximum temperature of each rabbit is taken to be its response and the sum of differences of each group were calculated (Table I).

Table I: Results of rabbit pyrogen test of infusible platelet membrane (IPM)

Number of rabbits* (C°)	Dose of IPM injection (mg/kg)	Summed response** (C°)	Material passes if summed response does not exceed* (C°)	Pass/Fail status
3	1.0	0.1	1.15	Pass
3	2.0	0.2	1.15	Pass
3	4.0	0.5	1.15	Pass
3	6.0	0.7	1.15	Pass
3	9.0	1.0	1.15	Pass

* Test for pyrogens was performed according to the EU Pharmacopoeia monograph (Ph. Eur. general chapter on Pyrogens (2.6.8).
** The sum of difference between the mean initial temperature (before injection) and the maximum temperature (after injection) of each rabbit

Interpretation of results

The test was performed on a group of three rabbits. The summed response of each group does not exceed 1.15° C and therefore all various doses of IPM were passed according to the EU Pharmacopoeia limits. Statistical analysis showed that there is a strong correlation between the amount of administrated dosage and summed response temperature of each group (r =0.996).

Characteristics of IPM

The lyophilized IPM was prepared in an aseptic condition with acceptable solubility test during reconstitution and mean particle size of 380 nm (Table II). The sterility test was passed and confirmed our efficient pasteurization procedure as a pathogen inactivation method (Table II). Haemostatic effect of IPM has previously been demonstrated by Nasiri et al. in animal studies[11,13]. The maximum decrease in the percentage of bleeding time was observed at 2 h after and this haemostatic effect was no longer detectable after 24 h of IPM administrations.

Table II: Characteristics of lyophilized infusible platelet membrane (IPM)

Total protein (g/dL)	0.2
Solubility test (min)	Less than 10 min
Mean particle size (nm)	380
pH	7.1
Sterility test	Pass
In vivo haemostatic effect (bleeding time)	Shortening of the bleeding time in thrombocytopenic rabbits was observed after 2 hours of IPM infusion
Rabbit Pyrogen test	Pass

DISCUSSION

Parenteral preparations have to be pyrogen-free because administration of pyrogens may induce fever, shock or even death. The severity of the adverse reactions depends on the concentration and biological activity of the respective pyrogen. There is a broad spectrum of pyrogens which are classified into endotoxin and non-endotoxin pyrogens. Endotoxins, representing the lipopolysaccharides (LPS) of the cell wall of Gram-negative bacteria, are the best characterised and the most potent pyrogens. The structural identity of most non-endotoxin pyrogens has not yet been clarified. Examples of pyrogens from Gram-positive bacteria are the lipoteichoic acids and peptidoglycans which are constituents of the bacterial cell wall[20].

Currently, there are two tests described in the European Pharmacopoeia which are related to pyrogen testing of parenteral medicinal products: the rabbit pyrogen test (RPT) which is considered to detect most of the pyrogens, i.e. endotoxins and non-endotoxin pyrogens, and the bacterial endotoxin test, i.e. Limulus polyphemus amoebocyte Lysate-test (LAL-test) which is used to detect or quantify endotoxins of Gram-negative bacteria.

Lyophilized IPM may have both endotoxins and non-endotoxin pyrogens due to the presence of higher bacterial contamination of platelet concentrates in comparison with the other blood components and proinflamatory cytokines such as tumor necrosis factor (TNF), interlukin (IL-1, IL-6) that may be accumulated during storage of platelet concentrates and also microparticles or membrane fragments of residual leukocytes and platelets. In addition, numerous laboratory studies have shown that a wide variety of biologic response modifiers, including cytokines, chemokines, complement fragments, histamine, and lipids, accumulate in platelet products during storage[21].

Febrile non-hemolytic transfusion reactions (FNHTRs) are common in recipients of platelet concentrates. These can raise a fever by 1 °C or more within the first 4 h of transfusion and normalization of the temperature within 48 h, if transfusion of a bacterially contaminated blood product can be excluded and if no signs of hemolysis are found. Blumberg et al. studied a platelet-derived substance, soluble CD40 ligand (sCD40L, CD154), in supernatants of platelet concentrates as possible cause of FNHTRs[22]. These authors indicate that platelet-associated CD40L and sCD40L induce prostaglandin E2 synthesis (inducing fever) and synthesis of pro-inflammatory cytokines in a variety of cells in the recipient, including endothelial cells and fibroblasts[23,24].

Our experiment showed that IPM is safe, tolerable and it does not induce fever in rabbits even at high doses of IPM such as 9.0 mg/kg in comparison with the standard-dose[10] of 2.0 mg/kg because, higher doses of disintegrated platelets had undesirable side effects in the experimental dogs[18].

CONCLUSION

In this study, it may be concluded that IPM as a platelet substitute can demonstrate safety with the acceptable tolerability without pyrogenecity. In spite of challenges in demonstrating its efficacy, the investigations should continue on different aspects of the novel IPM product to provide maximal clinical benefits with minimal risk of complications[8,25,26]. On the other hand, recent concerns of various complications have been raised about transfusion with platelet concentrates.

The advantages of IPM over conventional platelet concentrates include:

• improved shelf life, ease of storage and use

• reduced endotoxin and non-endotoxin pyrogens

• reduced viral and bacterial load due to an applied pasteurization method

• possible fever reduction induced by post-transfusion reactions due to removal of contaminating red cells, white cells, intracellular and extracellular proinflamatory mediators and other biologic response modifiers during freeze-thawing and washing steps.

It should be considered that platelet substitutes which aim to be alternatives to the current platelet products should demonstrate a clear benefit-to-risk ratio before they are considered for clinical trials. However, further animal studies are required to more fully define the safety or efficacy of IPM as a platelet substitute. Although much remains to be investigated, anyway, it should be considered that the success of these efforts will affect patient care in transfusion medicine.

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