Safety Assessment of Blood Bags Using Animal Cell Culture

Chaiwat Ritsuk* Nuananong Jirakarnjanakit**
Kalyanee Jirasripongpun***

ABSTRACT

Blood bag is a medical device that must be safe and acquires acceptable standard for clinical used. The import blood bags from oversea must pass the safety assessment according to ISO 10993-5 or the guideline from public health ministry and TIS 1298-2555. This study aimed to investigate the safety of blood bags which are quadruple blood bags from 3 manufacturers. The safety test was performed according to ISO 10993-5 emphasizing on acute cytotoxicity assay using mouse fibroblast L929 cell line. All blood bag materials demonstrated toxicity level at level 1 and 2 for agar diffusion and direct contact, respectively. MTT assay also exhibited cell viability more than 70% of all 3 sample manufacturers and gave reactivity grade at level 2 at the concentration 0.2 g/ml. On the whole, it can be concluded that all blood bags had acceptable safety criteria according to the ISO 10993-5 and are safe for clinical used.

Keywords: Blood bag, Safety assessment, Cytotoxicity

* *Student, Master of Science Program in Biotechnology, Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University*

** *Researcher (Specialists), Institute of Molecular Bioscience, Mahidol University*

*** *Associate Professor, Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University*

Introduction

Blood is a fluid connective tissue in body that contains red blood cells, white blood cells, platelets, and plasma. It has essential functions in the body. These include nutrients and oxygen transportation, body temperature regulation and excess blood loss prevention (Docherty, 2012). Blood transfusion is therefore necessary to replace the blood lost in patient. As a result, blood container or blood bag is an important device for blood transfer. In general, it is a plastic container which may be designed as single bag or combined unit according to the purpose of its use. The bag usually contains several sterile solutions for blood processing (Kitgrianggaikul, 2012). These include anticoagulant or blood preservation agent (Ministry of public health, 2016) . Polyvinyl chloride (PVC) is normally a plastic material used in a wide variety of medical devices including blood bag. It has been claimed safe, reliable and cost-effective material (PVC Med Alliance, 2016). However, PVC material processing and molding required plasticizer to obtain the desired properties of soft and pliable . The popularly used of Di-2-ethylhexyl phthalate (DEHP) plasticized in polyvinyl chloride (DEHPPPVC) of medical devices turned out a suggested evidence that DEHP migration can be harmful, and toxicity of DEHP has been demonstrated in vitro (Li et al. 2015). Therefore, alternative plasticizers such as TOTM, DEHT, DINCH, DINP, DEHA, and ATBC has been replaced. Furthermore, blood bag manufacturer usually have quality control for the safety assessment of DEHP level in the products, as well as getting standard properties such as physical, chemical, and biological properties according to ISO 10993-5.

The import blood bags from oversea must pass biocompatible test, according to ISO 10993-5 or the guideline from public health ministry (Ministry of public health, 2016) and TIS 1298-2012 (TIS:1298, 2012), before distribution and sale in Thailand. The quadruple blood bags from 3 manufacturers were cytotoxic tested using animal cell culture of mouse fibroblast L929 cell line. The acute cytotoxicity assays and sample preparation were done according to ISO 10993-5. The leachable toxic molecule from material was done using agar diffusion, while direct contact could be determined either leachable or non-leachable toxic molecule. Furthermore, toxic chemicals extracted from plastic bag material were also tested on cell viability using MTT assay. All these different methods could provide sufficient data on acute cytotoxicity for determination the safety of blood bag materials.

Objectives of the study

To investigate the safety of blood bag material using animal cell culture, by monitoring acute cytotoxicity on L929 cells line.

Methodology

The sample preparation and assay methods by agar diffusion, direct contact and MTT elution assay were done according to ISO: 10993-5. The positive and negative control was included in agar diffusion and direct contact assay. While MTT elution were determined based on untreated condition.

Sample preparation

The sterile quadruple blood bag products from 3 manufacturers were coded as A, B, and C. Samples (RCB bag, CPD bag, platelet bag and satellite bag) were cut into small pieces approximately 0.25 cm^2 by aseptic technique in laminar

flow cabinet. They were used directly in agar diffusion assay and direct contact assay. For MEM elution assay, the sample was immersed in 5 % fetal bovine serum MEM medium for 24 h extraction at 37 ºC.

Cell culture

L929 mammalian fibroblast cells were used as cell model in acute cytotoxicity assay. They were grew and maintained in Minimal Essential Medium supplemented with 5% fetal bovine serum. The cultures were maintained in a humidified 5% CO_2incubator at 37 ºC.

Agar diffusion assay

The cell suspension (3×10^5cell/ml) in MEM medium and plated on petti-dish. After 24 h of incubation, cell monolayer was stained with 50 µg/ml of neutral red for 4 h and then replaced with MEM without phenol red medium added with 2% agar. The test samples including positive and negative control were placed on solidified agar layer and further incubated for 24 h. After that, cell morphology and clear zone were determined under the inverted microscope in comparison with positive and negative control. The toxicity levels of specimens were classified based on cell morphology and clear zone or reactivity grade as showed in Table 1.

Direct contact assay

Cells L929 (1.5×10^5 cell/ml) were seeded in 24 well tissue culture plates and incubated for 24 h. Then specimens of test sample were placed on cell monolayer by contact directly in culture condition and further incubated for 24 h. Subsequently, cells were incubated with 50 µg/ml of neutral red and observed the morphological change under inverted microscope compared with positive and negative control. The toxicity levels of specimens were classified based on cell morphology and clear zone or reactivity grade as showed in Table 1.

Table 1. Reactivity grades for agar diffusion test and direct contact test (ISO:10993)

Grade	Reactivity grade	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Few malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending from specimen up to 0.5-1.0 cm
4	Severe	Zone extending beyond 1.0 cm around specimen

MEM elution assay (cell viability assay)

Cell viability was evaluated using the MTT assay. Briefly, L929 cells were seeded into 96-well cell culture plates at a density of 1.3×10^5 cells/ml. After 24 h, the culture medium was replaced with serial solutions of blood bag extract at varying dilutions. After incubation for 24 h, 50 µl of MTT solution was added into each well and incubated for 4 h. The purple formazan product was dissolved in 50 µl dimethylsulfoxide and quantitated by a plate reader at wavelengths of 570 nm. The percent relative cell viability based on control condition without extract sample was calculated using the formulation below. Reactivity grade was also determined based on cell condition as in Table 2.

([A] test/[A] control)* 100.

Table 2. Qualitative morphological grading of cytotoxicity of extracts (ISO:10993)

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50 % growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

Data analysis

Data of triplicate sample from three experiments were expressed as mean ± SD. The SPSS version 16 Statistical software was used to perform the statistical analysis by the One-way ANOVA and Post Hoc. P-values less than 0.05 were taken as statistically significant.

Results

Safety assessment of material based on agar diffusion

A quadruple blood bags is normally used without any further treatment. Therefore, the material of blood bag from manufacturer should be safe. Cytotoxicity results by agar diffusion assay of blood bags material from 3 manufacturers were showed in Fig. 1. Based on criteria of ISO 10993-5 in agar diffusion assay revealed that all four blood bag materials from each manufacturer had slight toxicity at level 1 reactivity grade. The cells demonstrated few malformed and degenerated cells under the specimens of all tested samples (1d-1o) as shown by few non stained red cells. Negative control showed non-toxicity by giving all red vital fibroblast cells under and beyond the specimen. Conversely, cell under positive control ZDEC (polyurethane film containing 0.1 % zinc diethyl dithiocabamate) (1a) showed clear zone in red lawn of vital cells extending up to 0.5 cm. The cells around positive control were damaged with shrink and disintegrating cell membrane. This large clear zone of cell lysis was classified as level 3 of reactivity grade.

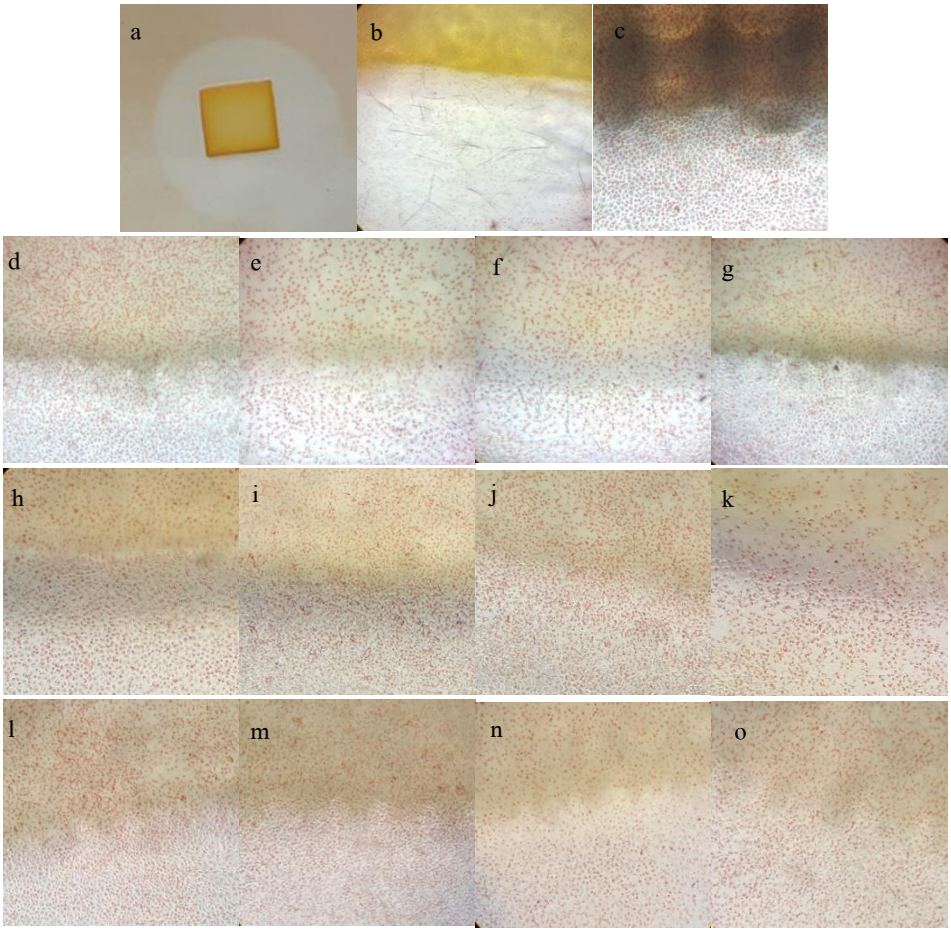

Fig.1 Cell morphology change in agar diffusion method of all four blood bags from 3 manufacturers, as compared to positive control (a-b) and negative control (c). The blood bag material of manufacturer A (d-g); (d- AS-5 bag, e-CPD bag, f-platelet bag, g-satellite bag),blood bag material of manufacturer B (h-k); (h-SAGM-2 bag, i-CPD bag, j-platelet bag I, k-platelet bag II), and blood bag material of manufacturer C (l-o); (l-AS-5 bag, m-CPD bag, n-platelet bag I, o-platelet bag II) were demonstrated.

Safety assessment of material based on direct contact

Direct contact test results of all four materials from 3 manufacturers (2c-2n) including negative control (2b) and positive control (2a) were shown in Fig. 2. Direct contact gave more cytotoxic result in all materials than agar diffusion. The cells contacted with positive control were obviously dead, giving clear zone to all well surface area in culture plate. The reactivity grade of positive control was at level 4, while that of negative control was classified as level 2 toxicity which had clear zone only under the sample. The tested materials from four blood bags of three sources also showed similar result to negative control, and were graded for toxicity at level 2.

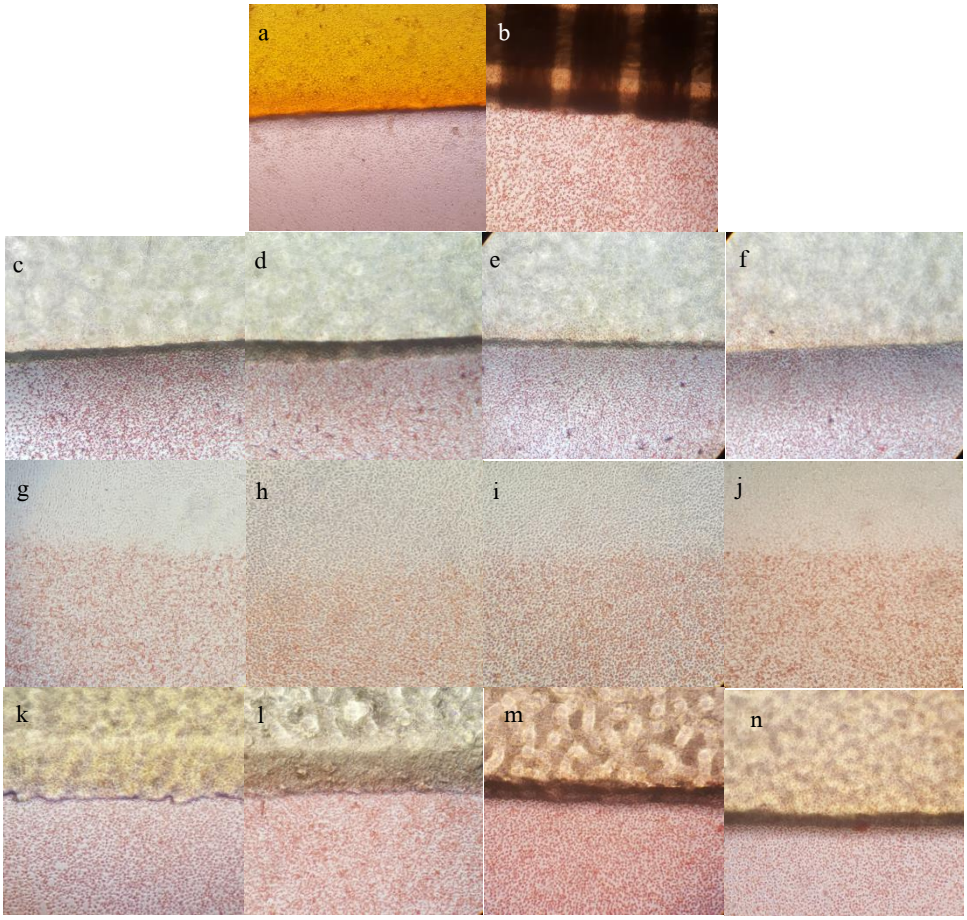

Fig.2 Cell morphology change in direct contact method that contain with positive control (a), negative control (b), blood bag material from manufacturer A (c-f); (c-AS-5 bag, d-CPD bag, e-platelet bag, f-satellite bag), blood bag material from manufacturer B (g-j); (g-SAGM-2 bag, h-CPD bag, i-platelet bag I, j-platelet bag II) blood bag material from manufacturer C (k-n); (k-AS-5 bag, l-CPD bag, m-platelet bag I, n-platelet bag II)

Determination of cells viability

The extracts from blood bag materials were tested for cell viability assay. As shown in Figure 3, all extract samples from 3 sources still giving high cell viability, at all tested concentration at 0.05, 0.1 and 0.2 g/ml after 24 hours incubation. Viability of cells was approximately at 90%, 80%, and 75% of sample bags from manufacturer A, B, and C, respectively. The cell morphology at 0.2 g/ml samples that used to determine the reactivity grade were also presented in Fig. 4. The cells from 3 sample sources of all blood bags material showed less than 50% of rounded or lysed cells which demonstrated the reactivity grade at level 2, while cells exposed to platelet bag extracts demonstrated reactivity grade at level 1.

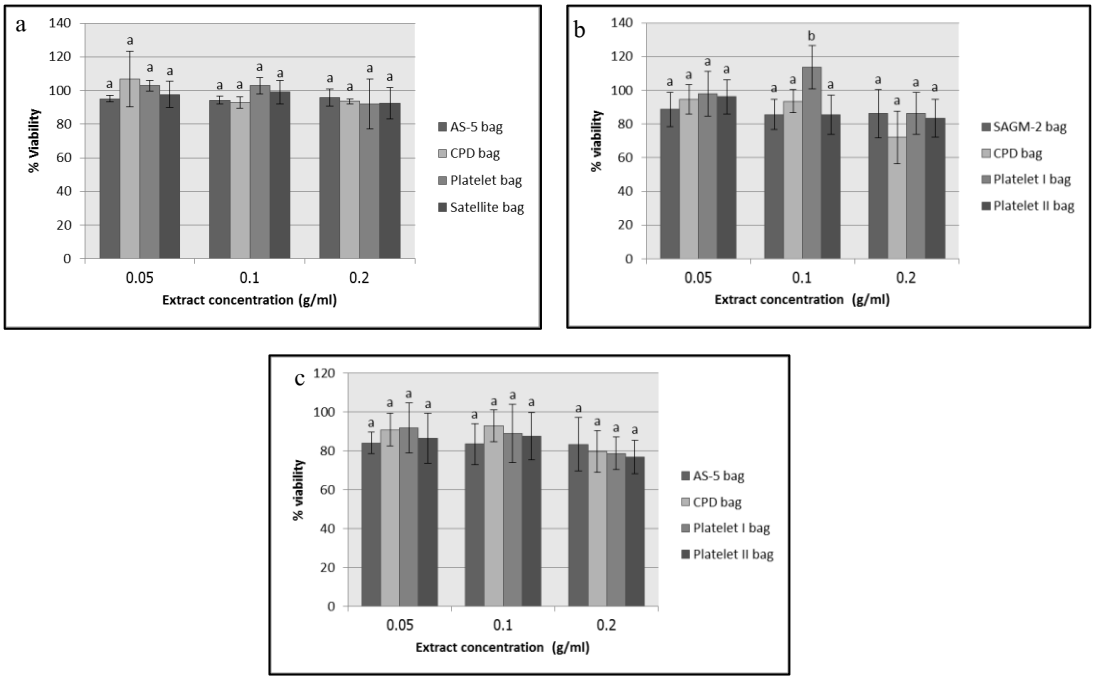

Fig. 3 Percent cell viability by MTT assay of extracts from manufacturer A (a) manufacturer B (b) and manufacturer C (c), after 24 h incubation(n=3), a and b represent statistical different at P<0.05 within each manufacturers.

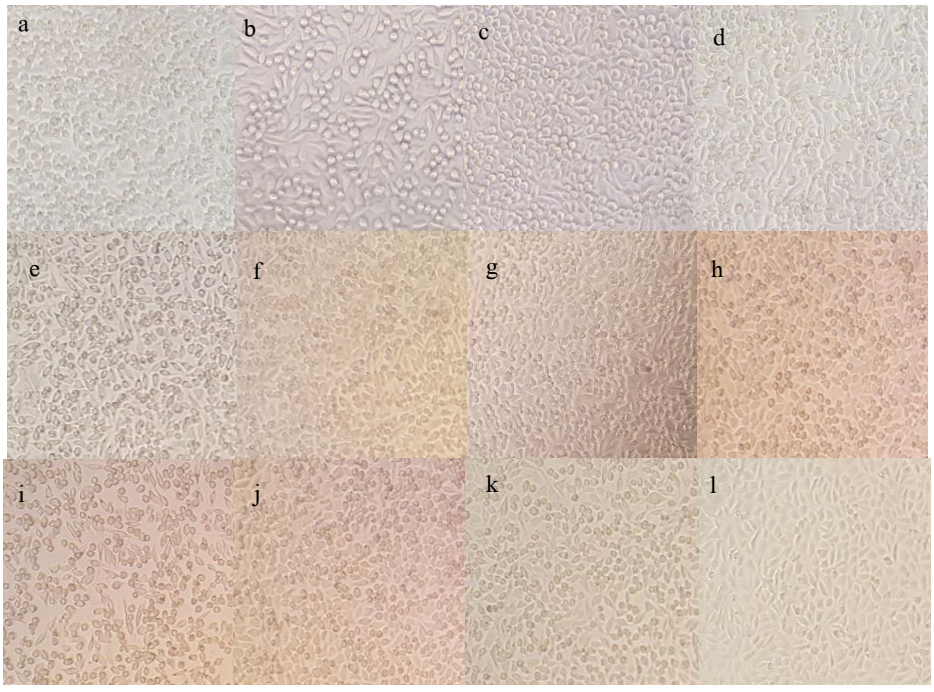

Fig.4. Cell morphology in MEM elution assay of 0.2 g/ml extract from blood bags of manufacturer A (a-AS-5, b-CPD bag, c-platelet bag, d-satellite bag), from blood bag of manufacturer B (e-RBC Bag, f-CPD Bag, g-Platelet I bag, h-Platelet II bag), and from blood bag of manufacturer C (i-AS-5 bag, j-CPD bag, k-Platelet I bag, l-Platelet II bag)

Discussion and conclusions

Quadruple blood bags manufactured from 3 difference sources were determined for safety using three methods of acute cytotoxicity based on the international standard ISO 10993-5. Agar diffusion assay was used to determine the leachable toxic chemicals from the tested materials that migrated or diffused through the agar to the cell monolayer. It has been used to determine the toxicity of high density material of medical device, while the direct contact assay usually used for safety assessment of low density material that give leachable or non-leachable toxic chemicals. The results from both assays of all materials tested in this study showed that direct contact assay demonstrated higher toxicity level of material than agar diffusion assay. This was different from cytotoxicity testing of plastic parenteral container using agar diffusion and direct contact by Charoensit and Chaisomboonpan (Charoensit and Chaisomboonpan, 2015) that gave the same non-toxicity of material at slight grade (level 1) by both assay. However, they also reported a higher toxicity level in direct contact than agar diffusion in some samples similar to this work. The higher level of toxicity test by direct contact than the agar diffusion assay was probably due to the weight of material as the negative control also provided same reactivity grade. While, the positive control clearly demonstrated for material toxicity, by the release of toxic substance Zincdiethyl dithiocarbamate (ZDEC) from material and cause cell death. Based on the result of 2 assays compared with negative and positive control, the materials from 3 sources could be classified as slight toxicity at level 1. According to ISO 10993-5, all samples were considered to be non-toxic if the scoring level was less than 2.

For MEM elution assay, the extract samples provided cell growth to more than 70% . Therefore, the cell viability at 90%, 80%, and 75% from sample bags of manufacturer A, B, and C, respectively, were considered safe. Furthermore cell morphology at 0.2g/ml showed different results among materials. The platelet bag extract provided more normal dense cells than CPD bag and AS-5 or SAGM-2 bag extract, respectively. The slight toxicity of samples was probably due to CPD and AS-5 or SAGM-2 solution that contaminated on blood bag material. The cytotoxicity assay of CPD solution has confirmed the higher toxicity of CPD solution than AS-5 or SAGM-2 solution, respectively.

In summary, safety assessment of blood bags from three manufacturers using L929 mammalian fibroblast cells by three methods of agar diffusion, direct contact and MEM elution assay according to ISO 10993-5, revealed slight reactivity grade (level 1) by agar diffusion, mild grade (level 2) by direct contact, and MTT assay. The materials could be considered as no-cytotoxic since none of the cultures exposed to the test item shows greater than mild reactivity (Grade 2). Therefore, all material of blood bags from 3 sources by manufacturer A, B, and C had acceptable safety criteria according international standard ISO 10993-5 and are safe to be clinical used. Additionally, chemical solution in each blood bag from A source seem to be no cytotoxic while, that from B and C manufacturer might need to be reconfirmed for their safety.

Acknowledgements

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