Viral safety of APOSEC™: a novel peripheral blood mononuclear cell derived-biological for regenerative medicine

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Background. Viral reduction and inactivation of cell-derived biologicals is paramount for patients' safety and so viral reduction needs to be demonstrated to regulatory bodies in order to obtain marketing authorisation. Allogeneic human blood-derived biological medicinal products require special attention. APOSEC™, the secretome harvested from selected human blood cells, is a new biological with promising regenerative capabilities. We evaluated the effectiveness of inactivation of model viruses by methylene blue/light treatment, lyophilisation, and gamma irradiation during the manufacturing process of APOSEC™.

Materials and methods. Samples of intermediates of APOSEC™ were acquired during the manufacturing process and spiked with bovine viral diarrhoea virus (BVDV), human immunodeficiency virus type 1 (HIV-1), pseudorabies virus (PRV), hepatitis A virus (HAV), and porcine parvovirus (PPV). Viral titres were assessed with suitable cell lines.

Results. Methylene blue-assisted viral reduction is mainly effective against enveloped viruses: the minimum $\log_{10}$ reduction factors for BVDV, HIV-1, and PRV were ≥ 6.42, ≥ 6.88, and ≥ 6.18, respectively, with no observed residual infectivity. Viral titres of both HAV and PPV were not significantly reduced, indicating minor inactivation of non-enveloped viruses. Lyophilisation had minor effects on the viability of several enveloped model viruses. Gamma irradiation contributes to the viral safety by reduction of enveloped viruses (BVDV: ≥ 2.42; HIV-1: 4.53; PRV: ≥ 4.61) and to some degree of non-enveloped viruses as seen for HAV with a minimum log10 reduction factor of 2.92. No significant reduction could be measured for the non-enveloped virus PPV (2.60).

Discussion. Three manufacturing steps of APOSEC™ were evaluated under Good Laboratory Practice conditions for their efficacy at reducing and inactivating potentially present viruses. It could be demonstrated that all three steps contribute to the viral safety of APOSEC™.

Keywords: viral safety, secretome, regenerative medicine, APOSEC, blood mononuclear cells.

Introduction

Although cellular secretomes have shown promising therapeutic potential in regenerative medicine, their viral safety has yet to be proven under Good Laboratory Practice (GLP) conditions in order to be approved by regulatory bodies for clinical trials. With regards to patients' safety, pooled allogeneic blood-derived products are of the highest priority[1,2]. APOSEC™ is a novel biological produced from gamma-irradiated human peripheral blood mononuclear cells[3,4]. Several preclinical studies showed that this product has a beneficial regenerative effect on tissues damaged by hypoxic conditions, such as myocardial infarction, stroke, and spinal cord injury[5-7]. APOSEC™ exerts cytoprotective effects, induces cell proliferation and migration of repairing cells including endothelial cells for neo-vascularisation, provides antibacterial activity and exhibits immunomodulatory effects[3].

The potency of APOSEC™ cannot be attributed to one or a particular subset of active ingredients. Several studies have proven that only the interaction of all constituents (proteins, lipids, exosomes) ensures the full regenerative potency of the secretome (Wagner *et al.*)[8,9]. None of the isolated fractions (proteins, lipids or exosomes alone) came close to having the same efficacy as APOSEC™ *in toto*[9]. Blocking single proteins hypothesised to contribute to the mode of action did not lead to a diminished potency[5,8,10]. These data showed that the beneficial effects attributed to APOSEC™ cannot be achieved by reproducing specific constituents in a xeno-free manner and justify the use of the whole secretome for clinical applications.

APOSEC™ is produced under Good Manufacturing Practice (GMP)-compliant conditions and has already been tested in a clinical phase I study (as an autologous product). This study (MARSYAS I) demonstrated the safety of the product[11]. Allogeneic APOSEC™ will be tested in a phase II study of diabetic foot ulcer (MARSYAS II) . Thus, validated viral clearance strategies are of the utmost importance according to the guidelines for biologicals[1,2]. Although the final product is cell-free, the secretome must undergo viral clearance before administration to patients. The production process includes four steps that lead to viral depletion or inactivation. After culturing peripheral blood mononuclear cells (PBMC), cells are removed by centrifugation and the secretome is filtered through a 0.22 μm sterile filter which removes possible cell-bound viral components, as well as host cells, capable of viral replication and possible bacterial contamination. Thereafter, the product is treated with methylene blue (MB) in combination with visible light (627 nm±10 nm), a standard procedure for viral clearance in therapeutic human plasma for transfusion. A further manufacturing step is lyophilisation, which has the potential to inactivate viruses. As a final step, the product is exposed to ≥25 kilogray (kGy) gamma (γ) irradiation.

MB is a phenothiazine dye, which intercalates into nucleic acids. Upon illumination with visible light it generates singlet oxygen leading to guanosine oxidation and destruction of viral nucleic acid preventing viral replication. MB/light treatment was established several years ago and is one of the virus inactivation methods recommended by the World Health Organization[1]. MB/light treatment is approved in several countries of the European Union as the MB-Plasma system for viral reduction of plasma products. The inactivation efficacy for enveloped viruses is typically 4-6 $\log_{10}$, while non-enveloped viruses are variably responsive. Ionising radiation (including gamma-irradiation)-assisted sterilisation is an approved method for terminal sterilisation according to the European Pharmacopoeia

(Ph. Eur. 5.1.1) with a reference absorbed dose of 25 kGy. This treatment is also recommended for the inactivation of viruses[1]. Its viral clearing capacity is believed to be based on direct and indirect mechanisms. The direct mechanism is attributed to radiolytic cleavage or crosslinking of genetic material. Indirect effects of gamma irradiation are principally ascribed to the actions of radicals such as OH• or ozone. These molecules can react with viral nucleic acids and proteins.

In this study, the main orthogonal viral clearance steps in the manufacturing of APOSEC™, MB/light treatment and gamma irradiation, as well as lyophilisation, are validated under GLP conditions for their efficacy to deplete and/or inactivate enveloped and non-enveloped viruses according to guideline Q5A R(1) of the International Conference on Harmonisation[12].

Materials and methods
Manufacturing of APOSEC™-intermediate and APOSEC™

APOSEC™ is manufactured under GMP conditions by the Red Cross Blood Transfusion Service of the Red Cross of Upper Austria, Linz, Austria. Blood donor selection and blood donations are in compliance with European and national regulations and standards regarding acceptance criteria, procedures, and documentation. Written informed consent was obtained from each donor. Every single blood donation is tested for hepatitis A, hepatitis B (HBV), hepatitis C (HCV), hepatitis E (HEV), human immunodeficiency viruses-1 and -2 (HIV-1 and HIV-2) and parvo B19 viruses as well as randomly for cytomegalovirus and herpes simplex 1/2 specifically for APOSEC™ production. PBMC are extracted from whole blood donations (extraction from buffy coat, followed by density centrifugation using lymphocyte separation medium) and washed twice with Dulbecco's phosphate-buffered saline and re-suspended in CellGenix® GMP DC Medium (CellGenix GmbH, Freiburg, Germany). Apoptosis/necroptosis is induced by 60 Gy (2×30 Gy) γ-irradiation. PBMC are cultured for 24±2 h at a standardised cell density of $25×10^6$ cells/mL. Cells are removed by centrifugation and the supernatant (the secretome of the PBMC) is filtered in a sterile manner through a 0.22 μm filter. About 12 secretomes that have been processed individually up to this step are pooled. Next, this APOSEC™-intermediate is subjected to a viral reduction step, i.e. MB/visible light treatment using a Macotronic B2 from Macopharma (Tourcoing, France). Afterwards, the product is pooled again, to combine secretomes from 90-100 donors in total, aliquoted in glass vials, lyophilised and sterilised by gamma irradiation with 25-35 kGy on dry ice. This drug product is called APOSEC™ and is stored at −80 °C.

All tests described below were conducted at the GLP laboratories of Charles River Laboratories Germany GmbH (CRL) in Cologne, Germany. No ethics committee vote was needed from the Medical University of Vienna.

Viruses and cell lines

In line with the "Note for guidance on virus validation studies"[13], this study was conducted with the viruses listed in Table I. A master virus stock was characterised, e.g. by sequence analysis, growth kinetics, stability, and capability to infect different kinds of indicator cell lines (phenotypic analysis). The master virus stock was used to prepare intermediate virus stocks. Virus working lots used in the studies were prepared from the intermediate virus stock and/or the master virus stock. A master cell bank of the indicator/propagation cell lines was prepared (Table I). The master cell bank was characterised, e.g. by purity, identity, and stability and then used to prepare a working cell bank of the indicator/propagation cell lines.

Culture of cell lines

Adherent cells were cultured regularly. Cells were seeded confluently into cell culture flasks and incubated for a cell line-specific incubation period. After reaching confluence, cells were washed with phosphate-buffered, trypsinised, centrifuged, counted and seeded into fresh culture flasks at a defined cell density. Suspension cell cultures were cultured regularly. The cells were seeded at a defined density into cell culture flasks and incubated for a cell-specific incubation period (Table I). After reaching sufficient density, cells were centrifuged (depending on the density of the cells), counted and seeded into fresh culture flasks at a defined cell density.

Pre-tests for test validation

Cytotoxicity of the test samples was assessed in a pre-test to define a non-toxic concentration for the assay to determine virus titre: in eight-fold replicates, 100 µL of APOSEC™-intermediate or lyophilised and reconstituted APOSEC™ (undiluted and/or diluted and then serially diluted 1:3 in cell culture medium) were added to one well of the 96-well plates containing the indicator cells in 100 µL cell culture medium. After at least 3 days, all cultures were examined microscopically for cytotoxic effects.

To further validate the test, interference and recovery assays were performed: the virus stock solutions were titrated in the presence of cell culture medium and APOSEC™-intermediate or lyophilised and reconstituted APOSEC™, at least at one non-cytotoxic concentration in order to measure a potential interference with the virus during the incubation period. Viral titers were determined according to the Spearman-Kärber's formula[14,15].

Table I - List of tested viruses and corresponding indicator cells.

Bovine viral diarrhoea virus (BVDV)	
Family	*Flaviviridae*
Strain	NADL
Origin/supplier	American Type Culture Collection (ATCC), VR 534
Characteristics	Single-stranded RNA virus, enveloped, 40-50 nm
Indicator/propagation cells	MDBK, bovine kidney
Origin/supplier	European Collection of Animal Cell Cultures (ECACC), 90050801
Determination of CPE	After 5-8 days

Human immunodeficiency virus type 1 (HIV-1)	
Family	*Retroviridae*
Strain	Bru
Origin/supplier	Georg-Speyer-Haus, Frankfurt
Characteristics	Single-stranded RNA virus, enveloped, 80-100 nm
Indicator/propagation cells	C8166, human T-cell line, HTLV-I transformed
Origin/supplier	European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, UK (#88051601)
Determination of CPE	After 7-12 days

Pseudorabies virus (PRV)	
Family	*Herpesviridae*
Strain	Aujeszky
Origin/supplier	American Type Culture Collection (ATCC), VR-135
Characteristics	Double-stranded DNA virus, enveloped, 120-200 nm
Indicator/propagation cells	Vero, African green monkey cell line from kidney tissue
Origin/supplier	European Collection of Animal Cell Cultures (ECACC), 84113001
Determination of CPE	After 5-7 days

Hepatitis A virus (HAV)	
Family	*Picornaviridae*
Strain	HM175
Origin/supplier	FDA Feinstone, RKI, Analysis; Robert-Koch-Institute
Characteristics	Single stranded RNA virus, non-enveloped, 27 nm
Indicator/propagation cells	FRhK-4, foetal Rhesus monkey kidney cells
Origin/supplier	European Collection of Animal Cell Cultures (ECACC), 84120701
Determination of CPE	After 11-6 days

Porcine parvovirus (PPV)	
Family	*Parvoviridae*
Strain	NADL-2
Origin/supplier	American Type Culture Collection (ATCC), VR-742
Characteristics	Single-stranded DNA virus, non-enveloped, 20-26 nm
Indicator/propagation cells	PK13, a porcine cell from kidney tissue
Origin/supplier	American Type Culture Collection (ATCC), CRL-6489
Determination of CPE	After 10-14 days

CPE: Cytopath(ogen)ic effect.

The recovery assay was performed to determine the reduction of the model virus caused by the test items directly. Aliquots (2 mL) of test items, APOSEC™-intermediate and APOSEC™, and CellGenix® GMP DC medium (control) were spiked with a defined titre of each model virus, by adding 200 µL virus stock solution. After spiking, the samples were incubated in the dark for 60 minutes. All samples were diluted with cell culture medium and analysed for the virus titre by endpoint titration.

Preparation and spiking of test samples for methylene blue/visible light treatment

MB was added to APOSEC™-intermediate as well as to placebo (CellGenix GMP DC medium only), according to manufacturer's study protocol. The samples were spiked with a virus stock solution (10% of volume) of the model viruses (see Table I). After mixing, control samples were drawn before light treatment. The remaining samples were treated with light (627 nm±10 nm) at room temperature at an intensity up to 120 J/cm^2. After treatment, samples were diluted with culture medium and the virus titre was analysed.

**Preparation and spiking of test samples for lyophilisation and gamma irradiation**

Test samples (16 mL APOSEC™-intermediate after MB treatment) were spiked with 1.6 mL virus stock solution (10% of volume). The solution was mixed and 4 mL of virus spiked test item was placed in a lyophilisation glass vial. In total, four vials per virus/run were prepared (see Table I for the viruses tested). As

controls, unspiked test items (APOSEC™-intermediate without a virus spike) and placebo (CellGenix® GMP DC medium) were prepared. In total, four vials were prepared for analysis of the residual moisture after lyophilisation with 4 mL of unspiked test item, four vials were prepared with 4 mL of medium to monitor the temperature during lyophilisation and additional vials were prepared with 4 mL of medium to fill up the tray of the lyophiliser to the full capacity of 48 vials to ensure a full run. All vials were prepared at ambient temperature (spiked test item, unspiked test item and placebo solution) and then partially closed with stoppers and placed onto the tray in the lyophiliser. Subsequently, the lyophilisation programme was started. After freeze-drying, all vials, except those for temperature measurement, were sealed under vacuum, capped and stored at below −60 °C.

The study design is depicted in Figure 1.

Methylene blue/visible light treatment

The MB/light treatment (commercially available as THERAFLEX MB-Plasma system) is one of the virus inactivation methods recommended by the World Health Organization[1]. The system includes a membrane filter (which removes residual leucocytes, red cells, platelets and aggregates, intra-cellular viruses and reduces microvesicles and microparticles) as well as a MB removal filter. MB has a viral inactivation effect by intercalating into viral nucleic acids where it destroys the structure and prevents viral replication. The inactivation efficacy of enveloped viruses is typically 4-6 log$_{10}$, while non-enveloped viruses are variably responsive.
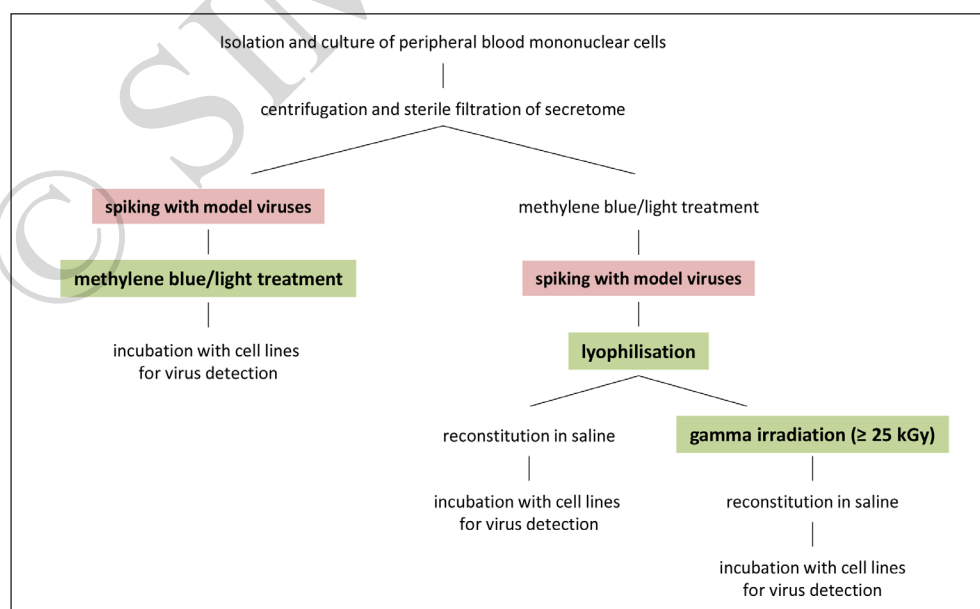
Figure 1 - Study design for methylene blue/light treatment, lyophilisation, and gamma irradiation.

The APOSEC™ samples were subjected to visible light treatment (627 nm±10 nm) in increments, i.e. cumulative doses of 20 J/cm^2, 40 J/cm^2, 60 J/cm^2, and 120 J/cm^2. Placebo was only treated with the standard operating dose of 120 J/cm^2.

Gamma irradiation

The vials with the test item were shipped from CRL (Cologne, Germany) on dry ice to Mediscan GmbH & CO KG (Seibersdorf, Austria) for gamma irradiation. The irradiation procedure was performed with <25 kGy (22.9-24.0 kGy) to assess a worst case scenario, given that the standard manufacturing procedure stipulates irradiation with >25 kGy. Control samples were also shipped to the facility but were not irradiated.

Reconstitution of lyophilised samples

For testing, the vials with virus-spiked APOSEC™ were reconstituted by addition of 4 mL 0.9% NaCl sterile solution. All samples were diluted with cell culture medium and analysed for virus titre.

Determination and calculation of the virus titre

Samples were analysed by endpoint titration. Briefly, to determine the virus titre of a sample, serial three-fold dilutions were made with cell culture medium. Aliquots (100 µL) of each dilution were added to eight wells of a 96-well plate with cells (in 100 µL cell culture medium per well). Cells were cultured for a specified incubation period (Table I) and inspected microscopically for virus-induced changes in cell morphology. If no virus-positive cultures were detected, a large volume of sample was analysed to improve the detection limit. For these large volume platings, 200 µL of the diluted sample were added to indicator cells in 100 µL culture medium. After a defined period of culture, the morphology of the cells was inspected microscopically. The virus titre (TCID50/mL) which caused a positive result in 50% of the test cultures (TCID50) of an endpoint titration was calculated according to the Spearman-Kärber's formula[14,15].

Results

Pre-tests

A non-cytotoxic MB concentration was titrated to a dilution factor of 1:18. The Vero cell line was sensitive to high MB concentrations in medium but not in APOSEC™. The interference assay was performed using a non-cytotoxic final dilution derived from the cytotoxicity assay (*data not shown*). A non-cytotoxic and non-interfering dilution was determined for each test item.

No reductions induced by the test items themselves were determined after an incubation period of 1 hour. All five test viruses, i.e. bovine viral diarrhoea virus (BVDV), HIV-1, pseudorabies virus (PRV), hepatitis A virus (HAV), and porcine parvovirus (PPV), were completely recovered from the different test items (*data not shown*).

Methylene blue and light treatment

The log$_{10}$ reduction factors are listed in Table II. The results are derived from non-interfering final dilutions. The enveloped viruses BVDV, PRV, and HIV-1 were effectively inactivated by MB and light treatment. No infectivity was detected in the highest dose in both runs of the APOSEC™-intermediate. Infectivity could be measured with PRV in the CellGenix® GMP DC medium in both runs and with HIV in the medium in one run. The non-enveloped viruses HAV and PPV were not inactivated by MB and light treatment and infectivity was detected in both runs of both test items.

Lyophilisation

The log$_{10}$ reduction factors after the lyophilisation step are listed in Table III. Remarkable virus titre reduction was found for BVDV and to lesser extents for PRV and HAV. In contrast, HIV-1 and PPV were not affected by lyophilisation.

Gamma irradiation

The log$_{10}$ reduction factors defined for the gamma irradiation (<25 kGy doses) after lyophilisation refer to the load fractions of the related process step and are listed in Table IV. Results are derived from non-interfering final dilutions.

The enveloped viruses BVDV and PRV were inactivated by gamma irradiation after lyophilisation. No infectivity was detected in both runs of the <25 kGy dose. The enveloped virus HIV was inactivated by gamma

Table II - Virus inactivation by methylene blue/light treatment.

	Log$_{10}$ reduction factor of					
Test item	**APOSEC™-intermediate**				**Medium**	
Dose	*60 J/cm^2*		*120 J/cm^2*		*120 J/cm^2*	
Run	1	2	1	2	1	2
Virus						
BVDV	≥6.48	≥6.42	≥6.48	≥6.42	≥6.48	≥6.30
PRV	≥6.82	≥7.24	≥6.82	≥7.24	7.17	7.29
HIV-1	≥6.18	≥6.36	≥6.18	≥6.36	6.41	≥5.94
HAV	-	-	0.84*	-	0.73*	1.55*
PPV	0.05*	−0.12*	−0.30*	−0.30*	0.17*	0.34*

BVDV: bovine viral diarrhoea virus; PRV: pseudorabies virus; HIV-1: human immunodeficiency virus type 1; HAV: hepatitis A virus; PPV: porcine parvovirus; -: not performed; *no significant reduction, not considered (ICH Q5A); ≥: infectivity below the limit of detection.

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Table III - Virus inactivation by lyophilisation.

Process	Log$_{10}$ reduction factor ± standard deviation for									
	BVDV		HIV-1		PRV		HAV		PPV	
	Run 1	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*
Lyophilisation	4.66 ±0.32	4.3 ±0.35	0.89* ±0.37	0.87* ±0.37	2.77 ±0.40	2.00 ±0.33	1.76 ±0.37	2.78 ±0.33	0* ±0.33	0* ±0.43

*No significant reduction, not considered (ICH Q5A). BVDV: bovine viral diarrhoea virus; HIV-1: human immunodeficiency virus type 1; PRV: pseudorabies virus; HAV: hepatitis A virus; PPV: porcine parvovirus.

Table IV - Virus inactivation by gamma irradiation after lyophilisation.

Process	Log$_{10}$ reduction factor ± standard deviation for									
	BVDV		HIV-1		PRV		HAV		PPV	
	Run 1	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*
Gamma irradiation (≥25 kGy dose)	≥2.42 ±0.12	≥2.54 ±0.20	5.54 ±0.24	4.53 ±0.23	≥4.61 ±0.29	≥5.20 ±0.28	3.30 ±0.26	2.92 ±0.23	2.60 ±0.35	2.78 ±0.45

BVDV: bovine viral diarrhoea virus; HIV-1: human immunodeficiency virus type 1; PRV: pseudorabies virus; HAV: hepatitis A virus; PPV: porcine parvovirus; ≥: infectivity below the limit of detection.

irradiation after lyophilisation. Residual infectivity was detected in both runs of the <25 kGy dose.

The non-enveloped virus HAV was inactivated by gamma irradiation after lyophilisation. Residual infectivity was detected in both runs of the <25 kGy dose. The non-enveloped virus PPV was not inactivated by gamma irradiation after lyophilisation. Infectivity was detected in both runs of the <25 kGy dose. Due to high infectivity, no titre was calculated for the large volume plating assay.

Discussion

This study examines three potential viral clearance steps in a novel cell-derived, yet cell-free biological medicinal product manufactured under GLP conditions. In human therapeutic plasma manufacturing, MB/light treatment is well established, and we could show that this method is also effective for viral reduction of enveloped viruses in the secretome of PBMC (APOSEC™). Our data lead to the conclusion that lyophilisation also has a certain potential to reduce the viral titre. Finally, as a third viral inactivation step, gamma irradiation not only inactivates enveloped viruses but also shows potential to inactivate non-enveloped viruses.

Given the nature of APOSEC™ (allogeneic, blood-derived, consisting of a complex mixture of components), specific attention is paid to controlling the starting materials and to viral safety aspects. Viral safety issues already start at the stage of selecting donors which is in compliance with European and national regulations and standards regarding acceptance criteria, procedures, and documentation for blood donors. All whole blood samples are tested for several viruses (e.g. HIV, HAV,

HBV, HCV, HEV, parvo B19 or seasonal viruses such as the West-Nile virus) and risk groups are excluded from donating based on patient history. In contrast to plasma donations, isolated irradiated PBMC are cultured for 24 hours which raises the theoretical possibility of viral replication during this period. As described by Op de Beeck and Caillet-Fauquet, viruses use the large pool of deoxynucleotides and many key enzymes present in actively replicating cells[16]. Moreover, some viruses actively drive quiescent cells into the S phase of the cell cycle or prolong this phase. It is, therefore, important that we were able to show that, after gamma irradiation with 60 Gy, more than 70% of cells remain in the G2/M phase and also the remaining cells are in the G0/G1 phase[17]. No cells were in S phase which means that there was no cell proliferation. The defined culture time, limited to 24±2 hours, also contributes to viral safety as several viruses need 1.2 days (HIV) or more than 30 hours (ds DNA duck hepatitis B virus) for one replication[18]. We, therefore, preclude significant viral replication during the culture period. Finally, theoretically possible secreted viruses or viral particles would be produced before the viral reduction and inactivation steps.

After culture, cells and debris are removed by centrifugation and sterile filtration and the secretomes of 90-120 donors are pooled. During its manufacturing process, the product is subjected to a first viral reduction step (MB/light treatment), is lyophilised and then subjected to a second, orthogonal viral reduction step (irradiation within 25-40 kGy). The latter also serves as terminal sterilisation.

The efficacy of an inactivation method needs always to be balanced against its detrimental effect on the

therapeutically active components. MB/visible light treatment is known to potentially reduce potency[19]. We, therefore, compared unprocessed secretomes of irradiated PBMC with viral-reduced APOSEC™. The quantity and quality of biological components were comparable in both groups, as described in Beer *et al.*[17]. Furthermore, we compared unprocessed and inactivated secretomes for their *in vivo* effects in an experimental model of acute myocardial infarction in domestic pigs. Both reagents could significantly reduce ischaemia/reperfusion injury to a comparable extent, as shown in Table I of the article by Beer *et al*. Cardiac output and left ventricular stroke volume were among other cardiac parameters improved in both treatment groups. These data indicate that double viral clearance of APOSEC™ did not alter the product's clinically relevant potency.

In this study, the MB/light treatment method was validated with five model viruses in accordance with current guidelines on virus validation studies[2,12,13,20]. The three enveloped test viruses HIV, PRV, BVDV were inactivated by >6 $\log_{10}$ both at the recommended light energy input of 120 J/cm² as well as half the recommended light energy input of 60 J/cm². The two non-enveloped test viruses PPV and HAV were not inactivated, even by the standard light dose (120 J/cm²). The test was carried out with commercially available equipment by Macopharma (Macotronic B2). For its operating range established by Macopharma a fixed amount of MB (85 µg) is dissolved in 220 to 315 mL of product (MB 1.2 µmol/mL to 0.8 µmol/mL). The efficacy of the system is determined predominantly by the MB concentration, the light energy input, and translucency of the product. There is a considerable safety margin with respect to the recommended settings (concentration and light energy). The validation runs were carried out within the specifications of the operating ranges of the CE certified equipment for MB/light treatment of therapeutic human plasma, and the results are in line with data published on the MB/light treatment of plasma[21,22]. It is, therefore, considered safe to operate within the entire recommended volume range (220 mL to 315 mL) during the manufacture of APOSEC™ as long as the recommended light dose of 120 J/cm² is applied. MB has been used for therapeutic plasma for more than 15 years and is a widely used method for viral reduction in Europe. However, attention must be given to the possibility of anaphylactic reactions to MB, which have been reported[23,24]. We are aware that MB can induce unintended side-effects and exclude patients for clinical trials with known hypersensitivity to MB. However, the filter eliminates about 90% of MB, so that only about 0.04 µg/mL of residual MB blue might be present in the product. The highest anticipated APOSEC™ dose is the equivalent of 4 mL, i.e. 0.16 µg MB/day. Recipients of plasma are exposed to >50 times higher MB levels per application.

The final gamma irradiation of the lyophilised secretome is a second, effective viral inactivation step, orthogonal to the MB/light treatment. As described above, ionising radiation sterilisation is a recommended method for the inactivation of viruses[1] and also for terminal sterilisation according to European Pharmacopoeia Monograph 5.1.1. On the one hand, viruses can be inactivated by radiolytic cleavage or crosslinking of genetic material. Other targets of direct damage by gamma irradiation include proteins and viral envelopes, although these effects are generally believed to play a minor role in inactivation[1]. On the other hand, indirect effects of gamma irradiation are mediated by the action of radicals and ozone, created from the radiolytic cleavage of water or O_2. These molecules can react with viral nucleic acids as well as proteins. However, irradiating samples at lower temperatures has been shown to limit the indirect mechanisms of gamma irradiation, potentially reducing the damage to proteins and virion structure while still efficiently inactivating the virus[25]. Therefore, irradiation performed on APOSEC™ is carried out on dry ice (−80 °C). A study by Hume *et al*. on RNA viruses found that 3 Mrad (=30 kGy) effectively inactivated the RNA viruses under investigation[25]. The study further suggests that sample product volume and solute protein content appear to have an effect on virus reduction while sample air volume does not. The virus reduction rate proved to be independent of the initial virus titre. In 2012 Moore reported, that enveloped viruses are more susceptible to irradiation than non-enveloped viruses in human tissues[26], which is in line with our findings.

The potential risk of bacterial contamination is minimised from the beginning of the manufacturing process by excluding donors with high levels of markers of immune activation. Furthermore, the whole manufacturing process outside the closed bag system is done in clean rooms category A in B with environmental monitoring. As mentioned above, gamma irradiation also serves as terminal irradiation and is controlled by addition of spore strips. Finally, the final product is tested for sterility and endotoxins.

The viral clearance potency of lyophilisation was not the main objective of the study. However, it is known that lyophilisation can contribute a more than one log reduction in the titre of some viruses, especially HAV[27]. Consequently, virus titres were determined after lyophilisation in order to distinguish the effect of this process on virus inactivation from that of gamma irradiation. As presented in Table III, lyophilisation did indeed inactivate individual viruses significantly, i.e.

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Table V - Cumulative virus inactivation (calculated combination of methylene blue/light treatment, lyophilisation and gamma-irradiation).

	BVDV	PRV	HIV-1	HAV	PPV
Methylene blue/light treatment	6.5	7.0	6.3	0.8*	0*
Lyophilisation	4.5	0.9*	2.4	2.3	0*
Gamma-irradiation	2.5	5.0	4.9	3.1	2.7
Cumulative mean $\log_{10}$ reduction factor	13.4	12.0	13.6	5.4	2.7

BVDV: bovine viral diarrhoea virus; PRV: pseudorabies virus; HIV-1: human immunodeficiency virus type 1; PRV: pseudorabies virus; HAV: hepatitis A virus; PPV: porcine parvovirus; * no significant reduction, not considered (ICH Q5A).

>1 log for PRV and HAV and even >4 log for BVDV, but had no effect on others. In those cases, in which lyophilisation had an effect, the virus titre was reduced accordingly. This in turn meant that the viable virus load was already reduced before the gamma irradiation so that in those cases the full potential of the gamma irradiation could not be demonstrated (particularly for BVDV and PRV).

The summative (calculated) viral reduction potency of all three methods mentioned above is presented in Table V. Neither of the main virus reduction steps (MB treatment and gamma irradiation) was able to inactivate the non-enveloped parvovirus to the limit of detection. However, the starting material (whole blood donation) is tested for Parvovirus B19, i.e. if present, it would be detected and parvovirus positivity represents an exclusion criterion. This donation would be discarded before further processing. In a study, characterising the secretome of PBMC, it could be shown that APOSEC™ contains antimicrobial peptides, such as calprotectin, RNase3, and cathelicidin, which are known to act as antiviral, antifungal and antibacterial agents[10]. In the medium group, both viruses were inactivated but still had infectious potency. Moreover, the known cell-protective effect of APOSEC™ could be observed in the cytotoxicity pre-test as the indicator/propagation cell line for PRV (Vero) did not react that negatively to less diluted MB concentrations in APOSEC™ compared to the corresponding MB concentration in medium only, further corroborating cytoprotective effects ascribed to APOSEC™[3].

Conclusions

In conclusion, the risk that APOSEC™ is contaminated by viruses is extremely low as the starting material adheres to the same quality controls as blood products which are administered in much higher volumes to patients. As part of the manufacturing process, potential host cells for viruses, as well as potential bacterial contaminants, are removed by centrifugation and sterile filtration. In addition, the two main orthogonal virus inactivation steps MB/light treatment and gamma irradiation are suitable for reducing the titre of viruses, which further mitigates the risk of viral contamination and safeguards the viral safety of APOSEC™. We, therefore, consider APOSEC™ a safe product for human use with acceptable safety margins for application in patients in a clinical phase II study.

Authorship contributions

AG, DS, JR, AP, TO, MM and HJA planned the studies. JR conducted the GLP experiments. AP, SS and CG were responsible for manufacturing APOSEC™. AG, DS, ML, JR, DB and BM evaluated the data. AG, DS, ML, JR and HJA wrote the manuscript. All Authors reviewed and agreed to the manuscript.

Disclosure of conflicts of interest

The Medical University of Vienna has claimed financial interest. APOSCIENCE AG holds patents related to this work (EP20080450198, EP20080450199, and EP17209165). HA is shareholder of APOSCIENCE AG. The other Authors declare no conflicts of interest.

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