Comparative Biochemical Characterization and Advantages of Spray-Dried Plasma vs FFP

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ABSTRACT

Entegrion Inc., is developing a spray-dried plasma under the trade name of Resusix® as a plasma derived resuscitation therapy with equivalent efficacy to Fresh Frozen Plasma (FFP), but with significant differences in physical, bio-chemical and proteomic composition when compared to banked FFP. Resusix® is prepared from pooled solvent-detergent treated (SD) plasma with the intention of having a superior safety profile, and can be shipped and stored at ambient temperature. FFP is prepared from single units of donated whole blood without pathogen reduction or delipidation, and requires cold chain dependency in transport and storage. This presentation demonstrates the differences in microparticle loads between solvent detergent treated, spray dried and reconstituted plasma and banked FFP, and reports the initial data on proteomic analysis of Resusix®. There is evidence in the literature of disease transmission and aggravation of acute lung injury in certain patients receiving FFP infusion, but no reports of TRALI in clinical use of SD plasma. The pooling of source plasma for Resusix® may confer an IvIG-like effect, and the removal of MP may further reduce pro-inflammatory activity. These findings support clinical investigation of Resusix® as an advantageous alternative to FFP.

INTRODUCTION

The introduction of solvent-detergent treated plasma into the European clinical experience has been associated with an apparent improvement in the transfusion safety record over banked fresh frozen plasma (FFP)[1,2] . The process of solvent-detergent treatment (S/D) is known to be an effective pathogen reduction step and also accomplishes a significant level of delipidation, both of which may be responsible in some measure for the decrease in adverse reactions and pro-inflammatory consequences associated with infusion of plasma products[3]. There is at present no published study that demonstrates a difference in clinical efficacy between S/D plasma and FFP in spite of noted variances in levels of clotting factor or other constituents[4]. Proof of clinical non-inferiority and a significantly greater safety profile for S/D plasma over FFP seems likely but awaits directed clinical trials.

Dehydration of plasma greatly increases the ease of its transport and length of storage, and has been practiced in several forms since World War II. However, lyophilization of frozen plasma may induce changes in proteins or other constituents, as evidenced in turbidity at reconstitution[5]. Spray-drying as a dehydration technique for citrated plasma is the intellectual property of Entegrion, Inc[6] , and the subject of commercial development in partnership with Kedrion, Inc (Naples, Italy) using their S/D plasma as the source material. The resulting dried plasma product has been given the trade name of Resusix© and has proven effective in comparison to FFP and superior to colloidal fluids in resuscitation protocols in a swine polytrauma model[5,7] . Elsewhere in this conference is presented characterization of coagulant activities and properties of Resusix©[8], as well as demonstration of recovery of clotting factor activities after infusion in a swine resuscitation model[9]. Here is presented initial proteomic analyses of Resusix© versus source S/D plasma to investigate changes related to spray-drying, including quantitation of microparticle content versus FFP. The ultimate goal of Entegrion is to obtain sufficient information to apply for IND approval of Resusix© for initiation of clinical trials in Q4 2011.

References

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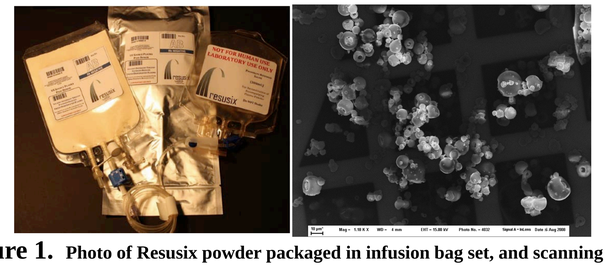


Figure 1. **Photo of Resusix powder packaged in infusion bag set, and scanning electron micrograph illustrating dispersion of Resusix powder particles.**

METHODS – ISADE MICROPARTICLE ANALYSIS

Invitrox Surface Antigen Detection Enumerator is a patented unique light scattering device. This technological breakthrough delivers rapid and accurate microparticle detection and analysis. Invitrox's Sizing, Antigen Detection and Enumeration (ISADE) is a Mie scattering method that does not require calculation of a diffusion coefficient to determine particle size. The sample is diluted in an appropriate solvent and passed though a flow cell where the particle encounters a focused laser. The amount of light scattered is proportional to the size of the particle. ISADE can assess particles from 0.15 to 20µm. A size frequency distribution is then calculated. The currrent ISADE device is equipped with three scatter detectors optimized for different ranges of signal to discriminate smaller (0.15 - 0.29 micron), medium (0.30 - 0.70 micron), and larger MP (0.71 – 4.99 micron) and can report quantitation information within each such bracket of size. Calibration is run with NIST standard particles.

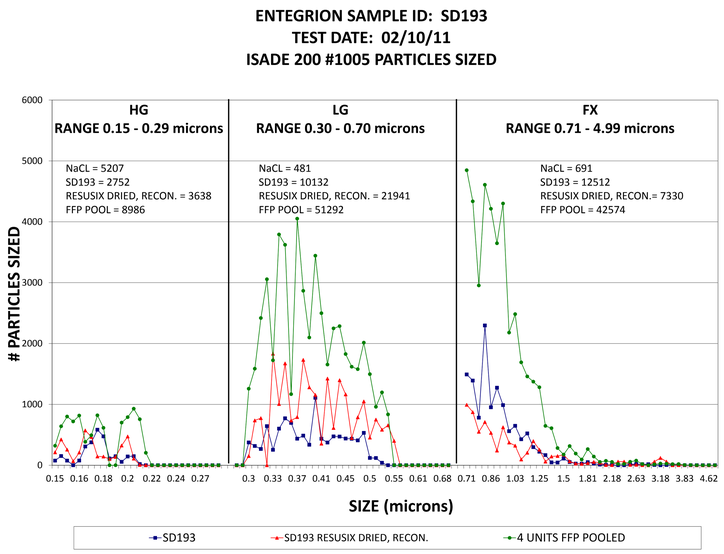


Figure 2. **Graph of ISADE particle sizing in samples of an S/D plasma preparation (blue) or its reconstituted spray-dried product (red) versus a pool of four units of FFP from local blood bank (green. There was a low number of MP in all samples in the 0.15 to 0.25 micron range, but the S/D plasma and the Resusix® seemed to have fewer of these MPs than FFP. The distribution in the intermediate size range (0.3 to 0.7 micron) showed much more in the way of sized MPs with significant differences among all three samples; FFP had the highest numbers of MPs and especially in the 0.33 to 0.42 micron range; Resusix® had more MPs in this size than the solvent-detergent treated plasma but much less than the FFP. Differences in the largest MPs (0.7 to 5.0 micron) between the three samples were also observed, with FFP showing clearly the highest numbers of MPs in this size.**

RESULTS – ISADE MICROPARTICLE ANALYSIS

In the analysis shown in Figure 2, **the FFP pool had an overall MP count of 102,852 which was 3-fold higher than the MP count of 32,909 in the Resusix** sample and 4-fold higher than the MP count of 25,396 in the S/D plasma.
The greatest contribution to this difference in MP count appeared to come from the particles in the size range of 0.7 – 2.0 microns, which would be consistent with larger cell fragments or formed microparticles from platelets or other blood cells. This finding was confirmed in ISADE analysis of other Resusix lots (see graph inset Figure 3) suggesting that these larger MP are likely removed from the S/D plasma and the Resusix by the solvent-detergent treatment but are retained in the FFP. Further analysis is continuing to identify the phenotype of the observed MP, especially in the larger size range where the greatest differences were observed.

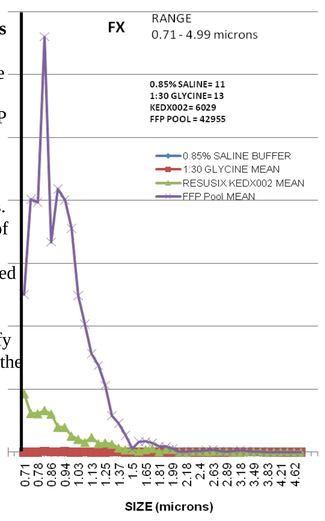


Figure 3. **Graph of ISADE particle sizing from high range detector for second lot of Resusix vs same FFP pool.**

METHODS – iTRAQ PROTEIN ANALYSIS

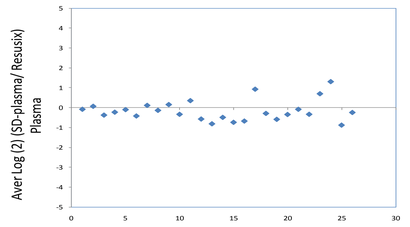
iTRAQ labeling (isobaric tagging for relative and absolute quantitation, Applied Biosystems) is an approach to analysis of the relative abundance of proteins in different samples. In this method, samples of interest are reduced and alkylated per standard protocols. Following this, each sample is digested with trypsin and the tryptic peptides are labeled with a unique iTRAQ tag. These are low molecular weight isobaric tags (MW 114, 115, 116, and 117 daltons) that have peptide reactive groups which bind the peptide N-terminus and lysine side chains. Following iTRAQ labeling, the study samples are combined, peptide sequencing is performed using mass spectrometry, and proteins identified. In addition to identification of proteins present in the test samples, the unique iTRAQ label associated with each sample allows comparison of the relative abundance of proteins between samples. The protein abundance is generally expressed as a ratio of relative abundance between the samples compared. The iTRAQ approach is able to reliably detect differences in protein abundance of greater than 50%. iTRAQ labeling is increasingly used for measurement of relative protein abundance and there are a number of reports of its use in the literature.

RESULTS – iTRAQ PROTEIN ANALYSIS

In order to assess the changes that may be induced by spray-drying from its solvent-detergent treated source plasma, samples were submitted to the UNC Proteomics Core Laboratories for analysis by the iTRAQ system of co-labeling of digestion products. A sample of reconstituted Resusix (lot#03310D) and a sample of its SD-source plasma (lot#P03909C) were digested and labeled with redundant iTRAQ peptide probes, then identified by mass spectrometry. The ratio of intensities of the most abundant identified proteins were plotted as log(2) ratios, where a value of "1" indicates a difference of 50% high or low, a value of "2" indicates 75% difference, etc.

None of the 26 proteins in this preliminary analysis exceeded a 50% difference between the samples, which is the approx error of measurement in this initial run.

Figure 4: Average log (2) diff-score for 26 most abundant proteins in plasma (S/D plasma versus Resusix)



METHODS/RESULTS – 2D DIGE GEL ANALYSIS

A sample of reconstituted Resusix (lKedx002) and a sample of its SD-source plasma (lot#P03909C) were subjected to labeling (Cy5 and Cy3 respectively) and electrophoresis on two dimensional DIGE gel in the UNC Systems Proteomics Center, and then analyzed by coincident coloring of spots.
The initial gel was dominated by the plasma proteins of highest abundance and thus yielded little information. The samples were then subjected to depletion of high abundance proteins by chroma-tography ~~on "pick" gels~~ and re-analyzed. There was also a preliminary analysis using the Decyder 2D DIA (Differential in-Gel Analysis) software module to give an estimate of significant differences in volume of protein a spot/protein is increased or decreased if there is more than a 2 fold difference in expression.

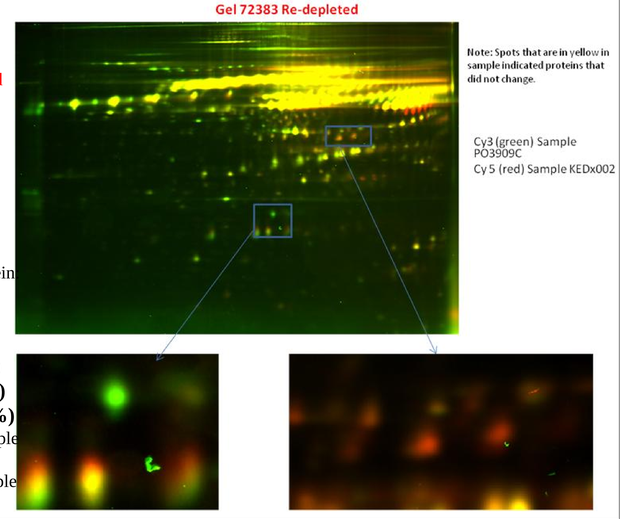


Figure 5: Overall results:
Decreased = 52 spots (4.4%)
Similar = 1010 spots (84.6%)
Increased = 132 spots (11.1%)
"Increased" refers to an increase in the KEDx002 sample compared to SD plasma P03909C
"Decreased" refers to a decrease in the KEDx002 sample compared to SD plasma P03909C

Table 1. **Identification of the thirty spots with the greatest volume differences from the depleted 2D DIGE gels shown in Figure 5. Many of the spots proved to be multiple peptides from one originating protein, with serum albumin and apolipoprotein A-IV being the greatest sources.**

Spot ID	Spot #	Abundance	Volume Ratio	UNIPROT #
Leucine-rich alpha-2-glycoprotein	2355	increased	2.14	P02750
	2268	increased	4.52	
	2334	increased	1.87	
Zinc-alpha-2-glycoprotein	2451	increased	14.74	P25311
Serum albumin	2324	increased	5.31	P02768
	2837	decreased	-2.04	
	2856	decreased	-2.11	
	3034	decreased	-2.01	
	2597	decreased	-2.12	
	2521	increased	3.93	
	3043	decreased	-1.74	
	2463	decreased	-1.58	
	2393	increased	1.52	
	3083	decreased	-1.62	
	2399	decreased	-1.62	
Apolipoprotein A-IV and Haptoglobin (comigrating in some curcumstances but it appears both have increased)	2427	increased	6.43	P06727 and Q6NSB4
	2422	increased	2.25	
	2432	increased	12.3	
	2461	increased	8.09	
	2480	increased	7.4	
	3349	increased	5.9	
	2425	increased	1.92	
Apolipoprotein A-I	3072	decreased	-1.57	P02647
Pigment epithelium-derived factor	2336	decreased	-1.54	P36955
Various Immunoglobins	2976	increased	2.91	various
	2889	decreased	-1.93	
	2943	decreased	-1.69	
	3014	decreased	-1.56	
	3023	decreased	-1.52	

CONCLUSIONS - MICROPARTICLE AND GEL ANALYSIS

The ISADE technology revealed that Resusix and its S/D source plasma contain far fewer microparticles than banked FFP, especially in the larger size range of 0.7 to 2.0 microns. Phenotyping of these MP in future studies will identify their cellular origins and potential pathological properties.

2dimensional gel electrophoresis revealed that there were only minimal differences induced in solvent/detergent-treated plasma by the Entegrion process of spray-drying; there was 85% homology of protein spots, and of those that showed significant increases or decreases most were redundant expressions of original proteins. None of those identified as yet are considered critical to the functionality of Resusix as a stabilized replacement for FFP in therapeutic plasma infusion.