Pathogen Inactivated Plasma Concentrated: Preparation and Uses[*]

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ABSTRACT

Background and Significance: Plasma transfusion is a crucial element of casualty care. Simple to perform, plasma transfusions restore the clotting factors, electrolytes, nutrients, immune factors and water that are lost in severe trauma. Unfortunately, two plasma components sharply limit the use of this life-saving fluid: contaminants and excess water. Contamination arises because plasma must be obtained from human donors, and these donors may be infected with a wide variety of pathogens. To reduce the risk posed by these pathogens, advanced screening and testing procedures have been developed. These procedures, however, are not entirely effective even in the civilian blood bank system. For the military, the situation is much worse, particularly for advanced field units operating in third world countries. If their supply lines are cut, such units may be forced to collect plasma from a local population suffering from high infection rates of malaria, HIV, or possibly even unknown pathogens. To reduce the risks of infection from plasma collected from either domestic or foreign sources, an effective means of plasma decontamination is therefore necessary. The second plasma problem, excess water, makes plasma difficult to freeze, transport, store, and thaw. Furthermore, this excess water limits the amount of clotting proteins that can be transfused without overloading the kidneys, and also lengthens the time required for lyophilization and other processing. An effective means of reducing the excess water in plasma is therefore necessary.

Approach: Ultraviolet-C (UVC) irradiation is quite effective at decontaminating blood products. Unfortunately, UVC also has sufficient energy to split dissolved oxygen into radicals, which then severely damage the plasma proteins. The solution to this problem is simply to degas the plasma before exposing it to UVC. Without dissolved oxygen, no radicals can form under UVC exposure, thereby sparing the plasma proteins. As an additional benefit, the degassed liquid is highly susceptible to ozone, another technique that is quite effective at decontamination. This high susceptibility ensures rapid, uniform treatment. The next step is to reduce the excess water in the decontaminated plasma. The limiting factor here is that plasma proteins rapidly clog conventional filtration systems. The solution to this problem is to apply ultrasound to cold plasma. The ultrasound generates pure ice crystals, which are then removed to leave concentrated plasma.

Testing: Porcine parvovirus (PPV) was spiked into the plasma to determine decontamination effectiveness. Factor VIII and fibrinogen concentrations were then measured before and after decontamination and cryoconcentration to determine protein quality.

Results: *Both UVC and ozone yielded a PPV logarithmic reduction factor (LRF) of 6, for a combined LRF of 12. UVC treatment reduced Factor VIII by 10% and fibrinogen by 7%. Ozone reduced Factor VIII by 12% and fibrinogen by 6%. Cryoconcentration up to a factor of 10 showed no measurable protein damage.*

Conclusions: *UVC and ozone yield high levels of decontamination with minimum protein damage. Cryoconcentration greatly reduces the excess water, without damaging the plasma proteins. The process can be applied to single donor units, thus avoiding the risks of pooled plasma. No additives are necessary.*

1.0 INTRODUCTION

Blood products are absolutely essential at advanced field locations. Whole blood, red blood cells (rbc's), platelets, plasma and plasma derivatives all have vital roles in on-site treatment, as well as in patient stabilization for transport to better equipped facilities.

There are three possible sources for these valuable components. First, they can be brought in with the rest of the mobile hospital equipment, but this approach is limited by the available space and the need for refrigeration for many products. When these initial supplies are exhausted, it is necessary to restock them by a supply line. If the supply lines are cut, however, it is then necessary to collect them locally, either from the forces themselves or from the local population. While soldiers donating to their fellow soldiers is a time-honored military tradition, it is inherently limited. The only remaining option is therefore to collect blood components from the indigenous populations, but this approach carries a great risk of dangerous infections, particularly in third world countries.

For an ideal supply of blood products, it is therefore necessary to consider all three of the above situations. In each situation, technologies currently under development at CryoFacets, Inc. can make great improvements over what is currently in use. Many of these advances are summarized below, starting with a single plasma unit with double pathogen inactivation. Next, a device to concentrate this plasma for shipment and direct transfusion is described. Lyophilization options are then described. Finally, a few comments on cellular components are provided. Because a great number of topics are discussed, the material on each topic is necessarily brief. CryoFacets, Inc., however, welcomes questions or other inquiries about all of these technologies.

2.0 PLASMA DECONTAMINATION

2.1 Need for Decontamination Technology

Plasma is the straw-colored liquid component of blood that remains after the cellular components have been removed. Consisting mainly of water, salts, nutrients, immune globulins and clotting factors, plasma is often transfused in cases of severe blood loss. Unfortunately, plasma can also contain a wide variety of pathogens, including parasites, bacteria, yeasts, viruses and possibly even prions.

The net result is that plasma is a high demand item, but plasma transfusion itself poses the risk of transmitting a deadly disease. In the case of domestically sourced plasma, these risks are minimal because of donor screening and extensive laboratory testing for pathogens such as HIV and hepatitis. Even then, there is still some risk due to screening failures, laboratory mistakes, the lack of specific tests for all pathogens, and the "window period" that exists between the time of infection and the generation of sufficient pathogens or antibodies to be detected.

A much more serious problem, however, occurs if it is necessary to collect plasma from an indigenous population. In this case, sophisticated laboratory testing will likely not be available, and even worse, there may be a locally high prevalence of a known or even unknown pathogens for which no test is available. For example, malaria is quite common in tropical climates, but there is no approved blood test for any of the four distinct parasite species responsible for the various forms of this debilitating disease [1].

2.2   Previous decontamination techniques

For these reasons, several different approaches to decontaminating plasma have been tested. One of these techniques, the use of a solvent-detergent, is the first wide-spread blood product decontamination technology to be marketed; the industry name is Plas+SD [2]. This process functions by attacking the lipid sheathes that surround enveloped viruses: viruses such as HIV cannot function without their envelopes. Of course, such an approach cannot attack non-enveloped viruses, but because the most dangerous currently known viruses have such envelopes, the solvent-detergent process can provide some degree of safety from infection. Unfortunately, the Plas+SD product suffers from high material and process costs and also poses the risk of bulk infection due to the pooling of multiple units of plasma in the manufacturing process. Finally, this product has been withdrawn from most markets because of adverse reactions.

Several other companies have since begun their own decontamination efforts, primarily Baxter/Cerus [3], Gambro/Navigant [4] and the Red Cross [5]. The common feature in all of these groups is the addition of some type of reagent, typically followed by exposure to ultraviolet light, either UVA and/or UVB. The light activated compound then binds the pathogen DNA and/or RNA, thereby decontaminating the product.

Note that it is not necessary to remove the pathogens: the above processes only prevent the pathogens from replicating and thus causing disease. Conversely, it is necessary to remove any toxic additives, and this requirement is a major problem with many of these technologies. It is of course not necessary to remove apparently harmless additives, such as the riboflavin used in the Gambro/Navigant process, but this still leaves questions over possible byproducts.

Unfortunately, all useful decontamination technologies also share the problem of being imperfect: not all pathogens are killed. Beyond the inability of solvent-detergent techniques to treat non-enveloped viruses, this rule holds even for pathogens for which the technique is most effective. The currently accepted standard for effective decontamination is a Logarithmic Reduction Factor (LRF) of 6, which means that one pathogen in a million survives. Note that this value is based only on conjecture, but it is so strict that it is difficult to measure for many pathogens. For comparison, even strong household cleaners claim to eliminate "99% of household germs" which is only an LRF of 2, or 10,000 times less effective than accepted plasma treatments.

Of course, such high levels of decontamination come only at the expense of protein quality: the agents that attack DNA and RNA also attack to some extent the chemically similar plasma proteins, notably the clotting factors that are crucial for hemostasis. The net result is that a balance must be struck between decontamination effectiveness and acceptable protein losses. Fortunately, disrupting the helix in DNA or RNA at even a few points completely destroys the ability of a pathogen to reproduce, while clotting proteins such as fibrinogen can still function well with even several damaged segments.

Under this scenario, many of the above groups have been somewhat successful in developing their respective technologies. On the other hand, what is a "safe" dose of HIV or Ebola? Note that there is some indication that HIV inactivation may require an LRF of 8, which requires 100 times the removal of an LRF 6 technology [6]. Furthermore, a unit collected shortly after infection may be at peak viral loads because no antibody

response has yet been established. Such cases could therefore overwhelm a process with only LRF 6 capability.

For these reasons, the Paul Ehrlich Institute mandates that European products be treated by at least two independent techniques [7]. Under this approach, the pathogens that escape one process may not escape the second, etc. Note that for combined processes, the FDA allows the LRF values to be added, so that 2 processes that each have an LRF of 6 thus have a combined LRF of 12, corresponding to the survival of 1 virus copy in a trillion.

2.3   CryoFacets, Inc. Plasma Decontamination

The CryoFacets, Inc. technology produces just such high levels of decontamination, while still maintaining low levels of protein damage. These unmatched results are obtained by a combination of ultraviolet-C (UVC) light and ozone.

2.3.1   Ultraviolet-C

Ultraviolet light is classified into four different components, according to energy (See Figure 1). The lowest energy band is UVA, followed by UVB. These bands are commonly used in tanning booths, as well as the previous decontamination techniques described above. Because these bands lack sufficient energy to attack the pathogens directly, UVA and UVB systems use the light to activate some chemical additive, which is then actually responsible for providing decontamination. At the opposite energy extreme is vacuum ultraviolet. Slightly less energetic than soft x-rays, vacuum ultraviolet is so readily absorbed that it cannot penetrate the sample deeply enough to perform useful decontamination.
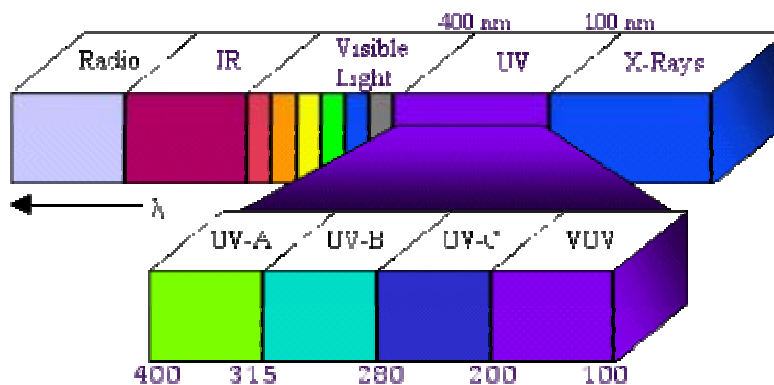

Figure 1: UV Segment of the Electromagnetic Spectrum

Lying between vacuum ultraviolet and UVB is the UVC band. A particularly interesting feature of this band is that light in the 250 to 260 nm light is strongly absorbed by DNA and RNA. This absorption yields *cis,syn*-cyclobutane pyrimidine dimers (CPD), primarily of thymine but also cytosine, as well as noncyclic pyrimidine(6-4)pyrimidone photoproducts [8,9]. CPD accounts for about 75% of the absorption product, while the (6-4) products comprise the remainder. Because of the formation of these compounds, UVC is highly mutagenic at low exposures. At higher exposures, the DNA and RNA are so severely damaged that they cannot function, which is the goal of effective decontamination.

Fortunately, light in this range is less strongly absorbed by blood proteins, which provides some degree of selectivity. As noted earlier, further selectivity follows from the fact that only a very few "hits" by UVC can inactivate DNA or RNA, but protein molecules, such as fibrinogen, can function quite well even with multiple direct hits.

UVC is thus known to be an extremely effective and selective decontamination technique, and has therefore been used for decades for pathogen inactivation. These applications typically employ mercury discharge lamps. These lamps are preferred because they emit most of their light at 254 nm, which is the middle of the peak DNA and RNA absorption range.

Unfortunately, these lamps are difficult to apply to blood work because 254 nm light also has sufficient energy to split any dissolved oxygen molecules into two free radicals. Although these radicals are uncharged, they still have sufficient energy to "burn" any proteins that they encounter. Furthermore, as they react, they also produce multiple other reactive oxygen species (ROS) that are also extremely damaging to blood proteins.

One way to reduce such damage is to add a chemical "quenching" agent that traps the radicals and other ROS before they can cause excessive damage [10]. Unfortunately, these quenching agents can be expensive, and it is typically necessary to remove them in an expensive, time-consuming process before the treated plasma can be used.

Figure 2. Vacuum Chamber and Plasma Processing Bag

The CryoFacets, Inc. solution to this problem is to apply ultrasound to the plasma under vacuum prior to UVC exposure. The purpose of the ultrasonic vacuum treatment is to remove much of the dissolved gasses, including oxygen, from the plasma. The underlying mechanism is a process called "rectified diffusion" in

which the successive compression and decompression causes bubbles beyond a minimum critical size to grow and then leave the liquid [11]. Without dissolved oxygen, no oxygen radicals can form under UVC illumination, thereby sparing the plasma proteins.

The vacuum chamber for this process is shown in Figure 2. Figure 3 shows the ultrasonic processor, the top of which accommodates the vacuum chamber. The plasma is contained in a bag placed in the vacuum chamber. Under vacuum, the evolved gasses leave the bag through a sterile vent.

Figure 3. Ultrasonic Vacuum Degassing Unit

Having thus degassed the plasma, the next problem is to illuminate it. There are two problems, however, that must be addressed here. First, UVC is so energetic that it has a quite limited effective depth in plasma. As a result, it is necessary to treat at most a thin film of fluid. As a further enhancement, it is desirable to expose this film from both sides, thereby producing a more uniform exposure.

Unfortunately, thin films also restrict the amount of fluid that can be processed at any one time. Therefore, some type of flow system is necessary to treat practical volumes. One type of flow is the simple laminar case, in which the center of the flowing liquid moves much more quickly than the boundaries. This kind of flow is commonly seen in rivers and streams, where the current in the middle of the flow is much more rapid than the current along the banks. While common, such a flow is undesirable because an exposure sufficient to treat the center of the stream will over-treat the liquid nearer the boundaries.

The conventional means to overcome this problem is to use a turbulent flow. At sufficiently high Reynolds numbers, the flow will make the transfusion from laminar sections to turbulent eddies. These eddies ensure rapid mixing, and thus uniform exposure. Unfortunately, highly turbulent flows can also damage plasma

proteins. Furthermore, the necessarily high velocities also require long flow paths for sufficient residence time, thereby requiring a large piece of equipment, multiple bulbs, and a great deal of disposable materials. For these reasons, high turbulence systems are unacceptable for this application.

The remaining type of flow field is the plug. In this case, the entire fluid mass moves together at one velocity, thereby ensuring uniform exposure. Plug flows, however, while desirable, rapidly convert to laminar flows due to drag along the flow boundary. To prevent this conversion, the CryoFacets, Inc. system utilizes ultrasound to eliminate the wall drag, thus maintaining a plug flow distribution throughout the entire flow field.

The net result is high pathogen decontamination levels with minimum protein damage. To assess the degree of decontamination, porcine parvovirus (PPV) was selected as a model virus; B19 is the form that infects humans. PPV is an interesting pathogen because it is small and non-enveloped, and thus particularly hard to kill. Note that the porcine form is in fact considered to be more robust than the human form. Thus, any technique that is effective against PPV is most likely even more effective against less robust pathogens, such as HIV. The CryoFacets, Inc. technology inactivates PPV to LRF 6 in 0.4 seconds, at a target thickness of 75 microns.

To assess the level of protein damage during the decontamination process, it is necessary to measure the concentrations before and after exposure. Two proteins of particular interest are Factor VIII and fibrinogen. Factor VIII is useful because it degrades readily, and thus provides a sensitive measure of protein damage. Fibrinogen is useful because this protein is critical for strong clots, such as those made from fibrin glues. Fibrinogen testing thus provides a measure of the expected functionality of the treated products. Factor VIII losses at LRF 6 decontamination levels were only 10%, compared to the 30 to 40% levels often seen with other techniques [12, 13].
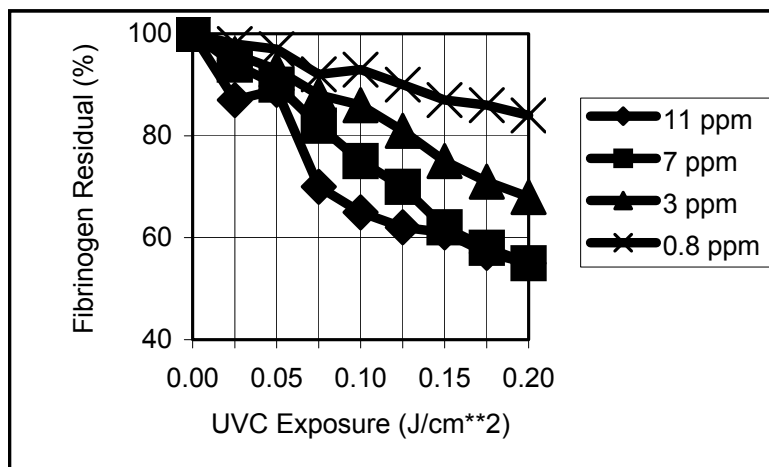

Figure 4. Fibrinogen Degradation as a Function of Exposure Time and Oxygen Concentration

Figure 4 shows the degree of fibrinogen damage associated with this level of decontamination for different dissolved oxygen concentrations. The overall trend is less damage with progressively less residual oxygen. Note that the highest concentration curve (11 ppm) intersects the next highest curve (7 ppm) because (7 ppm) is the saturation concentration. Thus, the intersection occurs when the supersaturated curve spontaneously approaches saturation.

Even though these results are quite favorable, they are at the limit of the measurable effectiveness of UVC. Specifically, it may be that UVC in this configuration is even better than LRF 6, but the PPV doping is at its technical testing limit at this point. Alternatively, it may be that UVC is at its practical limit at this point, and any further treatment would only damage the proteins. In either case, another technique is necessary to reach LRF 12 and thus meet the requirements of the Paul Ehrlich Institute.

2.3.2 Ozone

Ozone is one such alternative. Ozone is the triatomic oxygen molecule, formed when the conventional diatomic form is split and one atom bounds to another diatomic molecule. Ultraviolet light, electric arcs, and some electrolytic processes can be used to provide the initial split. Because all of these processes are high in energy, the resulting ozone molecule is also high in energy, and is in fact the second strongest oxidizing agent, following only hydrogen fluoride.

This high reactivity has a number of practical applications, including decontamination. The most common such work is water treatment, including waste disposal, municipal water supplies and even expensive bottled water. Ozone is also used in a variety of food and pharmaceutical processes, mainly because it is effective across a wide variety of pathogens, and it leaves no residue to contaminate the treated product.

The common feature in all of these applications is the ability of ozone to work by multiple mechanisms. One of these mechanisms is the direct oxidation of the pathogens on contact. This mechanism is effective against all pathogens, but it is particularly effective against enveloped viruses. Another mechanism is the splitting of lipids at the site of unsaturated bonds [14, 15]. The resulting aldehydes are effective across a broad range of pathogens. A wide variety of less energetic reactions can also occur, depending on the details of the local chemistry, the type of pathogens, etc. The net result of these multiple processes is thus a quite high level of effectiveness.

In terms of selectivity, the essential element of this technology is that, like the UVC process, the pathogens can survive only a few "hits," while the proteins can function quite well even with multiple hits. Even with this quite high level of selectivity, however, ozone applications in the blood industry have been somewhat limited to date. CryoFacets, Inc. has therefore developed an ozone processor that achieves high levels of plasma decontamination without excessive protein damage.

Like all other ozone systems that treat liquids, the first consideration in the CryoFacets, Inc. technology is to guarantee a thorough mixing of the gas with the plasma to be treated. Noting that the strong contact oxidation reactions cannot occur unless the gas touches the pathogens, it follows that the ideal situation is a uniform distribution of dissolved ozone throughout the entire treatment volume. Conventional systems, however, employ large bubbles that provide a high ozone concentration only at their surfaces.

Conversely, the CryoFacets, Inc. mixing chamber consists of a tube with multiple small holes. This tube, in turn, is attached to an ultrasonic driver. The ozone gas enters along the axis of the tube. As the gas escapes through the small holes, the motion of the tube shears the bubbles while they are still quite small. Specifically, the bubbles are sheared before they reach the critical size needed for ultrasonically assisted growth [11]. Thus, the bubbles are driven into solution, as desired. The driver is shown in Figure 5.

Figure 5. Ultrasonic Driver for Ozone Mixing Nozzle

While this approach works well for any gas and liquid combination, note that the plasma from the previous UVC process is degassed. As such, this liquid has a great deal of empty space to absorb gasses, and the ozone uptake is thus extremely rapid. As a further improvement in gas uptake, the system can be operated at elevated pressures, and at low temperatures, as shown in Figure 6; both higher pressures and reduced temperatures improve the gas solubility.
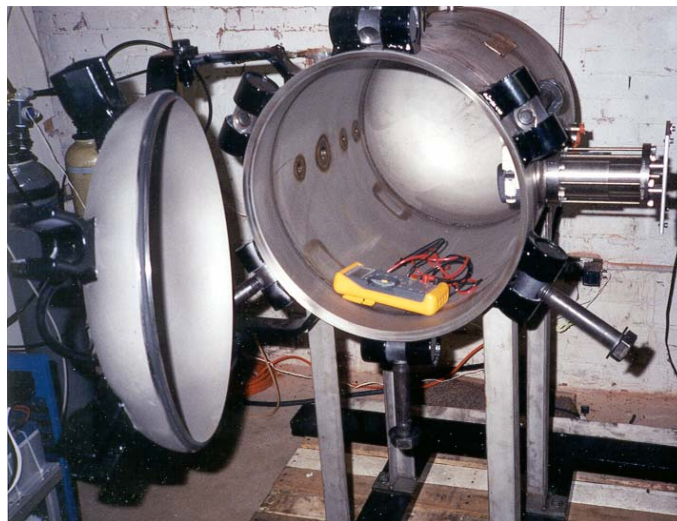

Figure 6. Pressure Vessel

Finally, many conventional ozone systems try to treat the entire product volume in one container. The net result is that the material near the gas inlet is over-treated, while the material in the more stagnant zones is
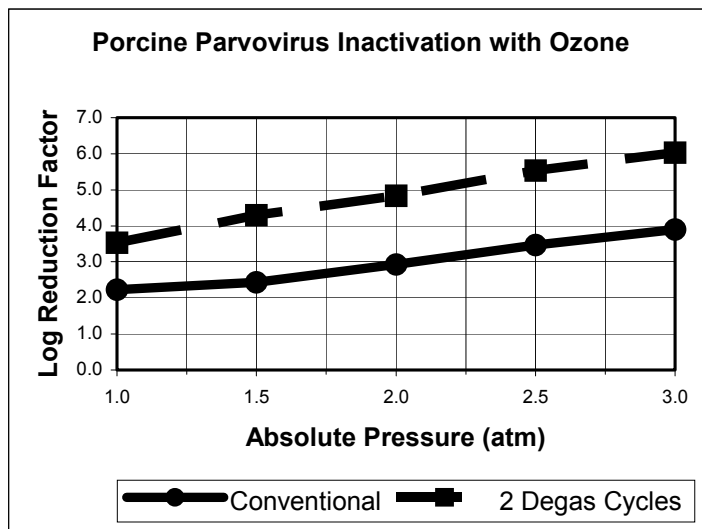
Porcine Parvovirus Inactivation with Ozone

Figure 7. Ozone Decontamination Effectiveness as a Function of Pressure for 1 and 2 Degassing Cycles

Figure 7 shows the ozone decontamination of plasma spiked with PPV. Note the increasing effectiveness with pressure, as expected from Henry's law. The corresponding Factor VIII loss was 12% and the fibrinogen loss was 6%.

3.0   PLASMA CONCENTRATION

3.1   Need for Plasma Concentrates

Plasma is mostly water. Because plasma must be stored and shipped frozen, most of this effort amounts to storing and shipping just water. Furthermore, water has a high heat of fusion and a high specific heat. Freezing, thawing, and warming all of this water is thus expensive. Even worse, the prolonged time for thawing and warming can be deadly if the plasma is needed quickly, as it often is.

The obvious answer to this problem is to concentrate the plasma. Unfortunately, conventional concentration techniques, such as evaporation and filtration, are not effective for plasma and can even damage the plasma proteins.

3.2   Cryoconcentration

The CryoFacets, Inc. solution to this problem is based on a technique called cryoconcentration. The underlying principles of this technology follow immediately form the phenomena of thawing and freezing. Specifically, when pure ice is warmed, the temperature gradually increases until the melting point is reached, which is 0 °C. The temperature remains at this point until all of the ice melts. The temperature of the liquid

water then continues to rise as more heat is added. Conversely, the freezing process is the reverse procedure, except that liquid water often still exists below the melting point. This phenomenon is called supercooling. This supercooled state is maintained until ice crystal formation starts at a surface irregularity or due to agitation; the initiation process is called nucleation.

For biological materials such as plasma, however, the situation is much more complicated. In this case, the salts make ice formation much more difficult, so the freezing point is proportionally lower. Then, as the ice crystals begin to form, they are quite pure and thus displace the salts and other solutes. As a result, the freezing point is depressed even further. This process continues until the displaced salts and remaining liquid solidify together at the eutectic point.

In the CryoFacets, Inc. technology, the region of interest extends from the point of freezing, up to concentrations of a maximum of about 10:1. The first concern here is that extensive supercooling leads to nearly instantaneous bulk freezing when nuclei are finally provided. A common example of this phenomenon is opening a bottle of beer that has been left in a freezer too long. In this case, the rapidly advancing freeze front entraps all of the solids, thereby bypassing much of the concentration process.

To prevent this undesirable result, the CryoFacets, Inc. technology begins with the application of 20-50 kHz ultrasound to initiate the freezing process at the melting point, thereby avoiding any significant supercooling. Furthermore, because the resulting nuclei are distributed throughout the freezing volume, the process proceeds uniformly.

The next concern is that the advancing freeze front invariably develops a branched, or dendritic, form (Figure 8). These dendrites are undesirable because they entrap the increasingly concentrated solution. In the CryoFacets, Inc. technology, the ultrasound sources are kept on to break these dendrites, thus freeing the concentrated pools. As an additional benefit, the dendrite fragments thus become additional nucleation sites. Ultrasound also has the additional benefit of aiding heat transfer and diffusion, thereby further accelerating the overall process.
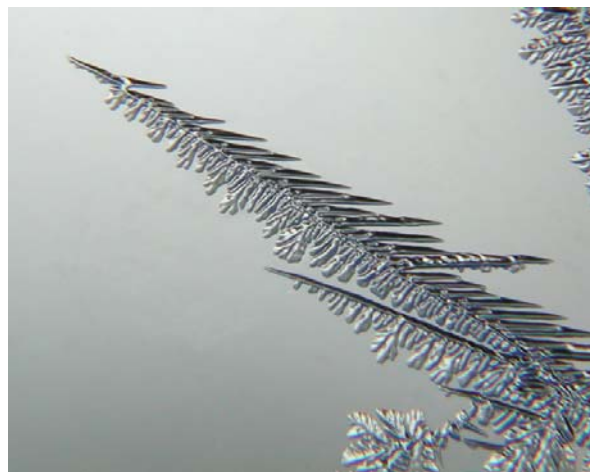

Figure 8. Dendritic Ice Crystal Forms

As these processes continue, however, the salt concentrations will eventually become so great that they will either damage the proteins or cause them to precipitate out of the solution. Salt reduction techniques, such as

dialysis, tangential flow ultrafiltration, or salt exchange columns, must therefore be used. The ice crystals can be removed before, during, or after this salt reduction. All that is required is a special collection bag and centrifuge rotor.

The cryoconcentration process is thus rapid and effective. The resulting product is salt balanced, with little or no protein damage, even at concentrations of up to 10:1.

3.3   Uses of Cryoconcentrated Plasma

Plasma cryoconcentrates are obviously of some value in terms of logistics: after all, even a 3:1 concentrate would reduce the number of freezers, transports, etc., by 2/3. On the other hand, the cryoconcentration process itself requires some effort as well, thus diminishing this advantage. Logistically a 10:1 concentrate is obviously much better than a 3:1 concentrate. Unfortunately, such high concentrates are quite labor and time intensive. As such, cryoconcentrates have some clear advantages in terms of logistics, but the advantages may not be worth the effort in some cases.

Another interesting use of cryoconcentration is rapid thawing. For comparison, even the best commercial plasma thawing equipment requires several minutes to thaw a single unit. Additional time is then necessary to raise the thawed material up to transfusion temperature. Conversely, adding 50 °C water to frozen plasma at dry ice temperature yields a unit of plasma at transfusion temperature in less than 1 minute. Because all that is required is a bottle of warm water, the whole procedure is thus quite fast and simple.

Incidentally, plasma concentrates thawed under this approach will sometimes have unmixed protein aggregates that resist mixing; a similar problem is commonly observed with lyophilized products. A simple solution to this problem is to apply an ultrasonic transducer directly to the bag surface. In several seconds the ultrasound will disrupt the aggregates and mix the freed proteins rapidly throughout the entire volume.

The net result is that rapid thawing is helpful, but because not all cases require such rapid processing, the advantages are again not always worth the effort.

Cryoconcentrated plasma, however, has significant clinical advantages that are well beyond just logistics and thawing improvements. These advantages follow directly from the reason that plasma is transfused in the first place. Of course, one benefit of a plasma transfusion is to restore volume and salts, but this requirement is preferably met with just saline solution. Instead, in the case of trauma, the transfused plasma also restores some of the clotting proteins required for hemostasis.

In normal plasma, however, clotting proteins comprise only a small part of the total volume. For example, fibrinogen concentrations are typically in the range of the low hundreds of milligrams per deciliter, so the entire circulating mass is about ten or so grams. Such a small amount is rapidly depleted in the event of the loss of a limb due to a mine, or the impact of a high velocity round. In such cases, transfusing a unit of plasma augments the body's limited reserve of clotting proteins, but even these additional materials can be lost rapidly.

As a further complication, compression and other techniques are commonly used to prevent massive fluid loss in such cases. Additional plasma transfusions, without reduced fluid loss, thus rapidly exceed the ability of the body to process the excess water. At this point of fluid overload, no further transfusions can be given, and without adequate clotting proteins, the patient is in danger of severe haemorrhage.

The immediate solution to this solution is to transfuse a concentrated plasma, not one that has been diluted back to its original state. Under this approach, the patient can receive large amounts of clotting proteins without the risk of hypervolemia. For example, a 3:1 concentrate provides the proteins of 3 units of plasma, but only the water volume of one unit.

Of course, increasing the protein concentration also increases the viscosity. In practice, viscosity increases can be either good or bad, and there are many factors to consider. First, the increase in viscosity is not directly proportional to the concentration factor. For example, the normal viscosity of plasma is about 1.5 cP, but a 3:1 concentrate has a viscosity of only about 3.1, not 4.5 cP. The reason for this apparent discrepancy is that water at body temperature has a viscosity of about 0.7, so each increase in concentration factor raises the viscosity about 0.8 cP, within reasonable limits.

The second factor to be considered is that even this elevated viscosity decreases over time as the clotting proteins are lost from circulation. Notably, the decrease in free fibrinogen markedly decreases the effective viscosity.

The third factor to be considered is that the total blood viscosity depends strongly on the presence of the cellular blood components. Quantitatively, whole blood has a viscosity of about 4.5 cP at moderate flow rates, and up to about 20 cP at low flow rates, versus 1.5 cP for plasma. Thus, as the red cells decrease due to haemorrhage, the viscosity drops rapidly.

The fourth factor to be considered is that the body can withstand elevated viscosities for at least a short time without adverse effects. Even extremely high viscosities, about 8 to 9 cP, or twice the normal level, are commonly seen in hyperviscosity syndrome. Note, however, that prolonged high viscosity conditions are associated with the risk of thromboembolism, retina damage, and other problems.

Fifth and most interesting, higher viscosity fluids are currently being studied for resuscitation fluids. This approach is in direct contrast to the long-held assumption that low viscosity fluids are preferable over high viscosity fluids for such purposes. The rationale has been that because lower viscosity fluids can more easily flow through the confined spaces of capillary beds, etc., saline solutions would be preferable over a fluid with the consistency of motor oil. On the contrary, Tsai and Intaglietta [16] have shown numerous that high viscosity blood substitutes have many benefits at the microcirculation level. Likewise, Bertuglia and Giusti [17] have shown the benefits of reduced reactive oxygen species damage with high viscosity fluids.

The net result here is that the higher viscosity of plasma concentrates is not only tolerable, but actually quite helpful. Research is therefore currently underway to determine the ideal concentration, given the above constraints. Several options are emerging in this effort. First, it may be preferable to formulate a very high concentration. Under this approach, the most severely injured patients would receive the maximum possible dose, with the understanding that preserving the life of the patient justifies the risks. As a further justification, the peak viscosity would soon drop in such cases anyway due to rapid loss of blood cells and clotting proteins. Incidentally, such an approach would also provide the maximum benefit from the improved logistics and thawing properties of high concentrates.

As an extension of this approach, the next option is to dilute the highly concentrated plasma with variable amounts of saline. Still more concentrated than normal plasma, the partially reconstituted material would have the clinical advantages described above in proportion to the degree of dilution. This variability would allow the product to be fine-tuned to individual needs if desired in a hospital setting; alternatively, the broad range of safety would allow a given dilution, such as an endpoint of 3:1, to be used for all intermediate patients near the field.

As a further extension of a "one size fits all" approach, another option is to pool small numbers of concentrates. The main advantage of such an approach is that the product would be more uniform on the basis of simple averaging. Thus, the effects of the transfusion would be more predictable. Also, if one donor happened to be extremely high or low in any given component, any consequences would be greatly diminished. Of course, these benefits would have to be carefully weighed against the increased risk of infection, but the use of the two decontamination processes described above largely offset this risk.

Finally, high concentration plasma could also be merged with the various blood substitutes currently under development. Although the ideal formulation would have to be determined for each product, the net result would be an extremely powerful resuscitation fluid.

4.0 LYOPHILIZATION

As noted above, one of the advantages of concentrates is the ability to reduce the number of storage facilities and transport operations. Even the highest concentrations of plasma, however, still require refrigeration, and this requirement can be a problem in isolated areas. An obvious solution to this problem is to lyophilize the plasma, and thus reduce or even eliminate the need for refrigeration.

For this reason, CryoFacets, Inc. began a series of experiments on the lyophilization of human plasma. The underlying concept was that the reduced water component would provide more rapid freezing and more rapid sublimation, thereby greatly accelerating the overall lyophilization process. These experiments have had mixed results.

First, in terms of speed, the ideal situation would first appear to be the maximum possible concentration, i.e., the maximum possible removal of water as ice in the concentration step. Unfortunately, such an approach also yields a quite thick liquid, which amounts to a paste. Freezing this material leaves few channels for the escape of water vapor, thus slowing the overall lyophilization process.

To overcome this problem, it is possible to use a vacuum process that essentially produces a foam. Because the pores in this foam produce large channels for the escape of water vapor, the overall lyophilization process is thus greatly accelerated, as desired. Unfortunately, subsequent legal work has revealed that another group had recently patented the process, so this effort has been discontinued.

Alternatively, it is possible to lyophilize plasma at lower concentrations, but this involves adding a separate step to the lyophilization process. This approach is thus not of great economic value over conventional lyophilization.

Despite these setbacks, CryoFacets, Inc. still has some interest in lyophilization, in particular the ability to handle individual plasma units. As noted above, the overall goal is to avoid pooling; furthermore, it is necessary to maintain the plasma in a closed system for regulatory reasons. In keeping with this goal, the lyophilization process must be completed in a "sealed" bag as well. One option is a gas permeable bag, but this approach is inherently limited because no bag material is capable of providing a reasonable processing speed.

CryoFacets, Inc. has therefore developed a unique segmented bag and coupling device that provides the necessary speed and meets the closed system requirement. This system is significantly faster than even conventional lyophilization devices. Planned improvements should make the system faster still, even without any prior concentration at all.

On the other hand, CryoFacets, Inc. has conducted extensive testing on one lyophilized plasma component: fibrinogen. The reason for these tests is that fibrinogen is the main component of "fibrin glue," a surgical adhesive.

The results are shown in the following figure, where the strength required to rupture the glue is plotted as a function of the fibrinogen concentration. The overall result is that the glue strength follows roughly the square of the fibrinogen concentration, which is intuitively reasonable because the strength depends on the available surface area, which is a squared term by definition. Note that other researchers have reported a linear dependence. Their findings are actually in agreement with the square law, however, as long as only limited segments are considered.

Not shown on this graph is the data for Tisseel, a commercial fibrin glue. According to the manufacturer, Tisseel has a fibrinogen concentration in excess of 10,000 mg/dl. As such, the clot strength should be (***) according to the square law. Instead, the observed strength is only (****), which is less than the strength of the (***) concentration.

The reason behind this apparent discrepancy is that the TIsseel product is provided in lyophilized form, while the points on the curve are for glues that have not been lyophilized. These results are important because the strength of the clot is a measure of the function of the treated protein. Conversely, simply measuring the fibrinogen concentration would not have predicted these results.

Because similar results are expected for other proteins, and because there have been no technological breakthroughs in the lyophilization process that might change these results, it has been necessary to re-evaluate the overall lyophilization strategy. From this re-evaluation, it appears at this time that a two-tier approach may be required. Specifically, non-lyophilized products would be used if possible due to their greater efficacy. Lyophilized products would thus be used only as "last resorts" if non-lyophilized products were not available.

Under this scenario, there is limited incentive to continue with plasma lyophilization efforts. Some of the technology, however, is of interest in ongoing cellular product efforts.

5.0 CELLULAR PRODUCTS

Finally, CryoFacets, Inc. is currently applying the above processes to the treatment of cellular blood products, specifically platelets and red blood cells (rbc's). Like plasma, the concern here is that the supply lines to advanced field units could be cut. Unlike plasma, however, cellular products are typically stored in liquids, and thus have a quite limited shelf life. Even a brief interruption in supplies would therefore require collection of cellular blood products from the local population. The net result is that while plasma decontamination is important, the ability to decontaminate cellular products is absolutely crucial.

Unfortunately, decontaminating cells is much more difficult than decontaminating plasma. The underlying problem is that cells are complex, fragile, and they have membranes that separate them from the surrounding plasma. It is therefore difficult to introduce any decontaminating agents into the cells, difficult to attack the pathogens without also attacking the cell contents, and difficult to remove the waste products after the decontamination reactions are finished.

To resolve these problems, the above ozone and UVC plasma decontamination processes have been essentially re-engineered. The overall approach is to use ozone primarily as an extracellular agent, although

there is some research that indicates some intracellular effects may also be occurring. Conversely, UVC is used for both extracellular and intracellular treatments.

Of the two processes, ozone has more difficult to develop because previous work shows that red blood cells are prone to Heinz body formation [18] and ozone is not selective against pathogens versus cells [19]. A new disposable and its associated process have eliminated both of these problems, and have likely reduced the incidence of Transfusion Related Acute Lung Injury (TRALI) as well.

Likewise, the UVC modifications also requires a new disposable and associated process.

The system is still under development, but preliminary results indicate combined LRF's of 12 or more, with only a few percent cell damage. As a spin-off application, the disposables can also be used to perform a conventional cell wash in 15 minutes, in a closed system, automatically, and with less than 1% cell loss.

Additional work is underway to refine these technologies. If successful, they will then be used as a basis for the development of decontaminated, lyophilized cellular products.

6.0 CONCLUSIONS

The CryoFacets, Inc. technology thus consists not of new processes or procedures *per se*. Instead, the technology consists of applying a combination of known pieces from a variety of disciplines to make new products. This use of known components has greatly reduced the development costs and time, and has ensured success at each step. Thus, CryoFacets, Inc. has two world leading plasma decontamination processes, with a combined LRF of 12 and protein losses less than 25% even for Factor VIII. The technology also includes a unique cryoconcentration process, capable of concentration up to a factor of 10:1, as well as lower concentrations that could be transfused directly as high viscosity resuscitation fluids. This concentrated plasma can also be used in a unique lyophilization system, although protein losses may be unacceptable. Finally, the decontamination processes are being modified to form the basis of a cellular product decontamination technology. If successful, this approach will avoid the use of the additives that have limited all other cellular decontamination attempts.

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