Pressure cycling technology: a novel approach to virus inactivation in plasma

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BACKGROUND: Hydrostatic-pressure virus inactivation is a novel approach to the inactivation of pathogens in plasma and blood-derived components, that retains the therapeutic properties of these products.
STUDY DESIGN AND METHODS: A custom-built apparatus was used to pressurize human plasma samples spiked with lambda phage. Phage titer and plasma protein activities were monitored after pressure treatment.
RESULTS: Pressure-mediated inactivation of lambda phage was found to be an effective means for virus inactivation, particularly when performed at near-zero (0°C) temperatures, rather than at temperatures above 20°C and below –40°C. The efficiency of inactivation was improved by an increase in applied pressure and repeated cycling from atmospheric to high pressure. In contrast, activities of plasma proteins alkaline phosphatase and total amylase did not vary with temperature and remained within 29 percent and 6 percent, respectively, of starting values after the same pressure treatments. By combining cycling, near-zero temperatures, and high pressure, phage titers in serum were reduced approximately 6 log after 10 to 20 minutes of treatment. Activities of plasma proteins IgG, IgM, and factor X were at 104 percent, 89 percent, and 80 percent, respectively, of starting values after 20 minutes of the same temperature and pressure treatment.
CONCLUSION: High-pressure procedures may be useful for the inactivation of viruses in blood and other protein-containing components.

The risk associated with the transfusion of blood and blood components has been significantly reduced by the screening of donors and the testing of blood components for specific pathogens.[1] There are, however, several limitations to screening: 1) current tests may miss certain infectious units, particularly during the early phase of infection before seroconversion; 2) effective tests are not available for all known pathogens; 3) newly emerging viruses or microorganisms cannot be detected[2]; and 4) the marginal benefit-to-cost ratio becomes prohibitively high for new tests.[3] Thus, screening and testing must be combined with inactivation procedures to obtain a lower-risk blood supply.[4]

A variety of inactivation procedures, such as pasteurization, solvent/detergent treatment,[3,6,7] irradiation,[8] and others,[9,10] are currently used to lower the residual risk of infection. All current inactivation methods, however, are problematic. For example, solvent/detergent treatment requires the pooling of plasma from over 2000 donors and is ineffective against nonencapsulated virus particles, which lack a lipid bilayer. Additives such as detergents or chemicals also must be removed before treated blood components are used therapeutically.[3,6,7] Filtration is slow and expensive and is suitable only for highly purified blood

ABBREVIATIONS: ALP = alkaline phosphatase; CMV = cytomegalovirus; PCT = pressure-cycling technology; PFU(s) = plaque-forming unit(s); T-AMY = total amylase.

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derivatives.[11,12] Heat treatment denatures proteins and requires the addition of stabilizers.[11] Furthermore, all current treatment procedures potentially reduce the therapeutic properties of proteins within plasma and blood derivatives.

An ideal inactivation method for blood components would be highly effective against a broad range of pathogens, while still preserving the functional activity of plasma proteins. In a consideration of production issues, the optimal method would be 1) capable of being taken from a prototype model to a full production model; 2) have high throughput capability; 3) be fully contained, minimizing cross-contamination; 4) verifiable (capable of validation); 5) be accessible, having low barriers to implementation; and 6) be free of chemical additives.

Elevated hydrostatic pressure has the potential to fulfill many of the above criteria. First, pressure is a physical process, as opposed to a chemical process, and so chemicals need not be introduced into the sample. In addition, hydrostatic pressure—unlike temperature treatment, which depends on the transmission of heat through the sample—can be applied and released quickly, thus enhancing high throughput capability. Moreover, the industrial applications of hydrostatic-pressure inactivation of microorganisms include food processing[13-19] and, potentially, vaccine production.[20-25] Despite these potential advantages, current approaches utilizing elevated hydrostatic pressure are too slow for blood-processing applications: at ambient temperatures, 100 hours of 150-megaPascal (MPa) pressure treatment was the estimated condition for an 8 to 9 log reduction in simian immunodeficiency virus titer.[20]

In this investigation, we examined the effects of low temperature, high pressure, and pressure cycling on the inactivation of a model test virus in plasma. We demonstrated the feasibility of rapid virus inactivation under conditions that preserved therapeutic and functional properties of several plasma proteins.

MATERIALS AND METHODS

Lambda phage pressurization experiments

Lambda phage stock ($2\text{-}5 \times 10^9$ plaque-forming units [PFUs] / mL) was diluted 1-in-10 in ACD human plasma. Heparin was added to a final concentration of 50 USP (United States Pharmacopeia) units per mL to prevent coagulation during postpressurization processing. Samples were frozen on dry ice and placed individually within the pressure chamber of a device for pressure-cycling technology (PCT) (BaroCycler Version 2.2, BBI-BioSeq, Woburn, MA). Samples were equilibrated to the indicated temperature for 4 minutes and then subjected to constant or alternating cycles of hydrostatic pressure. After processing, samples were stored temporarily on dry ice. To measure phage titer, samples were thawed at room temperature and atmospheric pressure. A series of 1-in-100 dilutions were made in suspension medium (0.05 *M* NaCl, 4.0 m*M* $MgSO_4$, 50 m*M* Tris-HCl [pH 7.5], 1.0 g/L gelatin), and the titer of lambda phage was measured as described and expressed as PFUs per mL.[21]

Protein function assays

After pressure treatment, human plasma samples were stored at –80°C until they were analyzed. Alkaline phosphatase (ALP) and total amylase (T-AMY) activities were measured by using a kinetic spectrophotometric assay with p-nitrophenyl phosphate and p-nitrophenyl-maltohep-taoside, respectively, as the substrates. Total protein was measured as an endpoint spectrophotometric assay, using the biuret reaction. Assays were performed on a chemistry analyzer (917, Hitachi, San Jose, CA) with reagents (Roche Diagnostics, Indianapolis, IN). The analytic sensitivity of the assays for ALP, T-AMY, and total protein was 3 U per L, 4 U per L, and 4 mg per dL, respectively. Each assay had a within-run CV of less than 5 percent. Activities of ALP and T-AMY were expressed as a fraction of the total protein to enable direct comparison of experiments using the same plasma lots, and they were plotted. Different lots of plasma were used for these experiments, so the enzyme activities cannot be directly compared from one figure to another.

For IgG and IgM studies, anti-cytomegalovirus (CMV) IgG- or anti-CMV IgM-positive human plasma (Boston Biomedica, West Bridgewater, MA) was diluted with normal plasma to yield results that fell within the range of the solid-phase enzyme immunoassay[22] (CMV Total AB EIA, Abbott Diagnostic Division, Abbott Park, IL). The plasma samples were pressurized as indicated and then frozen until they were analyzed. Data were expressed as a percentage of the nontreated control.

Factor X activity was assayed by using a chromogenic substrate (RVV-X, Pentapharm, Basel, Switerland). First, 20 µL of plasma, diluted 1-in-20 in assay diluent (American Diagnostica, New York, NY), was mixed with 20 µL of RVV-X and incubated for 5 minutes at room temperature. Then, 159 µL of diluent was mixed with 20 µL of substrate and added to the reaction. The optical density (405 nm) was monitored and found to increase at 0.056 per minute for the positive control. The addition of 20 µL of 50-percent acetic acid stopped the reaction and maintained a stable signal. A standard curve was prepared by using 100-percent, 50-percent, and 0-percent plasma dilutions, in triplicate. Unknowns were assayed in triplicate.

Operation of the PCT device

Sample volumes of 280 µL were placed in polyethylene microcentrifuge tubes (#06333-10, Cole Palmer, Vernon Hills, IL), sealed with paraffin film, and placed within the pressurization chamber of the PCT device (1.9-cm in diameter, 7.6-cm in length). Ethylene glycol with rhodamine (50% ethylene glycol, 0.1% rhodamine), used as the pressure-transducing medium, was added as needed to minimize

the amount of trapped air within the chamber. The temperature of the sample was allowed to equilibrate for 4 minutes before pressurization. After the PCT device's pressure setting was adjusted, pressure was applied to the chamber by activating the hydraulic pump. Pressures for all experiments were monitored either manually or with a strip-chart recorder. After pressurization, the pressure lock was closed, which sealed the chamber, and the hydraulic pump was deactivated. After a small (5%) decrease during the first 20 seconds, pressure within the chamber was stable for up to 10 hours.

RESULTS

Hydrostatic pressure inactivation of lambda phage at near-zero temperatures

First, the effects of lower temperature and pressurization time on virus inactivation were examined. Lambda phage suspended in human plasma was pressurized to 275 MPa for 7.5 minutes at temperatures ranging from –62°C to 44°C. A marked 3 to 4 log decrease in phage titer was observed for samples held at near-zero temperatures (Fig. 1A). Below –40°C and above 28°C, only a slight decrease in titer was observed.

To assess whether pressure treatment at near-zero temperatures had an effect on the functional properties of plasma proteins, the enzyme activity of two test plasma proteins—namely, ALP and T-AMY—was examined. Human plasma samples in separate tubes were processed concurrently with phage samples. No near-zero temperature effect was observed (Fig. 1B). All T-AMY activities were within 3.2 percent of the average activity, 1.14 ± 0.02 U per g of total protein. All ALP activities were within 17 percent of the average activity, 0.71 ± 0.06 U per g of total protein. These average activities were close to the starting activities, which were 0.96 ± 0.15 U per g and 1.10 ± 0.07 U per g for ALP and T-AMY, respectively. The protein data are plotted on a linear scale, while a log scale was used for the phage data.

Increased inactivation at higher pressures

Next, the relationship between applied pressure and virus inactivation was investigated. Samples of lambda phage suspended in human plasma were pressurized for 7.5 minutes at –5°C. The pressure-induced inactivation of phage increased significantly at pressures above 200 MPa, and it approached 3 log at 400 Mpa (Fig 2A). Activities of ALP and T-AMY were monitored under the same conditions (Fig. 2B) and found to be unaffected. T-AMY activities did not deviate more than 6 percent from the starting activity, which was 2.1 U per g of total protein. ALP activities within samples pressure-treated below 345 MPa were within 12 percent of the starting activity, which was 0.98 U per g of total protein.

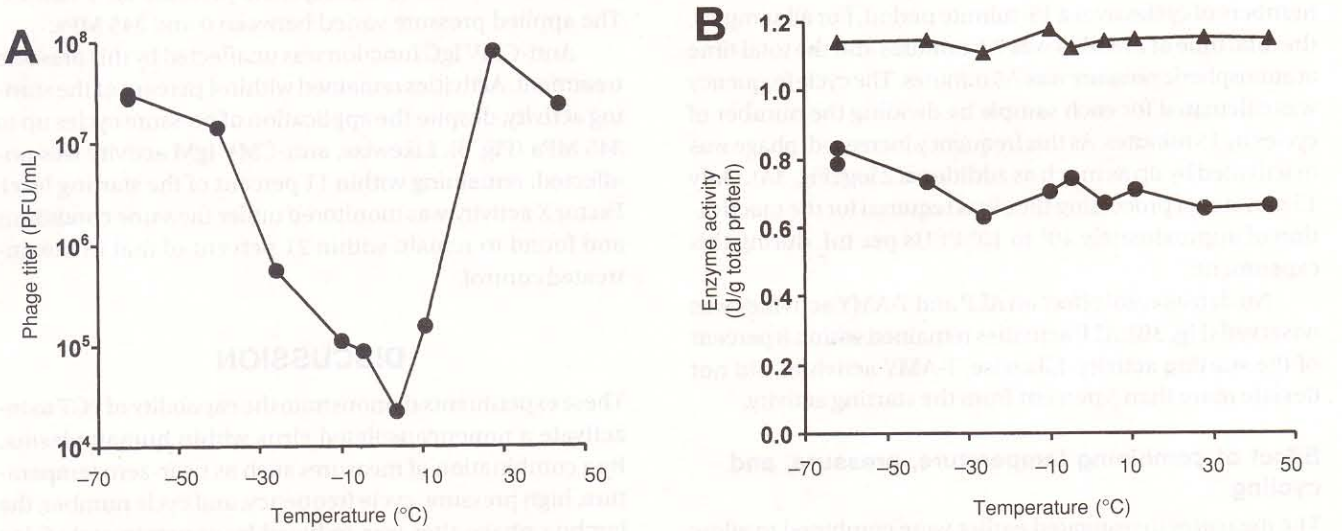

Fig. 1. Phage inactivation at near-zero temperatures. A) Human plasma samples spiked with lambda phage (final titer, 2-5 × 10^8 PFUs/mL) were placed within the pressure chamber, which was set to the indicated temperature. After temperature was equilibrated for 4 minutes, 275-MPa hydrostatic pressure was applied for 7.5 minutes. Phage titer was measured as described in Materials and Methods. Data were plotted from a single representative experiment. B) Human plasma samples were subjected to the same treatment. ALP (●) and T-AMY (▲) activities were measured as described in Materials and Methods. Data were plotted from a single, representative experiment.

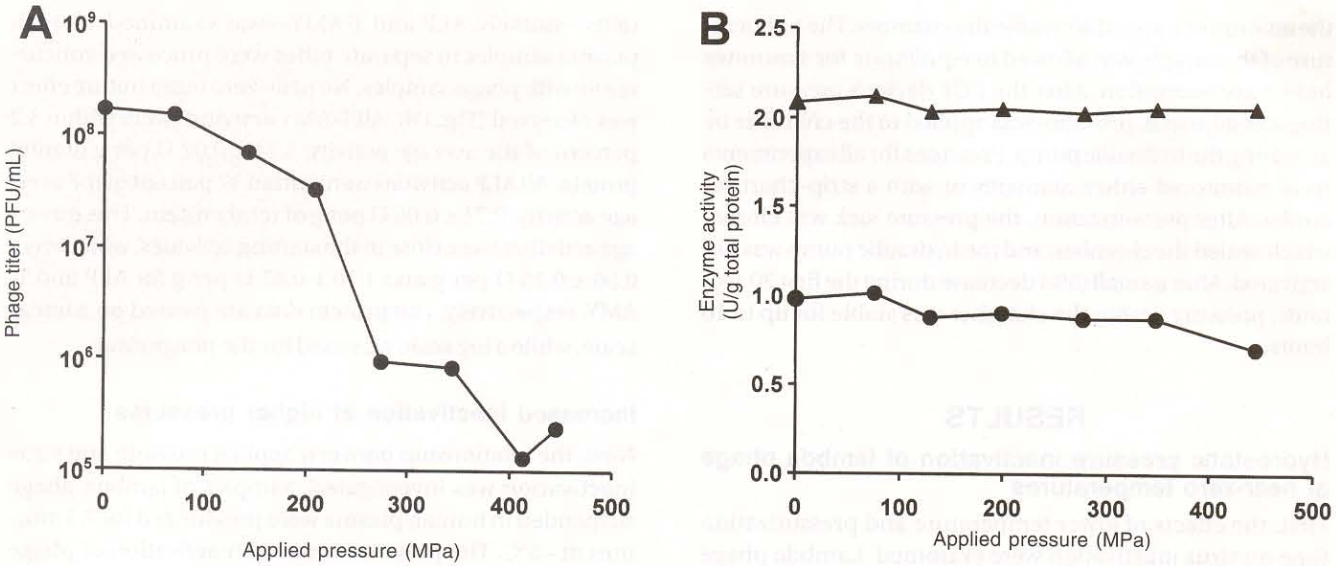

Fig. 2. Decrease in phage titer with increasing pressure. A) The pressure chamber temperature was set to –5°C, and then human plasma–lambda phage samples were pressurized as indicated for 7.5 minutes. Phage titer was measured as described in Materials and Methods. Data from two experiments were averaged and plotted. B) Human plasma samples were subjected to the same pressure treatment. ALP (●) and T-AMY (▲) activities were measured as described in Materials and Methods. Data from a single, representative experiment were averaged and plotted.

Effects of pressure cycling on inactivation

Cycling between high hydrostatic pressure and atmospheric pressure at near-zero temperatures further decreased the phage titers. Hydrostatic pressure was increased to 275 MPa and then decreased to atmospheric pressure for various numbers of cycles over a 15-minute period. For all samples, the total time at 275 MPa was 7.5 minutes and the total time at atmospheric pressure was 7.5 minutes. The cycle frequency was calculated for each sample by dividing the number of cycles by 15 minutes. As this frequency increased, phage was inactivated by up as much as additional 2 log (Fig. 3A). Only 15 minutes of processing time was required for the inactivation of approximately 10^4 to 10^5 PFUs per mL during this experiment.

No detrimental effect on ALP and T-AMY activities was observed (Fig. 3B). ALP activities remained within 8 percent of the starting activity. Likewise, T-AMY activities did not deviate more than 3 percent from the starting activity.

Effect of combining temperature, pressure, and cycling

The measures investigated earlier were combined to allow us to examine their additive effect on phage inactivation. Phage was diluted in plasma and then subjected to multiple 2-minute cycles of 275 MPa or 345 MPa at –5°C. After 20 cycles, the phage titer was reduced 4.2 log at 275 MPa or 6.9 log at 345 MPa (Fig. 4).

Preservation of blood constituent function

In addition to ALP and T-AMY, activities of three key therapeutic proteins—IgG, IgM, and factor X—were examined. Samples at –5°C were subjected to 10 cycles of pressure for 1 minute and then to atmospheric pressure for 1 minute. The applied pressure varied between 0 and 345 MPa.

Anti-CMV IgG function was unaffected by this pressure treatment. Activities remained within 4 percent of the starting activity, despite the application of pressure cycles up to 345 MPa (Fig. 5). Likewise, anti-CMV IgM activity was unaffected, remaining within 11 percent of the starting level. Factor X activity was monitored under the same conditions and found to remain within 21 percent of that in the untreated control.

DISCUSSION

These experiments demonstrate the capability of PCT to inactivate a nonencapsulated virus within human plasma. By a combination of measures such as near-zero temperature, high pressure, cycle frequency, and cycle number, the lambda phage titer was reduced by approximately 6 log units within 10 to 20 minutes. Phage inactivation was achieved under conditions in which common plasma proteins—IgG, IgM, and factor X—were at 104 percent, 89 percent, and 80 percent, respectively, of their starting values. Unlike the phage titer, ALP and T-AMY activities did not de-

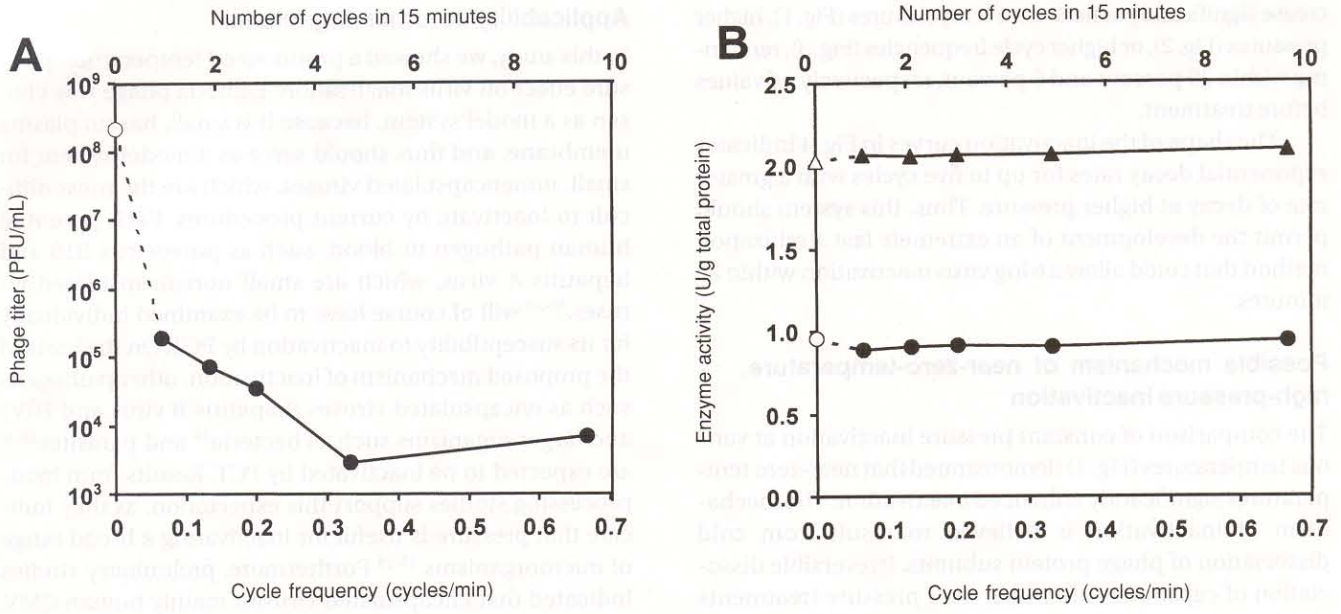

Fig. 3. Decrease in phage titer with increasing cycle frequency. A) The number of pressure cycles within a 15-minute period varied between 1 and 10 for samples composed of human plasma and lambda phage (upper abscissa). The frequency, shown on the lower abscissa, was calculated by dividing the number of cycles by 15 minutes. For each cycle, 275-MPa pressure was applied, and then pressure was released for an equivalent length of time (e.g., for a frequency of 1 cycle/min, the sample was pressurized to 275 MPa for 0.5 min, followed by 0.5 min at atmospheric pressure; the cycle was repeated 14 times). The total pressurization time for each sample was 7.5 minutes. Phage titer was measured as described in Materials and Methods. Data from a single representative experiment are shown (●). A nonpressurized control shows the original phage titer (○). B) Human plasma samples were subjected to the same pressure treatment. ALP (●) and T-AMY (▲) activities were measured and plotted.

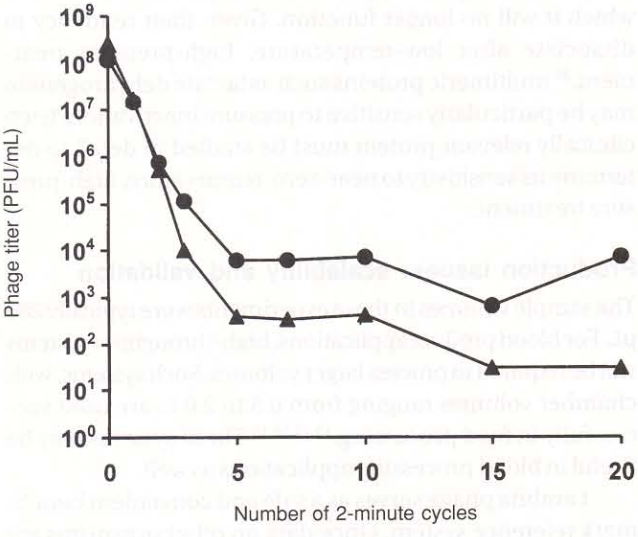

Fig. 4. Phage titer decreased with additional pressure cycles. After the chamber temperature was set to –5°C, pressure was increased to 345 MPa (▲) or 275 MPa (●) for 1 minute and then decreased to atmospheric pressure for 1 minute for the indicated number of cycles. Phage titer was measured as described in Materials and Methods. Data from a single, representative experiment were plotted.

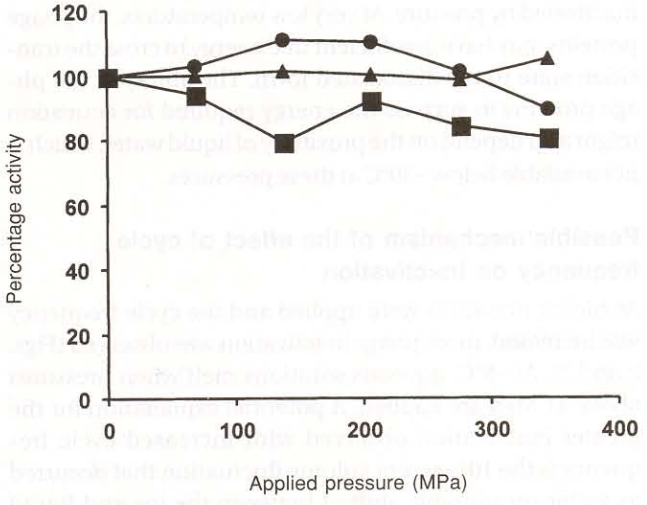

Fig. 5. Plasma protein activities after pressure cycling. Human plasma samples were placed within the chamber, which was set to –5°C. The indicated pressure was applied for 1 minute, and then the samples were at atmospheric pressure for 1 minute for a total of 10 cycles. Activities of IgG (▲), IgM (●), and factor X (■) were measured as described in Materials and Methods. Data from a single, representative experiment were expressed as a percentage of the nontreated sample activity.

crease significantly at near-zero temperatures (Fig. 1), higher pressures (Fig. 2), or higher cycle frequencies (Fig. 3), remaining within 29 percent and 6 percent, respectively, of values before treatment.

The shape of the inactivation curves in Fig. 4 indicates exponential decay rates for up to five cycles with a greater rate of decay at higher pressure. Thus, this system should permit the development of an extremely fast sterilization method that could allow a 6 log virus inactivation within 20 minutes.

Possible mechanism of near-zero-temperature, high-pressure inactivation

The comparison of constant pressure inactivation at various temperatures (Fig. 1) demonstrated that near-zero temperatures significantly enhanced inactivation. The mechanism of inactivation is believed to result from cold dissociation of phage protein subunits. Irreversible dissociation of capsomers after near-zero pressure treatments similar to those employed here was observed for the cowpea severe mosaic virus[23] and bacteriophage P22 coat protein.[24,25] In addition, the high-pressure dissociation of multiple-subunit proteins and protein-nucleic acid complexes (typically, between 100 and 200 MPa) is an empirical observation in numerous systems.[26-28] Once the capsid proteins dissociate, the virus genetic material apparently escapes from the particle and the virus is inactivated. Such a loss of DNA from the coliphage T-4 was observed after pressure treatment at 6°C.[29]

At temperatures below –30°C, phage was only slightly inactivated by pressure. At very low temperatures, the phage proteins may have insufficient free energy to cross the transition state to the dissociated form. The ability of the phage proteins to surpass the energy required for activation might also depend on the proximity of liquid water, which is not available below –30°C at these pressures.

Possible mechanism of the effect of cycle frequency on inactivation

As higher pressures were applied and the cycle frequency was increased, more phage inactivation was observed (Figs. 2 and 3). At –5°C, aqueous solutions melt when pressures above 41 MPa are applied. A potential explanation for the greater inactivation observed with increased cycle frequency is the 10-percent volume fluctuation that occurred as water presumably shifted between the ice and liquid phases. The repeated volume expansion and contraction with each pressure cycle may contribute to the physical dissociation of viral subunits. Because the ratio of spherical surface area to volume is inversely proportional to the radius (r) (i.e., $4\pi r^2/(4/3)\pi r^3 = 1/3r$), the dissociation of the viral subunits and hence the inactivation by PCT may be even more pronounced for larger viruses or other relatively large microorganisms, such as bacteria and parasites.

Applicability to other organisms

In this study, we showed a pronounced temperature–pressure effect on virus inactivation. Lambda phage was chosen as a model system, because it is small, has no plasma membrane, and thus should serve as a model system for small, nonencapsulated viruses, which are the most difficult to inactivate by current procedures. Each potential human pathogen in blood, such as parvovirus B19 and hepatitis A virus, which are small nonencapsulated viruses,[30-33] will of course have to be examined individually for its susceptibility to inactivation by PCT. On the basis of the proposed mechanism of inactivation, other pathogens such as encapsulated viruses (hepatitis B virus and HIV) and larger organisms such as bacteria[34] and parasites[35,36] are expected to be inactivated by PCT. Results from food-processing studies support this expectation, as they indicate that pressure is useful for inactivating a broad range of microorganisms.[13-19] Furthermore, preliminary studies indicated that encapsulated viruses, mainly human CMV, herpes simplex virus, and HIV were inactivated by hydrostatic pressure.[37]

Protein function after pressure treatment

These experiments clearly demonstrate pressure inactivation of a virus with concurrent maintenance of most activity of the studied proteins. The plasma proteins studied here—ALP, T-AMY, IgG, IgM, and factor X—remained active after equivalent pressure treatments (Figs. 1B, 2B, 3B, and 5). Theoretically, every protein has a pressure limit over which it will no longer function. Given their tendency to dissociate after low-temperature, high-pressure treatment,[38] multimeric proteins such as lactate dehydrogenase may be particularly sensitive to pressure inactivation. Each clinically relevant protein must be studied in detail to determine its sensitivity to near-zero-temperature, high-pressure treatment.

Production issues: scalability and validation

The sample volumes in these experiments were typically 280 µL. For blood product applications, high-throughput systems will be required to process larger volumes. Such systems, with chamber volumes ranging from 0.5 to 2.0 L, are used successfully in food processing.[13,14,18,19] These systems may be useful in blood processing applications as well.

Lambda phage serves as a safe and convenient benchmark reference system. Once data on other organisms are available, their inactivation can be expressed as a lambda phage equivalent. Lambda phage can be processed during procedure validation and control runs during production. The equivalent inactivation levels for other organisms can then be assessed.

In conclusion, PCT is a promising new method that may be useful for inactivating viruses in protein-containing therapeutic products. In the context of microorganism

inactivation within blood components, these findings should be considered a preliminary demonstration of a novel approach. The method may also be applicable to other biotechnology products, such as complex recombinant biopharmaceuticals, which are difficult to sterilize by conventional techniques. The method may be useful as a preliminary inactivation step in the processing of clinical samples for diagnostics.

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